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MASTERARBEIT

Titel der Masterarbeit

Development of a bicistronic expression system for generation of Multi-Hit transgenic mice

verfasst von

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Contents

1. Introduction

1.1. Principles of cancer development

Accumulation of genetic alterations is the main cause for cancer development. Regarding their role in tumorigenesis, genes could be divided into two groups – tumor-suppressors and oncogenes. Tumor suppressor genes such as the retinoblastoma protein (pRB) play a role in the control of cell cycle progression and proliferation, regulate survival pathways such as phosphatase and tensin homolog (PTEN) and are implicated in maintenance of genomic stability such as p53. Mutations in tumor suppressor genes may result in loss of function and promote cancer formation. In contrast, oncogenes are generally activated in tumors by gain of function mutations. Genetic alterations, affecting either protein expression or its structure, might lead to the constitutive activation of oncogene precursors (proto-oncogenes). Mutations in src, jun, Hras, Kras have been detected in many cancers and myc gene alterations have been found in myeloid leukemia. Although a single genetic change can influence normal cell functions like proliferation, growth and differentiation, it is not sufficient to give rise to cancer. Cancers have accumulated several genetic changes that act in a synergistic manner and lead to acquisition of characteristics known as hallmarks of cancer.

1.2. Ras signaling pathway

Ras is a signaling protein that plays a crucial role in the control of normal and transformed cell growth (Figure 1). It is a master regulator of many important cell functions – proliferation, migration, differentiation, transcription, membrane trafficking and endocytosis. There are three Ras proteins: Hras, Kras, and Nras[1]. In the inactive state, Ras protein is tethered to the plasma membrane and bound to guanosine diphosphate (GDP). Signaling via receptor tyrosine kinase (RTK) leads to conversion of Ras from the inactive GDP bound state to an active GTP bound state. The process is regulated by the action of two accessory proteins – GEFs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins). Ras proteins activate a plethora of different downstream effector

signaling pathways including MAPK, PI3K and RAL. Furthermore, Ras proteins are implicated in the regulation of E cadherin/β catenin signaling. Thus, Ras signaling is not only implicated in tumor initiation but also in invasiveness and metastasis[2]. A single point mutation at amino acid position 12, 13 or 61 can lead to constitutive activation of Ras[3]. Approximately 20% of human tumors carry such point mutations which lead to amino acid substitutions that stabilize the activated Ras-GTP complex.

1.3. The concept of the Multi-Hit mouse model

Cell growth and division are a result of signal transduction from the plasma membrane to the nucleus after mitogen exposure. The process involves coordinated actions of distinct signaling proteins that belong to different signaling cascades and their crosstalk with each other.
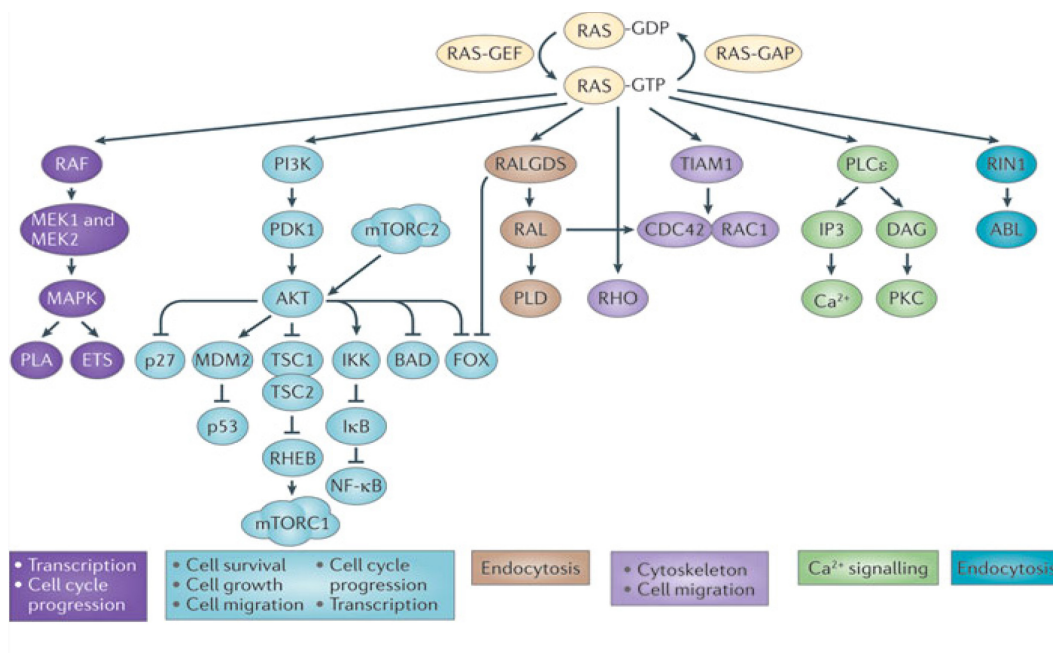
Figure 1: Ras signaling pathway. The figure illustrates the main downstream targets of Ras and their roles in cellular processes[4].

We have developed a Multi-Hit model[5] that enabled us to investigate the cooperativity between different signaling pathways in cancer formation (Figure 2).
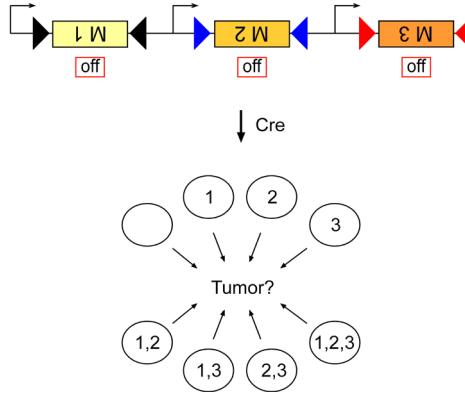
Figure 2: Principle of the Multi-Hit tumor mouse model. Scheme of a transgenic Multi-Hit construct for three hits (upper drawing). The hits represent expression modules for oncogenes (M1,2,3). The modules are cloned in inverted (*off*) orientations behind a promoter (arrow). Each module is flanked by loxP sites (triangles) that allow Cre-mediated activation (flipping). Incompatible, heterotypic loxP sites (black = canonical loxP; blue = lox5171; red = lox2272) are used to avoid deletions. Upon transient induction of Cre, modules flip until Cre activity terminates which leaves them either in *on-* or in *off*-orientations. Therefore, Cre induction in a Multi-Hit mouse results in mosaic activation of expression modules creating 8 genotypically different cell types (lower drawing). Combinations of synergistic hits are positively selected and result in tumors. Sheme adapted from *Musteanu, M. et al., 2012.*

The original BAC transgene used for generation of Multi-Hit mice contained three Ras mutants for selective activation of downstream effector pathways. The corresponding mice were called Ras Effector (RasE) Multi-Hit mice. For selective activation of the MAPK pathway, the Hras^{V12S35} allele was inserted into the first expression module. The Hras^{V12G37} allele was used in M2 for the constitutive activation of the RalGEF signaling pathway. The Hras^{V12C40} allele was used in M3 for the constitutive activation of the PI3K signaling pathway.

EYFP, dsRed, hCD2t reporter genes were integrated into the expression modules M1, M2 and M3, respectively, to monitor stochastic activation of H*ras* mutant genes in Multi-Hit mice. Each reporter was inserted after a 5xGtx IRES sequence (Figure 3).
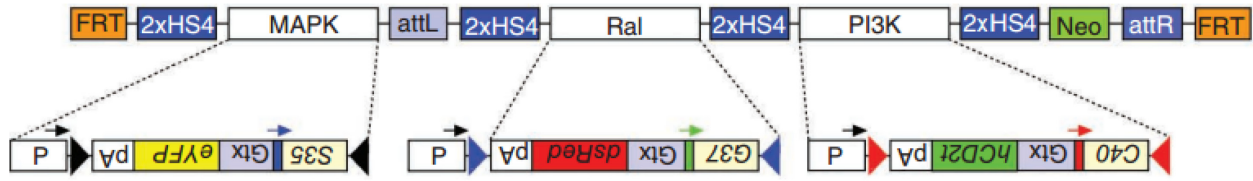
Figure 3: Components of the RasE Multi-Hit construct. The scheme shows the RasE Multi-Hit BAC construct. Each expression module was cloned in *off*-orientation and is flanked by heterotypic loxP sites (triangles). A CAGGS promoter is localized at the beginning of each expression module. The genes of interest (Hras^{V12G35}, Hras^{V12G37}, Hras^{V12C40}) are separated by an internal ribosome entry site (Gtx) from reporter genes EYFP, dsRed and hCD2t. The polyadenylation signal at the end of each module is marked as pA. attL-attR sites represent sequences that are recognized by PhiC31 recombinase and have been used for BAC assembly. The expression modules are separated from each other by chicken 2xHS4 insulators which prevent promoter interference. The whole BAC is flanked by FRT recombination sites which allows FLP-mediated deletion in vitro or in vivo.

It was demonstrated that interference between several promoters in an expression vector can impede gene expression[6,7]. Therefore, expression modules of the Multi-Hit construct were separated by insulator sequences. We utilized 2xHS4 insulators, which are derived from the 5′-end of chicken β-globin locus. Furthermore, the insulators prevent silencing of transgenes by chromosomal position effects.

In order to induce stochastic activation of MAPK, PI3K and RalGEF, the Multi-Hit mice were intercrossed with *Rosa26CreER*^{T2} mice. Corresponding *RasE*^{CreER} mice were treated with tamoxifen for transient activation of Cre recombinase. As anticipated, formation of Ras induced tumors was observed in all *RasE*^{CreER} mice[5]. However, expression of the reporter genes EYFP, dsRed and hCD2t was not detected. We argue that the 5xGtx IRES is not able to provide efficient re-initiation of translation leading to undetectable amounts of reporter proteins. To overcome these difficulties, we decided to replace IRES for viral P2A sequences. Moreover, we decided to replace the original reporter genes for memEYFP, mCherry and nucECFP. These fluorescent proteins are targeted to different cellular compartments which facilitates analysis of reporter gene activity indicative for stochastic activation of expression modules. The reviewers of our original manuscript criticized the use of the artificial Hras mutants. Therefore we decided to replace them for specific

activators of MAPK, PI3K and RalGEF signaling. The new sequence elements, genes of interest (GOIs) and reporter genes are described in the following sections in more detail.

1.3.1. Characteristic of the P2A sequence

Recently, several viral sequences P2A, E2A, T2A and F2A were discovered and investigated for their use in bicistronic expression systems. Contrary to IRES sequences, these sequences have a small size and lead to stochiometric production of both proteins in the bicistronic system. Their function within recombinant vectors is based on the "ribosome skipping" effect; i.e. the inability of the ribosome to form a bond between the last two amino acids at the end of the viral sequence, which leads to the synthesis of two polypeptides from a single reading frame.

It has been demonstrated that the most efficient viral sequence is P2A (*5'-ggaagcggagctactaacttcagcctgctgaagcaggctggagacgtggaggagaaccctggacct-3'*)[8,9,10]. Therefore, we used it for improvement of the Multi-Hit vector system.

1.3.2. Reporter genes – memEYFP, mCherry, nucECFP

Exposure of these proteins to UV light leads to emission of light with wave lengths ranging from 477 nm (nucECFP), 527 nm (memEYFP) and 610 nm (mCherry), which allows the simultaneous detection of all three colors within a cell with fluorescence microscopy or FACS (Figure 4). Furthermore, the reporter genes are genetically modified in a way, which ensures their different localization in the cell. Subsequent to the post-translational modifications memEYFP will be bound to the plasma membrane, mCherry will be localized in the cytosol and nucECFP contains a nuclear localization signal (NLS) at the C terminus for import into the nucleus.
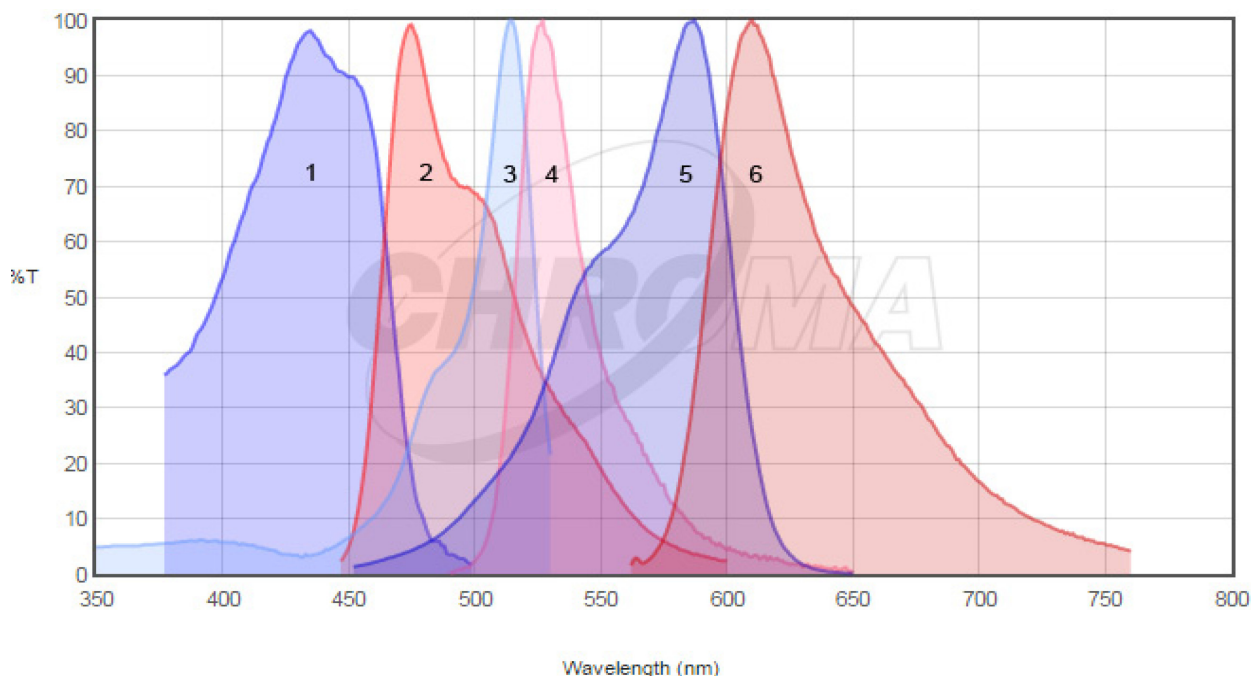
Figure 4: Spectral characteristics of memEYFP, nucECFP and mCherry. The differences in the excitation and emission peaks make the simultaneous observation of three colors (cyan, green and red) in cotransfected cells possible. 1: nucECFP excitation spectrum; 2: nucECFP emission spectrum; 3: memEYFP excitation spectrum; 4: memEYFP emission spectrum; 5: mCherry excitation spectrum; 6: mCherry emission spectrum. *(Modified picture by Chroma Thechnology Corp Website).*

1.3.3. Downstream effectors of Ras – C-RafCAAX, p110myr/H1047R, RalG23V

We exchanged the candidate genes, used in the original *RasE* Multi-Hit BAC (Hras[V12S35], Hras[V12G37] and Hras[V12C40]), for three genetically modified downstream effectors of Ras: C-RafCAAX, p110myr/H1047R and RalA[G23V].

C-RafCAAX

(1)   Characteristics of Raf proteins

Three Raf protein isoforms - A-Raf, B-Raf, C-Raf (Raf-1) – exist in mammals[11, 12]. They exert different non-overlapping functions, which are essential for embryonic development and survival[13]. Raf isoforms are not equally sensitive to activation by Ras[3]. C-Raf can bind only to farnesylated Hras, whereas B-Raf associates with considerable higher affinity with both, farnesylated (membrane-bound) and non-farnesylated (cytosolic) Hras. B-Raf is the most frequently mutated Raf isoform in human tumors. For example, the mutation rate of

B-Raf in human melanomas is 43%. In contrast, genetic alterations of the C-Raf gene are observed in only 1% of melanomas. However, constitutive C-Raf signaling contributes significantly to formation of many cancers. The Raf/MEK/ERK signaling cascade exerts an essential role in distinct physiological processes such as proliferation, cell cycle progression, cell death and neurological function. It has been reported, that its isoforms contribute to almost all biological capabilities, acquired from the cells during the multi-step process of tumorigenesis[11].

All the proteins from the Raf/MEK/ERK exert their functions upon phosphorylation. Raf phosphorylates substrate proteins on serine/threonine residues. By attaching phosphate groups to MEK, Raf induces a conformational change in this "dual-specificity-kinase" and contributes to its activation. MEK phosphorylates ERK proteins at serine/threonine and tyrosine residues. Phosphorylated ERK regulates many transcription factors such as Mnk1, RSK and TCFs.

Regulation of the Raf/MEK/ERK signaling cascade

The Raf/MEK/ERK signaling cascade is tightly controlled due to its important role in oncogenesis. Negative regulation is performed by a negative feedback loop through ERK, which inactivates Raf by phosphorylation of five proline residues. Hyperphosphorylation of Raf prevents binding to Ras[14]. Negative regulation is also achieved by inactivating phosphorylations of SOS and Mek1[15].

Another mode of negative regulation is based on the interaction between Raf and the inhibitor protein RKIP (Raf kinase inhibitory protein). RKIP prevents interaction between Raf and MEK. Phosphorylation of RKIP by protein kinase C results in dissociation from Raf[11]. Mitogenic stimulation of cells leads to a conformational change of inactive Raf proteins. Ras activation, preceded by replacement of GDP by GTP, results in dissociation of 14-3-3, dephosphorylation of S259 and S621 and additional phosphorylation on S338, Y340 and Y341. Due to these modifications the protein can bind to the plasma membrane, where it fulfills its signaling functions. Raf proteins can form hetero- and homodimers and diverse dimerization combinations were observed in cells. In these structures the proteins

possess catalytic activity when they are orientated in a way, which allows the formation of a catalytic cleft between their N-terminal regulatory and C-terminal kinase[11, 16, 17, 18].

(2) C-Raf active mutant: C-RafCAAX

The most common mutation in C-Raf, resulting in constitutive activation, is deletion of the N-terminal regulatory domain[12, 13, 17]. Artificial induction of constitutive activity can be achieved by an artificial CAAX sequence. We used a modified human C-Raf sequence with a CAAX myristoylation motif at the C terminal end (C-RafCAAX). Post-translational modification of C-RafCAAX leads to myristoylation, membrane localization and constitutive activation[19].

p110myr/H1047R

(1) Characteristics of Phosphatidylinositol 3-kinase

The PI3K pathway regulates cell growth, proliferation, survival, migration and metabolism. Hence, the proteins from the PI3K family are considered as oncogenes. Phosphatidylinositol 3-kinase (PI3K) is a dimeric enzyme composed of a catalytic and a regulatory subunit[20, 21]. The proteins comprising this family can be separated into three groups – Class I, Class II and Class III enzymes. It has been shown that only kinases belonging to the first class play a role in tumorigenesis. Class I enzymes can be further subdivided in two subclasses - Class IA and Class IB. Class IA proteins consist of three different catalytic subunits with the same size of 110 kDa (p110α, p110β and p110γ). They can form heterodimers with the regulatory proteins p85α, its alternative spliced isoforms, p55α or p50α, p85β and p55γ. Class IB proteins comprise p110δ and p87[22, 23].

(2) Localization of PI3K

PI3K can be detected in the cytosol of resting cells. Upon binding of ligands to RTKs and G protein-coupled receptors (GPCRs), PI3Ks get activated and bind to the membrane. Moreover, PI3Ks might be bound to intracellular membrane vesicles in fibroblasts, endothelial cells and adipocytes, where they fulfill the same functions as in the cytosol[24]. Nevertheless, PI3Ks were found in the nucleus. It is believed that they are important for the neuronal differentiation and growth arrest and also play a role in the transcription and RNA metabolism[25].

(3) PI3K; Functions and regulation

PI3Ks catalyze the formation of phosphatidylinositol phosphates (PIP$_s$) which are important intracellular messengers. The most important PIP is phosphatidylinositol triphosphate (PIP$_3$)[1].
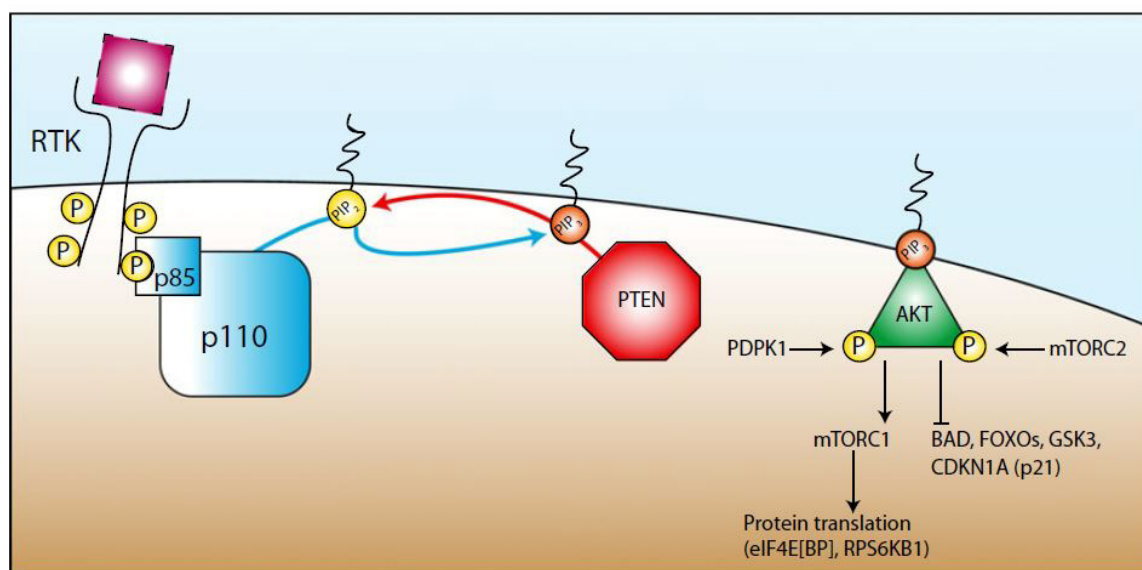
Figure 5: The PI3K/Akt signaling pathway. Activation of RTKs results in association of the regulatory subunit p85 to its cytosolic p110 domain. Interaction between p85 and p110 leads to activation of PI3K, which in turn adds phosphate groups at 3'position to PtdIns (4,5)P$_2$. Signal transmission to mTORC1 is important for protein translation. Association of membrane bound Akt to its downstream effectors BAD, FOXO and GSK3 results in regulation of the apoptosis, cell cycle progression, transcription, inflammation, migration and glucose metabolism[21, 26, 27].

The amount of PIP$_3$ is low in quiescent cells but binding of growth factors (GFs) to RTKs results in a significant increase in its levels. Interaction of PIP$_3$ with the downstream effector kinase Akt leads to its recruitment to the plasma membrane and activation. The signal is further transmitted to mTORC1, which controls translation of elF4E[BP] and ribosomal protein S6 kinase. Thus, activation of PI3K/Akt signaling by GFs stimulates protein synthesis and proliferation. The most important negative regulator of PI3K/Akt signaling is the lipid phosphatase PTEN. It converts phosphatidylinositol 3,4-biphosphate (PtdIns (3,4)P$_2$) to phosphatidylinositol 4-phosphate (PtdIns (4)P) and phosphatidylinositol 3,4,5-triphosphate (PtdIns (3,4,5)P$_3$) to phosphatidylinositol 4,5-biphosphate (PtdIns

(4,5)P_2). Due to its capability to inhibit signal transmission from PI3K to Akt, PTEN is regarded as a tumor suppressor. Inactivating mutations of PTEN are often observed in cancer cells[21, 25] (Figure 5).

(4) Active mutant of p110α: p110myr/H1047R

The p110α protein is encoded by the *PIK3CA* gene. As shown in Figure 6, it comprises five domains: (1.) an N-terminal domain that contributes to the bond formation between the regulatory subunit p85 and the catalytic subunit (ABD); (2.) a Ras-binding domain (RBD); (3.) a C2 domain (protein-kinase-C homology domain) that promotes the association of p110α to the plasma membrane; (4.) a helical domain; (5.) a C-terminal kinase domain.

Almost all cancer driver mutations detected in p110α are amino acid substitutions. In approximately 80% of all tumors with genetically altered p110α one of three hot spot mutations is present (marked on Figure 6). These mutations are associated with gain of p110α function. E542K and E545K substitutions can be detected in the helical domain. They have a positive effect on the p85-p110α interaction. The H1047R mutation is observed in the kinase domain of p110α. Cells carrying such genetic alterations can be characterized with constitutive PI3K signalization and increased levels of phosphorylated Akt. Furthermore, this mutation changes the activation loop conformation which affects the interaction of p110α with its substrate. It has been suggested that the unbound p110α, carrying this genetic alteration, has the same loop conformation as the non-mutated protein in the Ras-p110α interaction[28]. Consequently, the mutated p110α acquires Ras independence. Transmission of a signal occurs independently of the interaction with Ras and the presence of extracellular mitogens. One mutation in the *PIK3CA* is sufficient for cancer development. However, tumors with concomitant mutations in E545K and H1047R have also been observed in humans.
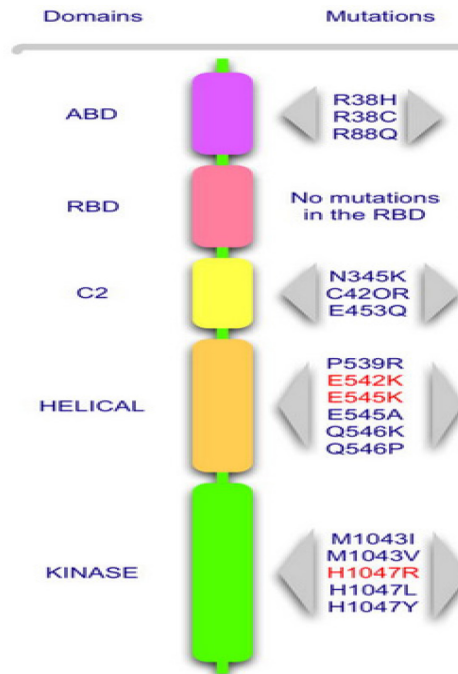
Figure 6: The most commonly occurring mutations in the *PIK3CA* gene encoding p110α. The scheme represents the domain localization of the most frequent genetic alterations in *PIK3CA*. 80% of all cancers, carrying mutation in p110α, contain one of the red labeled amino acid substitutions[28].

RalA^G23V

(1) Characteristics of Ral proteins

Ral signaling is crucial for endocytosis, exocytosis, filopodia formation and oncogenic transformation[29]. It has been recently shown that Ral, as a downstream effector of Ras, plays a pivotal role in Ras-mediated transformation of human cells. Ral proteins, encoded by the *rala* and *ralb* genes, belong to the family of small GTB-binding proteins. The highly homologous proteins RalA and RalB differ from each other by a 30 amino acid hypervariable region localized at the C terminus. The post-translational modifications in this sequence, triggered by aurora A kinase, determine the localization of these proteins[30, 31]. Mutations in RalA and RalB are rarely found in tumors but they are expressed at high levels. Cre recombinase – mediated inactivation of both genes in Ras-associated non-small cell lung carcinoma (NSCLC) prevented tumor formation[32]. Additionally, inactivation of RalB is associated with the inability of transformed cells to form metastasis.

(2) Activation of Ral proteins

Activation of Rals is achieved by six guanine nucleotide exchange factors (RalGEFs) - RalGDS, RGL, RGL2/Rlf, RGL3, RalGPS1 and RalGPS2. They promote release of GDP and binding of GTP which activates Ral. RalA and RalB inactivation is accomplished by two GTPase activating proteins - RalGAP1 and RalGAP2[31, 33]. They promote GTP hydrolysis and convert GTP-Ral into inactive GDP-Ral.

Ral is regulated by Ras-dependent and Ras-independent mechanisms (Figure 7). Stimulation of Ras signaling by binding of extracellular ligands to RTKs and GPCRs results in formation of GTP-bound Ras. The active RasGTP recruits RalGDS to the plasma membrane, where it could interact with the membrane-bound Ral protein. Furthermore, a positive effect on the RalGDP catalytic activity has also the PI3K signaling pathway. Ras independent activation depends on the interaction of the RalGDS proteins with β-arrestin[34].

(3) Ral proteins – role in the cell signaling

RalA and RalB are involved in the regulation of several transcription factors – c-Jun, ATF2, STAT3, NFAT and AFX[35]. Ral proteins are also capable of regulating CD24 expression, which is a metastasis driver. This emphasizes the role of RalA in regulation of cell morphology, cytoskeleton assembly, cell growth, anchorage independent proliferation and survival[33, 34].
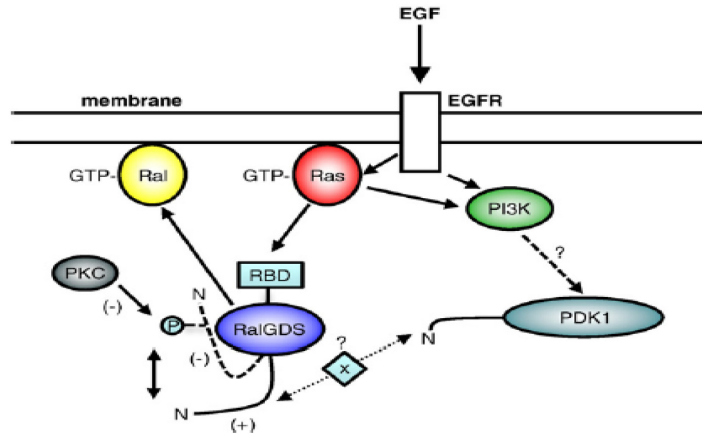
Figure 7: Regulation of RalGDS activity in epidermal growth factor (EGF) stimulated cells. Ral function depends on the activity of its upstream effector RalGDS. RalGDS stimulation is preceded by either binding to RasGTP or PDK1[34].

Ral proteins exert their diverse functions in normal and tumorigenic cells through several downstream effectors (Ral Binding Protein 1 (RalBP1), Sec5, Exo84, filamin, transcription factor ZONAB, phospholipase D and phospholipase C delta).

The binding to RalBP1 determines the potential of RalA and RalB proteins to impact actin dynamics, formation of filopodia and membrane refilling. Additionally, RalBP1 also controls receptor-mediated endocytosis.

Sec5 and Exo84 are subunits of the octameric exocyst complex. Association of Rals with these proteins determines their role in the formation of vesicles, polarized membrane trafficking, cytokinesis, tight junction formation, glucose metabolism and tumor cell invasion[29].

To investigate this cooperation between RalA and other downstream effectors of Ras within tumor cells we used a RalA sequence with a single amino acid substitution - G23V (RalA^{G23V}). It has been demonstrated that proteins, harboring this gene alteration, are constitutively active[36, 37].

2. Aims of the project

The aim of this master thesis was the technological improvement as well as the *in vitro* testing of the Multi-Hit vector system that was recently generated[5].

In this study, the 5xGtx IRES, incorporated into the original Multi-Hit construct, was exchanged by a self-cleaving viral P2A sequence. Furthermore, the original reporter genes (EYFP, dsRed, hCD2t) were replaced by memEYFP, nucECFP and mCherry. Instead of mutated Hras genes we used three modified downstream effectors of Ras - C-RafCAAX, $RalA^{G23V}$ and p110myr/H1047R.

At the beginning of this study we defined the following three aims:

- Assembly of the three improved expression modules in inverted (*off)* orientation.
- Inversion of the expression modules via transformation of *E.coli* cells harboring a temperature sensitive Cre mutant.
- Testing of the improved Multi-Hit Vectors *in vitro*: transfection of mammalian cells and evaluation of the P2A activity by Fluorescence microscopy, Western blot and FACS analysis.

3. Materials
3.1. Solutions

Name	Composition
LB Broth	1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, Sterilization by autoclave; Stored at RT
LB Agar	1.5% Agar, 1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl; Sterilization by autoclave
Ampicillin 100 mg/ml stock (working concentration 100 µg/ml)	1 g ampicillin dissolved in 10 ml ddH_2O; Filter sterilization; Stored at -20 °C
TFBI	30 mM potasium acetate, 10 mM CaCl2, 50 mM MnCl, 100 mM RbCl, 15% Glycerol pH=5.8; Filter sterilization; Stored at RT
TFBII	10 mM MOPS or PIPES (pH=6.5), 75 mM CaCl2, 10 mM RbCl, 15% Glycerol; pH=6.5; Filter sterilization; Stored at RT
100 mM $CaCl_2$ + 15% Glycerin / 1000 ml	$CaCl_2 \cdot 2XH_2O$ -14.7 g, 150 ml Glycerin, ddH_2O to 1L; Sterilization by autoclave; Stored at RT
10xPBS- 1000 ml	81.8 g NaCl (MW=58.44), 2 g KCl (MW=74.56), 2 g KH_2PO_4 (MW=136.09), 20.1 g $Na_2HPO_4 \cdot 12 H_2O$ (MW=177.99), 1.5 g $NaH_2PO_4 \cdot H_2O$ (MW=137.99), dd H_2O to 1L; Stored at RT
3 M Sodium Acetate pH=5.2 -100 ml	40.8 g Sodium acetate trihydrate dissolved in 100ml ddH_2O; pH=5.2; Sterilization by autoclave; Stored at RT
Laemmli Sample Buffer 2x	4% SDS, 10% 2-β-mercaptoehtanol, 20% Glycerol, 0.004% Bromphenolblau, 0.125 M Tris HCl; pH=6.8; Stored at – 20 °C
10xTBST – 1000 ml	100 ml 1M Tris.HCl pH=8.0, 300 ml 5 M NaCl, 10 g Tween 20 add 1%, ddH_2O to 1 L; Stored at + 4 °C
2 M $CaCl_2$ (20 ml)	5.88 g $CaCl_2$, 20 ml H_2O; Filter-sterilization; Stored at – 20 °C
100 mM Chloroquine (10 ml)	0.516 g Chloroquine diphosphate, 10 ml H_2O; Filter-sterilization; Stored at + 4 °C
2xHBS (1000 ml)	280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, ddH_2O to 1L; pH=7.0; Stored at + 4 °C
Ponceau S	0.2% Ponceau S in 3% TCA; Stored at RT

6xDNA-Loading dye (6xLD)	40% Sucrose (4 g), 0.25% Bromphenolblau (25 mg); ddH$_2$O to 10 ml; Stored at + 4 °C
10xRunning Buffer	144 g Glycine, 30 g Tris Base, 50 ml 20% SDS, ddH$_2$O to 1 Liter; Stored at RT
10xTransfer Buffer	140 g Glycine, 30 g Tris Base, ddH$_2$O to 1 Liter; Stored at RT
RIPA Buffer (Radio Immunoprecipitation Assay buffer)	150 mM Sodium Chloride, 1.0% NP-40 or Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH=7.6, 1 mM EDTA; Stored at +4 °C
Protein Isolation (PI) Buffer	25 mM HEPES pH=7.5, 0.1% Tween 20, 0.5% NP-40, 150 mM NaCl , 10 mM EDTA, 10 mM β-Glycerophosphate; Stored at + 4 °C
0.5 M EDTA	0.5 M Na-EDTA (M=372.2), pH=8.0 adjusted with 5 M NaOH; Stored at + 4 ˚C
10xTBE Stock Solution	890 mM Tris base, 890 mM Boric acid, 20 mM EDTA; Stored at + 4 ˚C
50xTAE Buffer	2 M Tris base (M = 121.14), 1 M Acetic acid, 50 mM Na-EDTA pH=8.0, ddH$_2$O; Stored at + 4 °C

3.2. Primers

Primer Name	Primer sequence
P2A_M1_for	5'-cgcgccggaagcggagctactaacttcagcctgctgaagcaggctggagacgtggaggagaaccctggacctcc-3'
P2A_M1_rev	5'-ttaggaggtccagggttctcctccacgtctccagcctgcttcagcaggctgaagttagtagctccgcttccgg-3'
P2A_M2_for	5'-cgcggaagcggagctactaacttcagcctgctgaagcaggctggagacgtggaggagaaccctggacctcc-3'
P2A_M2_rev	5'-ttaggaggtccagggttctcctccacgtctccagcctgcttcagcaggctgaagttagtagctccgcttccgcgat-3'
P2A_M3_for	5'-ccggaagcggagctactaacttcagcctgctgaagcaggctggagacgtggaggagaaccctggacctcc-3'
P2A_M3_rev	5'-ttaggaggtccagggttctcctccacgtctccagcctgcttcagcaggctgaagttagtagctccgcttccggccgg-3'
Sequencing primers	
p110alpha For	5'-GTGGCCTCACGGAGGCATTC-3'
p110alpha Rev	5'-GACAAAACTGAGCAAGAAGC-3'
Raf-1 For	5'-CCCGGCGCTGATAGCCAAAC-3'
Raf-1 Rev	5'-TGCTTCAGCACTCTCTGCCG-3'
RalA For	5'-CTGTCCGCTTTGGTAGGTTC-3'
RalA Rev	5'-GCGCGCCAACGTTGACAAGG-3'
Primers for PCR cloning	
RafCAAX_for	5'-AGCAGACCTAAGGACATGGAGCACATACAGGGAGC-3'
RafCAAX_rev	5'-AGCAGACTCGAGTTACATGATCACGCACTTTG-3'
memEYFP_for	5'-AGCAGAGGCGCGCCGACTCACAACCCCAGAAACACCACCATGCTGTGCTGTATGAGAAG-3'
memEYFP_rev	5'-AGCAGAGGCGCGCCACTTGTACAGCTCGTCCATGC-3'
p110myr_for	5'-AGCAGAGCGATCGCGACTCACAACCCCAGAAACACCACCATGGGGAGCAGCAAGAGCAA-3'
p110myr_rev	5'-AGCAGAGCGATCGCAGTTCAATGCATGCTGTTTA-3'
mCherry_for	5'-AGCAGACCTAAGGACATGGTGAGCAAGGGCGAGGA-3'
mCherry_rev	5'-AGCAGACTCGAGTTACTTGTACAGCTCGTCCA -3'
RalA-G23V_for	5'-AGCAGAGGCCGGCCGACTCACAACCCCAGAAACACCACCATGGCTGCAAATAAGCCCAA-3'
RalA-G23V_rev	5'-AGCAGAGGCCGGCCATAAAATGCAGCATCTTTCT-3'
nucECFP_for	5'-AGCAGACCTAAGGA**C**ATGGTGAGCAAGGGCGAGGA-3'
nucECFP_rev	5'-AGCAGAACCTGGTGATACATTGATGAGTTTGGA-3'
mCherry; sequencing primers (Vector pL1_M2_PmelΔ_off_p110myr_P2A_mCherry)	
PL1M2_PmelΔ_P2A_mCherry_1 For	5'-TGTTGACGTTGTAGGCGCCG-3'
PL1M2_PmelΔ_P2A_mCherry_2 For	5'-CGGGGTGCTTCACGTAGGCC-3'
PL1M2_PmelΔ_P2A_mCherry Rev	5'-CCTCCTCCGAGCGGATGTAC-3'
memEYFP; sequencing primers (Vector pL1_M1_off_memEYFP_P2A_RafCAXX)	
MemEYFP For1	5'-TCGTTGGGGTCTTTGCTCAG-3'
MemEYFP For2	5'-CCGTAGGTGGCATCGCCCTC-3'
MemEYFP Rev	5'-GGACGGCAACATCCTGGGGC-3'
C-RafCAAX; sequencing primers (Vector pL1_M1_off_memEYFP_P2A_RafCAXX)	
PL1 M1_P2A RafCAAX For1	5'-GTAGAGAGTGTTGGAGCAGC-3'
PL1 M1_P2A RafCAAX For2	5'-AGCGTGACTTTACTGTTGCC-3'

PL1 M1_P2A RafCAAX For3	5'-AGAGCCTGACCCAATCCGAG-3'
PL1 M1_P2A RafCAAX For4	5'-GTCAAAGAAGGTAGTGCTGG-3'
PL1 M1_P2A RafCAAX Rev	5'-CATCTTCATGGTGGGCCGAG-3'
RalA^{G23V}; sequencing primers (Vector pBS_M3_off_RalA^{G23V}_P2A_nucECFP)	
Ral A Rev	5'-GGGCAGGAGGACTATGCTGC-3'
Ral A For	5'-AGAAGACACAGAGGAAGCCC-3'
Ral A For2	5'-CATTCCACTGGTCAGCTCTG-3'
nucECFP; sequencing primers (Vector pBS_M3_off_RalA^{G23V}_P2A_nucECFP)	
NucECFP For1	5'-CGAAGCTTGAGCTCGAGATC-3'
NucECFP2 For1	5'-CTGAGTCCGGACTTGTACAG-3'
NucECFP For2	5'-CATGTGGTCGGGGTAGCGGC-3'
NucECFP Rev	5'-CAACATCGAGGACGGCAGCG-3'
p110myr/H1047R; sequencing primers (Vector pL1_M2_PmelΔ_off_p110myr_P2A_mCherry)	
P110 alpha myr For1	5'-TGGGCTCCTTTACTAATCAC-3'
P110 alpha myr For2	5'-AGTGTTAGCATATCTTGCCG-3'
P110 alpha myr For3	5'-CCCACATGCACGACAATAGG-3'
P110 alpha myr For4	5'-AGAGAGAGGATCTCGTGTAG-3'
P110 alpha myr For5	5'-CCACCTGGGATTGGAACAAG-3'
P110 alpha myr For6	5'-GTTCTGGTACACAGTCATGG-3'
P110 alpha myr For7	5'-GAAGAAGTTGATGGAGGGGG-3'
P110 alpha myr Rev	5'-TGTGCTGGATACTGTGTAGC-3'
Cre1	5'-CGGTCGATGCAACGAGTGATGAGG-3'
Cre2	5'-CCAGAGACGGAAATCCATCGCTCG-3'
Jun1_lox5	5'-CTCATACCAGTTCGCACAGGCGGC-3'
Jun2_lox6	5'-CCGCTAGCACTCACGTTGGTAGGC-3'
RALA_REV	5'-ACAACTACTTCCGAAGCGGG-3'
CAGGS For1	5'-TCCCAAATCTGGCGGAGCCG-3'

3.3. Restriction enzymes

Enzyme	Specificity [1] 5´ → 3´	Reaction Temperature °C	Buffer (B)	Firma	Catalog #
AscI	5´-GG/CGCGCC-3´	37°C	10xCut Smart B	NEB	R0558S
BstEII	5´-G/GTNACC-3´	60 °C	NEB 3.1 Buffer	NEB	R0162S
Bsu36I	5´-CC/TNAGG-3´	37 °C	10xCut Smart B	NEB	R0524S
DraI	5´-TTT/AAA-3´	37 °C	10xCut Smart B	Thermo scientific	ER0221
EcoRI	5´- G/AATTC-3´	37 °C	NEB 2.1 Buffer	Thermo scientific	ER0271
FseI	5´-GGCCGG/CC-3´	37 °C	10xCut Smart B	NEB	R0588S
HindIII	5´-A/AGCTT-3´	37 °C	10xBuffer R	Thermo scientific	FD0504
PmeI	5´-GTTT/AAAC-3´	37 °C	10xCut Smart B	Thermo scientific	ER1341
SacII	5´-CCGC/GG-3´	37 °C	10xCut Smart B	NEB	R0157S
SexAI	5´-A/CC(A/T)GGT-3´	37 °C	10xCut Smart B	NEB	R0605S
SfiI	5´-GGCCNNNN/NGGCC-3´	50 °C	10xCut Smart B	Thermo scientific	ER1821
SgfI	5´-GCGAT/CGC-3´	37 °C	B. C Promega	Promega	R7103
SrfI	5´-GCCC/GGGC-3´	37 °C	10xUniversal B	Stratagene	501064
XbaI	5´-T/CTAGA-3´	37 °C	B. D Promega	Promega	R6181
XhoI	5´-C/TCGAG-3´	37 °C	10xCut Smart B	NEB	R0146S
XmaI (Crf9I)	5´-C/CCGGG-3´	37 °C	10xCrf9I Buffer	Fermentas	ER0171

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[1] **A** - Adenine; **T**- Thymine; **C** - Cyosine; **G** - Guanine; **N** - any nucleotide

3.4. Bacteria

Bacterial Strain	Specifications	Company
DH5α	F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ–	Laboratory stock
DH10β	Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (Str^R) rph spoT1 Δ(mrr-hsdRMS-mcrBC)	Kindly provided by Katalin Zboray
Stbl2	F- endA1 glnV44 thi-1 recA1 gyrA96 relA1 Δ(lac-proAB) mcrA Δ(mcrBC-hsdRMS-mrr) λ$^-$	Laboratory stock
MAX Efficiency® Stbl2™ Competent Cells	F- endA1 glnV44 thi-1 recA1 gyrA96 relA1 Δ(lac-proAB) mcrA Δ(mcrBC-hsdRMS-mrr) λ$^-$	Life technologies #10268-019
CreLoxP	Cells expressing temperature sensitive CreLoxP mutant	Laboratory stock
dcm-	Grow plasmids free of dcm and dam methylation ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10) Tet^{S}endA1 rspL136 (Str^R) dam13::Tn9 (Cam^R) xylA-5 mtl-1 thi-1 mcrB1 hsdR2	Laboratory stock

3.5. Mammalian cell lines

Name	Specifications	Company
HEK293T	Human embryonic kidney cell line	Laboratory stock
NIH3T3	Mouse embryonic fibroblast cell line	Laboratory stock
Jurkat cells	Immortalized line of human T lymphocyte cells	Kindly provided by Maria Eisenbauer

3.6. Vectors/Plasmids

Name	Specifications
pCRII	Laboratory Stock
pBS KS+/-	Laboratory Stock
pmCherry	Kindly provided by D. Stoiber
pMembranes-EYFP	Kindly provided by H. Schmid
pNucleus-ECFP	Kindly provided by H. Schmid
pBABEpuro p110alpha-myr/H1047R	Kindly provided by Mariano Barbacid
pLPC RalA^{G23V}	Kindly provided by Mariano Barbacid
pBABEpuro c-RafCAAX	Kindly provided by Mariano Barbacid

3.7. Loading dyes, DNA and protein markers

Name	Company
GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bps	Thermo scientific # SM0322
GeneRuler 1 kb DNA Ladder 250 to 10.000 bps	Thermo scientific # SM0311
Lambda DNA/EcoRI+HindIII Marker	Thermo scientific # SM0193
Precision Plus Protein™ Unstained Standards	Bio Rad # 161-0363
Precision Plus Protein™ Dual Color Standards	Bio Rad # 161-0374
PageRuler Prestained Protein Ladder	Fermentas # 26616
PageRuler™ Prestained Protein Ladder	Fermentas # SM0671

3.8. Antibodies
Primary antibodies

Name	Concentration	Source	Company
Anti-Raf1	1:500	rabbit	abcam (ab32025)
Anti-Raf1	1:500/1:1000	rabbit	Santa Cruz (sc-133)
PI3 Kinase p110α	1:1000	rabbit	Cell Signaling (4255S)
PI 3-kinase p110 (H-239)	1:200/1:1000	rabbit	Santa Cruz (sc-7189)
Anti-mCherry	1:2000	mouse	abcam (ab125096)
Anti-GFP	1:1000	rabbit	abcam (ab6556)
Anti-RalA [EPR6468]	1:5000	rabbit	Abcam (ab126627)

Secondary antibodies

Name	Concentration	Source	Company
IRDye® 800CW	1:15000	Goat anti-rabbit	Li COR (925-32211)
Anti- β-Actin	1:5000	mouse	Sigma (A5316)

horseradish peroxidase (HRP)-conjugated	1:5000	mouse	GE Healthcare Life Sciences
HRP-conjugated	1:5000	rabbit	GE Healthcare Life Sciences

3.9. Equipment

Thermal cycler (BIO-RAD T100 Thermal Cycler); Fluorescence microscope Nikon Ti Eclipse Inverted; CASY® Cell Counter (Scharfe System, Reutlingen); Odyssay Clx Infrared imaging system; Apelex PS304II minipac; Severin 900 & Grill mikrowelle; Pharmacia Electrophoresis Power Supply EPS 500/400; Bio-Rad PowerPac 3000; Eppendorf thermomixer comfort BioShake IQ; VWR MinisStar silverline; Lab balance - Acculab Sartorius Group Atilon; Mettler AE163 balance; Magnetic stirrer - IKAMAG RH basic; HERAEUS Pico 17 Micro centrifuge-Thermo scientific; GelDocTM XR image system – Bio-Rad; GFL 3031 Shaking Incubator; Amersham Biosciences GeneQuant pro RNA/DNA Calculator Spectrophotometer; NanoDrop 1000 Spectrophotometer; Thermo Scientific Holten Horizontal Laminar Airflow ; Thermo Scientific Heracell 150i CO2 Incubator; Nikon inverted microscope ECLIPSE TS100; Heraeus™ Labofuge™ 400R Centrifuge - Thermo Scientific; Infinite® M1000 PRO - Tecan –plate reader; Thermo Scientific Multifuge 1S-R Refrigerated Centrifuge.

4. Methods

4.1. Bacterial cultures

4.1.1. Agar dishes

8.75 g of premixed LB agar powder (*Sigma # L2897*) were weighted out and brought into 250 ml ddH$_2$0 in a 500 ml glass laboratory bottle. The mixture was sterilized by autoclave. Then the agar was cooled to 50- 55 °C and supplemented with 250 µl ampicillin (amp) of a 100 µg/ml ampicillin stock solution (*Sigma # A951825G*). Subsequently, the agar was poured in Petri dishes under sterile conditions. The plates were stored at + 4 °C.

4.1.2. Overnight culture

To isolate plasmid DNA we incubated bacterial cells, carrying the vector of interest, overnight in Lysogeny Broth (LB) broth medium (*Sigma # L3022-1KG*) supplemented with ampicillin. The used from us antibiotic concentration equaled 100 µg/ml.

Small scale plasmid DNA isolation

2.5 ml of the ampicillin supplemented medium were inoculated with a bacterial colony picked from an agar Petri dish. All of the utilized bacterial strains, except CreLoxP strain, were incubated o/n at 37 °C under constant shacking (220 rotations per minute (rpm)). The most suitable incubation temperature of the CreLoxP strain is 30 °C.

Large scale plasmid DNA isolation

This method differs from the small scale isolation in the amount of the used LB medium. Instead of 2.5 ml we utilized 100 ml. The next steps were performed as described in the previous paragraph.

4.1.3. Transformation of bacteria

We achieved vectors amplification via transformation of gram-negative bacteria. In this study we made use of six *E.coli* strains (see 5.4.). By the selection of bacterial strains we took into consideration the characteristics of the DNA plasmids such as size and stability[38].

For preparation of competent bacterial cell strains (DH5α, dcm-, Stbl2 and CreLoxP) we applied the $CaCl_2$ method (see 6.1.4.). Generation of competent DH10β cells was performed using the $RbCl_2$ method (see 6.1.5.). Cells were thawed on ice and aliquoted in volume of 100 µl in 1.5 ml Eppendorf tubes. The bacteria were transfected either with an aliquot of a ligation mixture or with plasmid, isolated with mini-/midiprep kit. The samples were placed on ice for 20 minutes. Thereafter, they were incubated for 45 seconds at 42 °C (heat shock). The rapid increase in the temperature leads to creation of pores on the cell membrane. The high concentration of $CaCl_2$ in the cell environment contributes to DNA intake. After the heat shock, the samples were kept on ice for 2 minutes. 350 µl LB medium were added to each tube. Then they were incubated on thermoblock for 1 hour at 37 °C by constant agitation (220 rpm) and plated on ampicillin containing agar plates. An overnight incubation at 37 °C was conducted. Additionally, two control reactions were set up. Via a positive control we determined the transformation efficiency of the bacterial strain. For this purpose we incubated 100 µl cells with 1 ng PCRII vector (see 5.6.). 100 µl cells without plasmid served as a negative control. By the transformation of MAX Efficiency Stbl2 competent cells we followed the manufacturer's guideline.

4.1.4. Competent cells – $CaCl_2$

Several bacterial cells, picked up from a frozen glycerol stock of competent cells, were introduced into 3 ml LB Broth medium and incubated o/n at 37 °C and 220 rpm. On the next day 1 ml of this cell culture was transferred to a 250 ml flask, containing 100 ml LB Broth. The cells were grown to log phase (optical density (OD) equal to 0.130- 0.150). Thereafter, the bacteria were concentrated over centrifugation for 10 minutes at 3000 rpm and + 4 °C. After the supernatant was poured off, the pellet was gently resuspended in 15 ml chilled 100 mM $CaCl_2$ + 15% Glycerol. Immediately after that another centrifugation

for 10 minutes at 3000 rpm was performed. The pellet was resuspended in 15 ml 100 mM $CaCl_2$ + 15% Glycerol and incubated on crashed ice for 30 minutes. After the end of the incubation, the cells were pelleted over a 10 minutes long centrifugation. Then 2 ml 100 mM $CaCl_2$ + 15% Glycerol were added to the pellet. The resuspended competent bacteria were aliquoted in chilled 1.5 ml tubes and stored at – 80 °C. All steps of the protocol were performed closed to the flame of a Bunsen burner. In order to assess the competency of the cells, 100 µl of them were transformed with 10 ng PCRII plasmid, according to the described above procedure (see 6.1.3.). Another 100 µl were transformed with 1 ng from the same vector. The aim was to test the most suitable DNA concentration. A negative control was also set up – 100 µl cells without plasmid. The bacteria were spread onto agar plates with 100 µg/ml amp and incubated o/n at 37 °C and 5% CO_2.

4.1.5. Competent cells – $RbCl_2$

A single bacterial colony (DH10β strain) was picked from a LB plate and incubated in 2.5 ml LB medium, supplemented with 100 µg/ml amp, overnight at 37 °C under constant shaking at 220 rpm. On the next day the bacterial culture was subcultured - 2.5 ml from it were poured into 1 L flask, containing 250 ml LB and 20 mM $MgSO_4$. Thereafter cells were grown until O.D. 600 reached 0.4- 0.6. Then they were centrifuged at 5500 rpm for 5 minutes at + 4 °C. The pellet was gently resuspended in 0.4 original volume of ice-cold TFB1. Then the cells were incubated on ice for 5 minutes and centrifuged at 5500 rpm and + 4 °C. 1/25 original volume ice cold TFB2 was added to the bacteria. Subsequently, DH10β cells were left on ice for 15 minutes and aliquoted in chilled 1.5 ml tubes. Then they were frozen in liquid nitrogen and stored at – 80 °C until further use.

4.2. Mammalian cells

4.2.1. Thawing of mammalian cells

Dulbecco's Modified Eagle's Medium (DMEM), supplied with 10% Fetal Bovine Serum (FBS) (*Sigma# Lot 123M3398 F7524*), 1% L-glutamine (Gln) and 1% penicillin/streptomycin (P/S) was pre-warmed at 37 °C for 30 minutes. The cryogen vial, containing the needed cells, was took out of the liquid nitrogen container and placed on ice. The vial was decontaminated with 70% ethanol (*VWR # 1.00983.2511*). In a laminar box

the cells were resuspended rapidly with 1 ml medium and the vial content was transferred into a canonical tube, containing 9 ml medium. The cells were pelleted for 5 minutes at 1500 g and then the supernatant was discarded. In accordance to the pellet size a suitable amount of DMEM was added. Cells were seeded in 10 cm tissue culture dishes. Incubation at 37 °C and 5% CO_2 was performed.

4.2.2. Propagation and splitting

To maintain 70- 80% confluency we split the mammalian cells every 3- 4 days. They were grown in sterile 10 cm tissue culture dishes in incubator at 37 °C and 5% CO_2. When cells had reached the required density, the DMEM medium was aspirated from the flask. The cells were then briefly washed once with sterile 1xPBS. Subsequently, 5 ml 0.05% Trypsin/ EDTA solution (*PAA# L 11-004*) were added to the plate and the cells were incubated for 2- 3 minutes at 37 °C. The detached cells were transferred to a canonical tube and pelleted at 1500 g for 5 minutes. The cells were resuspended in an appropriate volume of DMEM, seeded in tissue culture dishes and incubated in incubator at 37 °C and 5% CO_2. All experiments were carried out with cells between passages 3 and 30.

4.2.3. Freezing of mammalian cells

Mammalian cells were grown to 50- 60% confluency. After washing with 1xPBS and treatment with trypsin they were pelleted by 5 minutes long centrifugation at 1500 rpm. Thereafter the supernatant was discarded and the cells were resuspended in a pre-warmed mixture, composed of 90% DMEM and 10% DMSO. The cells were placed on ice for 20 minutes and then left o/n at − 80 °C. Subsequently, they were transferred to a liquid nitrogen container.

4.2.4. Transfection of mammalian cells

$7.5x10^6$- $10x10^6$ HEK293T cells were seeded in 9 ml DMEM (10% FCS + 1% P/S + 1% Gln) in 10 cm cell culture dishes and incubated o/n in thermostat. On the next day the attached cell, which had reached 75- 85% density, were subjected to transformation. Two solutions were prepared and mixed dropwise and slowly – Solution A and Solution B.

Solution A (total volume 500 µl)		Solution B (total volume - 500 µl)	
Plasmid DNA	20 µg	2xHBS	500 µl
H₂O	437.5 – vol. of DNA (µl)		
2 M CaCl₂	62.5 µl		

The mixture of solution A and B was incubated at RT for 15 minutes. 2.5 µl 100 mM Chloroquin were added to each tissue culture dish. After the end of the incubation, the precipitate was transferred gently to the dishes.

We set up five control reactions.

Positive control	20 µg vector expressing GFP
Negative control	Without DNA
V1*off* positive control	10 µg V1*off* + 10 µg vector expressing GFP
V2*off* positive control	10 µg V2*off* + 10 µg vector expressing GFP
V3*off* positive control	10 µg V3*off* + 10 µg vector expressing GFP

The cells were incubated in incubator o/n at 37 °C and 5% CO_2. 14- 15 hours after the start of the incubation the medium was changed. The transformed cells were observed under Nikon inverted fluorescence microscope (see 5.9.) and used for Western blot (WB) or flow cytometry analysis (FACS).

4.2.5.   Hoechst staining of live mammalian cells

For the staining of cell nuclei we dissolved 1 µg bisBenzimide H33342 trihydrochloride (*Sigma # B2261*) in 1 ml ddH₂O. Since the required dye concentration is 1 µg/ml, 10 µl of the solution were added to each 10 cm cell culture dish, containing transfected HEK293T cells. 5 minutes long incubation at RT was performed. The medium was changed and fluorescence was monitored under a fluorescence microscope[39].

4.2.6. Protein isolation

The transformed HEK293T cells were briefly washed with ice cold 1xPBS buffer. Subsequently, 1 ml from the same buffer was added to the cells and they were scraped. The cell-buffer suspensions were collected in 1.5 Eppendorf tubes and centrifuged for 5 minutes at + 4 °C and 1500 rpm. Prior to the extraction the tubes, containing the cell pellet, were submerged in liquid nitrogen for several second. Protein isolation was performed with Radio Immunoprecipitation Assay buffer (RIPA) / Protein isolation (PI) buffer (see 5.1.) and complex of inhibitors.

Inhibitor	Company	Concentration per 1 ml IP / RIPA buffer
Aprotinin	Sigma # 10820	10 µg/ml
Leupeptin	Sigma # L2884	10 µg/ml
NaF	Fluka/Sigma # 71519	1 µM
PMSF	Fluka/Sigma # 93482	1 µM
Na$_3$VO$_4$	Sigma # S6508	1 µM

Additionally, we added to the buffer a protease inhibitor cocktail tablet (*Roche # 11697498001).* By the determination of the required concentration we followed the manufacturer's protocol. The pellets were resuspended in distinct amount of the prepared solution, which depended on their size. Thereafter, the samples were incubated for 30 minutes at + 4 °C under constant rotation. The extracts were centrifuged at 13 000 rpm for 30 minutes. The protein lysates, concentrated in the supernatant, were collected into 1.5 ml tubes and stored at – 80 °C. We determined the protein concentrations with Protein Assay Dye Reagent Concentrate (*BioRad - #500-0006*) following the manufacturer's protocol.

4.3. DNA methods

4.3.1. Plasmid DNA preparation

For the preparation of large/small scale DNA we utilized mini- and midiprep kits, purchased from Thermo scientific (*#K0481, #K0503*). Isolation of plasmid DNA from competent *E.coli* cells was carried out according to the protocol, provided with the kit. DNA concentration was measured by NanoDrop.

4.3.2.   Separation of DNA by agarose gel electrophoresis

To separate, identify and purify DNA fragments we utilized agarose gels. The gels were prepared with an appropriate for the size of the fragment concentration of agarose (ranging from 0.7% to 2.5%). Before ligation, DNA fragments were separated on an agarose gel, stained with EtBr (*Sigma #E1510*)**,** or SERVA DNA Stain Clear G (*Serva Electrophoresis # 39804.01*). The electrophoresis was run in fresh 1xTBE buffer. To separate and identify DNA molecules we utilized 1xTAE buffer and gels stained with Midori Green Advance DNA Stain (*Nippon Genetics Europe GmbH # MG 04*).

4.3.3.   DNA purification

Prior to ligation the amplified fragments and their corresponding vectors were digested and loaded on a 0.9% agarose gel. After 1.5/2 hours long running at 130 V and 200 mA, the DNA bands were visualized with UV light and carefully excised from the gel with a scalpel. Purification of DNA fragments was performed with GENECLEAN Turbo Kit (*MP Biomedicals 111102200*). We used the manufacture's protocol. The pure DNA was stored at – 20 °C until further use.

4.3.4.   PCR products purification

Removal of contaminants, primers, dNTPs, salts, enzymes, impurities, EtBr, ails and detergents from PCR reactions was performed with QIAquick PCR Purification Kit (*Qiagen # 28104*). Purification, based on spin-column technology, was conducted in accordance with the manufacturer's recommendations.

4.3.5.   Phenol extraction and ethanol precipitation of DNA

Protein contaminants from DNA solutions, as polymerase chain reaction (PCR) and restriction digest mixtures, were removed through extraction with phenol/chloroform/isoamyl alcohol (24:1:1) (*Roti # A156.1*). We added an equal volume of the mixture to each DNA sample. After 20 seconds long vigorously vortexing, the samples were centrifuged at 13 300 rpm for 2 minutes. The aqueous phase, containing DNA, was removed carefully and transferred to a new clean 1.5 ml Eppendorf tube. 1/10 volume of 3 M sodium acetate (pH=5.2) was added to each DNA sample. They were vigorously

vortexed for 20 seconds and then precipitated with 2/2.5 volume of ice cold 100% ethanol (calculated after salt addition). The solution was mixed and incubated at – 20 °C for 1 hour. Thereafter, the samples were centrifuged for 5 minutes at 13 300 rpm. To remove the salts and small organic molecules we washed the pellets with 70% ice cold ethanol. After 5 minutes long centrifugation at 13 300 rpm the pellets were dried for 10 minutes at RT. Then they were dissolved in 10 µl water. 2 µl of each sample were loaded on an agarose gel for DNA concentration determination.

4.3.6. Fragment amplification

Amplification of the six GOIs is based on PCR. Q5® High-Fidelity DNA Polymerase (*NEB # M0491S*) was used. As a template we used 1 to 10 ng vector, which contains one of the GOIs (see 5.6.). As a negative control of each PCR amplification served a reaction with 1 µl H_2O instead of DNA.

PCR master mix for 1 reaction - Total Volume 25 µl

5xQ5 Reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
10 µm Forward Primer	1.25 µl
10 µm Reverse Primer	1.25 µl
Template DNA	1 µl (1 ng /10 ng)
Q5 high fidelity DNA Polymerase	0.25 µl
Nuclease-Free Water	15.75 µl

Thermal cycler program

STEP	TEMP	TIME
Initial Denaturation	98˚C	30 sec
	98˚C	10 sec
30 cycles	54˚C	30 sec
	72˚C	90 sec
Final Extension	72˚C	2 min
Hold	4˚C	∞

The extremely large size of p110myr /H1047R (approximately 3000 bps) necessitated increase in the initial denaturation time of template DNA and final extension time.

Thermal cycler program

STEP	TEMP	TIME
Initial Denaturation	94˚C	3 min
	94˚C	40 sec
35 cycles	55˚C	30 sec
	72˚C	4 min
Final Extension	72˚C	10 min
Hold	12˚C	∞

PCR screening was performed with Taq DNA Polymerase (*5 Prime Nr. 2200020*). As a template we used 1 to 10 ng vector, isolated with mini- or midiprep kit.

PCR master mix for 1 reaction - Total Volume 25 μl

10xPCR Buffer	2.5 μl
10 mM dNTPs	0.5μl
10 μm Forward Primer	1 μl
10 μm Reverse Primer	1 μl
Template DNA	1 μl
Taq Polymerase	0.20 μl
DMSO	2.5 μl
Nuclease-Free Water	16.3 μl

Thermal cycler program

STEP	TEMP	TIME
Initial Denaturation	94˚C	2 min
	94˚C	40 sec
30 cycles	54˚C	45 sec
	72˚C	3 min
Final Extension	72˚C	5 min
Hold	4˚C	∞

4.3.7. Colony PCR

Transfection of bacterial cells was followed by screening for recombinants. In order to identify the plasmids within the bacterial colonies we employed the colony PCR method.

The PCR master mixture was prepared as already described (see 6.3.6.; PCR screening). Instead of template DNA 1 µl H$_2$O was added to each reaction. Only well isolated colonies were subjected to analysis. They were picked directly from the agar dishes through pipette tips and transferred to the already aliquoted master mix. To examine if the vectors contain the insert of interest we used one forward and one reverse insert-specific primer (see 5.2.). In order to check the orientation of the fragment/module we used either one vector-specific and one fragment-specific primer or a combination of sequencing primers of gene of interest and reporter gene. After the amplification, samples were loaded on an agarose gel.

4.3.8. Restriction digest

For detailed analysis of a sequence in a distinct DNA region, single or double restriction digests with 1 µg DNA were performed. The volume of each reaction was adjusted to 20 µl. The enzyme volume was determined in accordance with its concentration in units (U)/µl and DNA concentration in the sample. Incubation at the specific required temperature was performed for 2 hours.

Prior to every ligation, the fragments and their corresponding vectors were digested o/n. In order to achieve higher final concentration we digested 4 µg from the vector of interest. The volume of the reaction mixture was 50 µl. The undigested DNA molecule served as a negative control by each restriction.

4.3.9. Ligation

To ligate inserts with their corresponding vectors we utilized Instant Sticky-end Ligase Mixture Mix (*NEB#M0370S*) or T4 DNA Ligase (*NEB #M0202*). By each procedure we set up several reactions, where the vector: insert molar ratio ranged from 1:1 to 1:6. We used 100- 200 ng digested vector per reaction. The needed amount insert was estimated with the following formula:

(1) ((ng vector) x (kb size of insert)) / (kb size of vector)) x (molar ratio of (insert/vector))
    = (ng insert)

Sticky end ligation mixture

5 µl from the kit were added to 5 µl vector: insert mixture and incubated for 15 minutes at RT. The ligation mixture was used for transfection of *E.coli* or stored at − 20 °C until further use.

Ligation with T4 Ligase

10xT4 ligation buffer   2 µl
enzyme   1 µl
Vector:insert mixture   Estimated with formula *(1)*
Nuclease-Free Water   to 20 µl

Incubation over the night at 21 °C was performed. The mixture was used for transfection or stored at − 20 °C. No-insert controls with water instead of DNA fragment were also prepared.

4.3.10. DNA dephosphorylation

Three of the cloned GOIs are flanked by the same restriction enzymes - memEYFP, p110myr/H1047R and RalA^{G23V}. Restriction digest of Vector 1 with AscI, Vector 2 with SgfI and Vector 3 with FseI results in the formation of compatible ends[40]. The re-legation of the opened vectors might lead to reduction of the vector-insert ligation efficiency. To avoid this we treated the digested vectors with alkaline phosphatase (AP) (*Roche # 10713023001*). It removes phosphate groups from the 5'-end of the DNA strand. The vectors were digested o/n (see 6.3.8.). Then AP and 10xDephosphorylation Buffer were added to each 50 µl sample and they were incubated at 37˚C for 1 hour. To inactivate the enzyme we left the samples on thermoblock at 75 °C for 10 minutes.

4.4. Activation of genes by Cre mediated recombination

Inversion of the expression modules, which are flanked by loxP sites, was performed by transformation of competent *E.coli* cells that express temperature sensitive Cre mutant. By each transformation 100 µl cells were mixed with 1- 3 ng V1*off,* V2*off* or V3*off.* After 30

minutes long incubation on ice, they were placed on thermoblock for 45 seconds at 42 °C. Subsequently, 400 µl LB medium were added to each tube and they were then incubated at 37 °C for 4 hours. The bacterial cells were spread onto ampicillin agar dishes and incubated o/n at 30 °C. On the next day several colonies were picked up and incubated in LB medium supplemented with 100 µg/ml amp o/n at 30 °C and 200 rpm. These bacterial cultures were used for small scale plasmid DNA preparation.

We checked the orientation of the expression modules and proceeded with the isolation of pure colonies, which contain the vector of interest with either *on-* or *off*-orientation of the expression module. For this purpose we performed transfection of DH5α cells according to the standard protocol (see 6.1.3.). After overnight incubation at 37 °C the grown colonies were subjected to PCR screening. To screen the orientation of the expression modules we utilized one vector-specific primer by all screenings - *CAGGS For1* and one insert-specific primer, which was unique to each vector. The successfully inversion of M1 was verified with *MemEYFP For2* primer; of M2 with *P110 alpha myr For7* primer; of M3 with *Ral A For2* primer (see 5.2.).

4.5. DNA sequencing

Sequencing of all fragments was performed after their successfully ligation with the corresponding vectors. The vectors were also subjected to sequencing after the inversion of the expression modules. The primers were purchased from VBC (See 5.2.). Sequencing was performed by Eurofine MWG Operon. The samples were prepared according to a standard protocol.

DNA	100 ng
10 pmol/µl primer	2 µl
Nuclease-Free Water	to 17 µl

4.6. Annealing of oligonucleotides

In order to generate double stranded P2A fragments we designed and purchased three pairs oligonucleotides, which are composed of the viral P2A sequence, flanked by different restriction sites (see 5.2.). The concentration of each primer was adjusted to 100 pmol/µl.

50 µl aliquots of the complementary primers were mixed in PCR tubes. After the three samples were prepared they were heated to 95 °C for 2 minutes in a thermal cycler. Subsequently, they were left at 25 °C for 45 minutes. The generated in this way P2A fragments were immediately inserted into their corresponding vectors.

4.7. Western blot

After the protein isolation (see 6.2.6.) 30 µg of each sample were loaded on a 10% SDS polyacrylamide gel. The electrophoresis was run for approximately 1 hour at 20 mA (1 Gel)/ 40 mA (2 Gels) in 1xRunning buffer. To transfer the proteins to a nitrocellulose membrane (*Whatman™ # 10401396 Protran™ BA83*) we performed blotting o/n at RT at 30 mV. For this purpose we utilized a BioRad blotting chamber and 1xTransfer buffer, containing 20% methanol. The successful transfer of the proteins was verified after 5 minutes long incubation with Ponceau S (*Sigma P 3504*) at RT. Thereafter, the membrane was washed with ddH$_2$O for 5 minutes. It was incubated for 1 hour at RT with blocking solution, composed of 5% BSA, 1xTBS and 0.1% Tween 20. This solution was also used for 3x10 minutes washing of the membrane. Thereafter the membrane was incubated for 1.5 hour with the primary antibody diluted in TBST buffer, containing 1% BSA. 3x10 minutes washing in 1xTBST was conducted. Then the membrane was probed with a secondary antibody, which is HRP-conjugated. After one hour incubation a washing was carried out. The membrane was developed with Pierce ECL Plus Western Blotting Substrate THP (*Thermo Scientific / Pierce 32132X3)*, which we applied according to the manufacture's recommendations. The proteins on the membrane, probed with IRDye® 800CW, were visualized immediately after the washing step using Odyssay Clx Infrared imaging system. The membrane was stored at + 4 °C.

Membrane stripping
The nitrocellulose membrane was subjected to 7 minutes long incubation with 0.2 M NaOH at RT. Subsequently, it was washed in ddH20 for 5 minutes and then in 1xTBST. The blocking and probing of the membrane were performed as described above.

10% Lower gel - 20 ml / for 2 gels		5% Stacking gel - 3 ml / for 2 gels	
H$_2$O	9.592 ml	H$_2$O	2.225 ml
40% AA	5 ml	40% AA	0.375 ml
1.5 M Tris (pH=8.8)	5 ml	1 M Tris (pH=6.8)	0.380 ml
10% SDS	0.2 ml	10% SDS	0.030 ml
10% APS	0.2 ml	10% APS	0.030 ml
TEMED	0.008 ml	TEMED	0.003 ml

We were interested in testing the capability of P2A to cleave itself. Thus, we conducted several Western blot experiments. They were preceded by transfection of mammalian cells with the modified V1 (*on/off*), V2 (*on/off*) and V3 (*on/off*). The transfection procedure was performed as already described in section 6.2.4.

In order to test the activity of the anti-Raf1 antibody we utilized untransfected mouse embryonic fibroblasts (NIH3T3 cells) treated with 2 M CaCl$_2$ and 100 mM Chloroquin. As a positive control of the reporter genes we considered HEK293T cells transfected with one of the vectors, which were used as a template for the fragment amplification: pMembranes-EYFP, mCherry and pNucleus-ECFP (see 5.6.). We set up three additional cotransfection reactions.

V1*off* positive control   10 µg V1*off* + 10 µg vector expressing GFP
V2*off* positive control   10 µg V2*off* + 10 µg vector expressing GFP
V3*off* positive control   10 µg V3*off* + 10 µg vector expressing GFP

To monitor the transfection we used a fluorescent microscope. Subsequently, cells were subjected to protein extraction (see 6.2.6.).

4.8. Fluorescence activated cell sorting

For the purpose of the study we utilized eleven 6 cm tissue culture dishes, containing 70- 80% confluent HEK293T cells. The following reactions were set up:

Tube # 1	20 µg V1*on*	**Tube # 7** C+ [2]	20 µg GFP expressing Vector
Tube # 2	20 µg V2*on*	**Tube # 8** C - [3]	Without DNA
Tube # 3	20 µg V3*on*	**Tube # 9 V1*off* C+**	10 µg V1*off* + 10 µg V1*on*
Tube # 4	20 µg V1*off*	**Tube # 10 V2*off* C+**	10 µg V2*off* + 10 µg V1*on*
Tube # 5	20 µg V2*off*	**Tube # 11 V3*off* C+**	10 µg V3*off* + 10 µg V1*on*
Tube # 6	20 µg V3*off*		

15 hours after the incubation start the cell culture dishes were observed under fluorescent microscope. Afterwards, they were briefly washed with sterile 1xPBS and scraped with CytoOne Cell Scraper, 220 mm, 11 mm Fixed Blade (*Starlab #CC7600-0220*). The cell suspension was filtered through a 70 µm cell strainer (*VWR#734-0003*) and transferred to a 50 ml falcon. A centrifugation at 1500 g for 5 minutes at RT was performed. Thereafter, the cells were resuspended in a suitable amount of sterile 1xPBS in accordance to the size of the pellet. The concentration was determined over CASY® Cell Counter according to the manufacture's protocol. 100 000 cells of each sample were subjected to FACS analysis.

2: positive control

3: negative control

5. Results

5.1. Cloning strategy for modification of the Multi-Hit vector system

The starting vectors of our study were: pL1_M1, pL1_M2 and pBS_M3, which contain the original expression modules M1, M2 and M3 with the Hras mutants in *off*-orientation, respectively (Figure 8). By modification of these Multi-Hit Vectors we replaced three sequences downstream of the CAGGS promoter in each vector.

Modification of expression module M1

We replaced the Hras^{V12S35}-5xGtx-EYFP sequence of pL1_M1 (Figure 8A) for a memEYFP-P2A-C-RafCAAX- sequence. Self-cleavage via the P2A sequence adds a few amino acids to the C-terminus of the upstream protein and to the N-terminus of the downstream protein. We avoided addition of extra amino acids to the C-terminus of C-RafCAAX because the CAAX motif is located at the C-terminal end. Therefore, C-RafCAAX was placed upstream of the P2A sequence in the *off* vector. After Cre-mediated activation, C-RafCAAX will be located downstream of P2A and no extra amino acids are added to the C-terminal end when the protein is expressed (Figure 9). MemEYFP was placed downstream of P2A in the *off* vector because the membrane localization sequence is located at the N-terminus (see Supplemental Figure 2 for a detailed scheme of the new Vector pL1_M1_memEYFP_P2A_RafCAAX. From now on, V1*off* and V1*on* will be used in this diploma thesis as abbreviation for pL1_M1_memEYFP_P2A_RafCAAX in *off*-orientation and pL1_M1_memEYFP_P2A_RafCAAX in *on*-orientation, respectively.
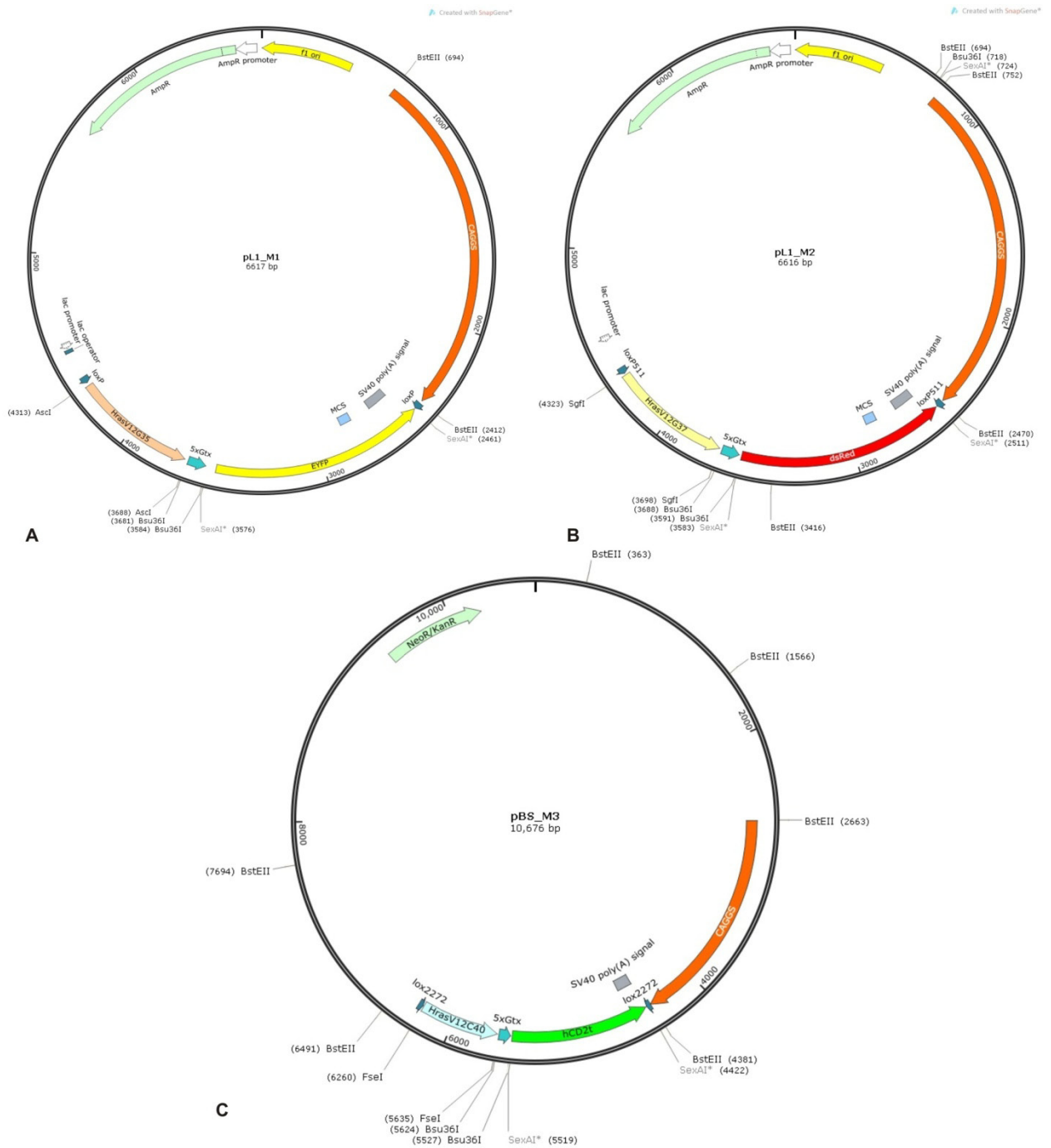
Figure 8: Shemes of the starting vectors. (A) pL1_M1, **(B)** pL1_M2 and **(C)** pBS_M3. Excision of the three genes of interest, 5xGtx and the reporters was performed by restriction digest with the illustrated enzymes.
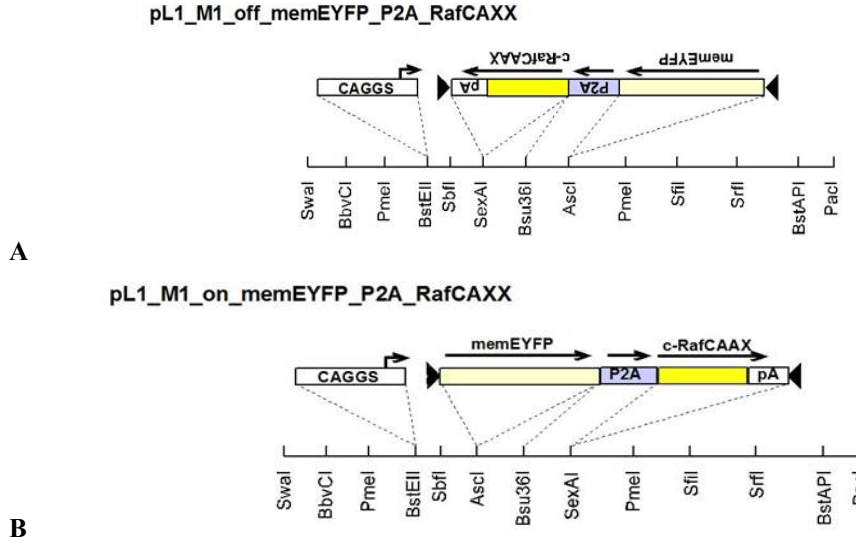
Figure 9: Modified expression module M1. Scheme **A** represents the inverted expression module M1 (*off-orientation*). Scheme **B** shows the expression module M1 after Cre-mediated activation (*on*-orientation).

Modification of expression module M2

In pL1_M2, we exchanged the Hras^{V12G37}-5xGtx-dsRed sequence (Figure 8B) for a p110myr/H1047R-P2A-mCherry sequence. Translation of the p110myr/H1047R mutant gene leads to the expression of a constitutive active PI3K subunit with a myristoylation signal at the N-terminus. In order to avoid addition of amino acids to the N-terminus after P2A self-cleaving, we inserted the mutated *PIK3CA* gene downstream of the P2A sequence in the *off* vector (Figure 10). After Cre-induced inversion of expression module M2, the p110myr/H1047R sequence will be placed upstream of P2A and no extra amino acids are added to the N-terminus (Figure 10). A detailed scheme of the new Vector with expression module M2 in *off*-orientation (V2*off*) and in *on*-orientation (V2*on*) is shown in Supplemental Figure 2.

pL1_M2_Pmel∆_off_p110myr_P2A_mCherry

A

pL1_M2_Pmel∆_on_p110myr_P2A_mCherry

B
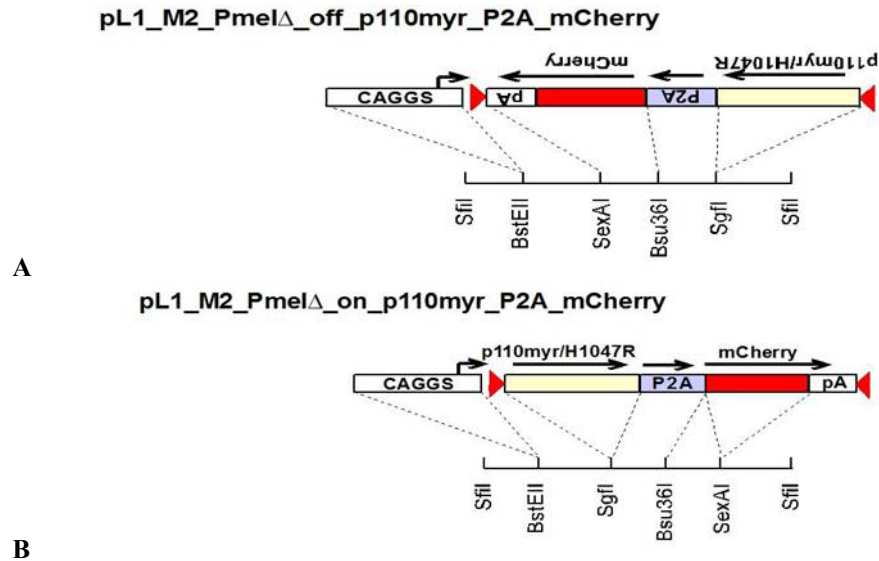
Figure 10: Modified expression module M2. Scheme **A** represents the inverted expression module M2 (*off*-orientation*)*. Scheme **B** shows the expression module M2 after Cre-mediated activation (*on*-orientation).

Modification of expression module M3

In pBS_M3, we replaced the Hras^{V12C40}-5xGtx-hCD2t sequence (Figure 8C) for a RalA^{G23V}-P2A-nucECFP sequence. The nuclear localization signal of the reporter protein nucECFP is placed at its C-terminus. Therefore, we inserted the nucECFP gene upstream of the viral P2A sequence in the off vector (Figure 11). Translation of the inverted (*on*-orientation*)* M 3 (Figure 11) results in expression of nucECFP protein with no extra amino acids at its C-terminus. A detailed scheme of the new Vector with expression module M3 in *off*-orientation (V3*off*) and in *on*-orientation (V3*on*) is shown in Supplemental Figure 2.

pBS_M3_off_RalAG23V_P2A_nucECFP

A

pBS_M3_on_RalAG23V_P2A_nucECFP

B
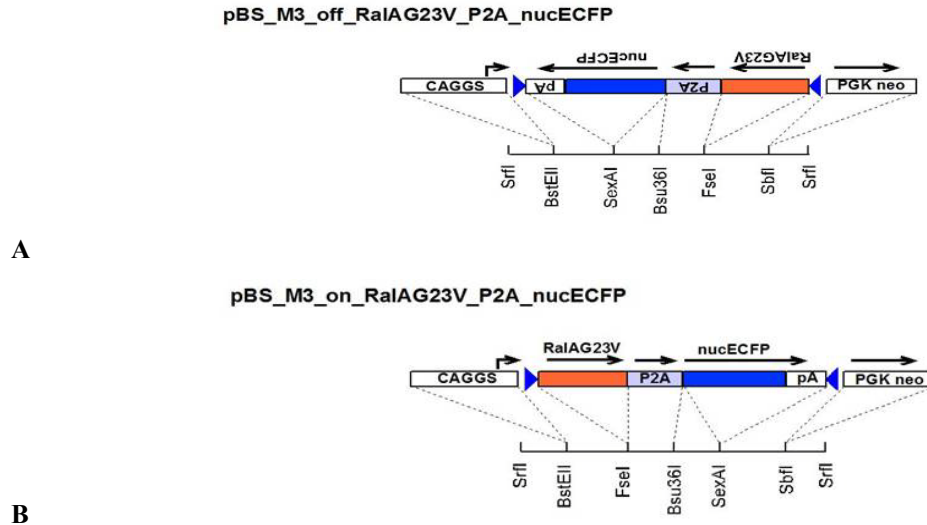
Figure 11: Modified expression module M3. Scheme **A** represents the inverted expression module M3 (*off-orientation)*. Scheme **B** shows the expression module M3 after Cre-mediated activation (*on-*orientation).

Cloning steps

The main techniques employed for the modification of the Multi-Hit vector system and their chronological order are shown in Figure 12.

In the first step, the 5xGtx IRES sequence was replaced for a P2A sequence in all starting vectors by linker cloning. We generated three double-stranded P2A fragments via annealing of oligonucleotides that contained compatible single stranded ends for cloning into the vectors after restriction digest. The procedure was performed according to the protocol (see 6.6.) (Figure 13).

The GOIs and the reporter genes were amplified by PCR (see 6.3.6.). Each PCR primer contained a restriction enzyme sequence that was used for restriction digest and cloning of the amplicons. Because many restriction enzymes are not able to cut at the end of a linear DNA molecule, we added a 6-bps long DNA sequence downstream of the restriction site of each cloning primer (*5'-AGCAGA-3')*.

Moreover, we included a 25-bps long sequence (*5'-GACTCACAACCCCAGAAACACCACC-3'*) that contained a Kozak sequence in the

~ 50 ~

upstream primer of the genes that are transcribed first in the bicistronic expression units after Cre-mediated inversion (memEYFP, p110myr/H1047R and RalA^{G23V}) to improve translation.

The amplicons (see 6.3.7.) were purified by extraction with phenol/chloroform/isoamyl alcohol (24:1:1) (see 6.3.4.) or with QIAquick PCR Purification Kit (see 6.3.5.), subjected to single or double restriction digests (see 6.3.8.), separated on agarose gels (see 6.3.2.), eluted and ligated with their corresponding vectors.
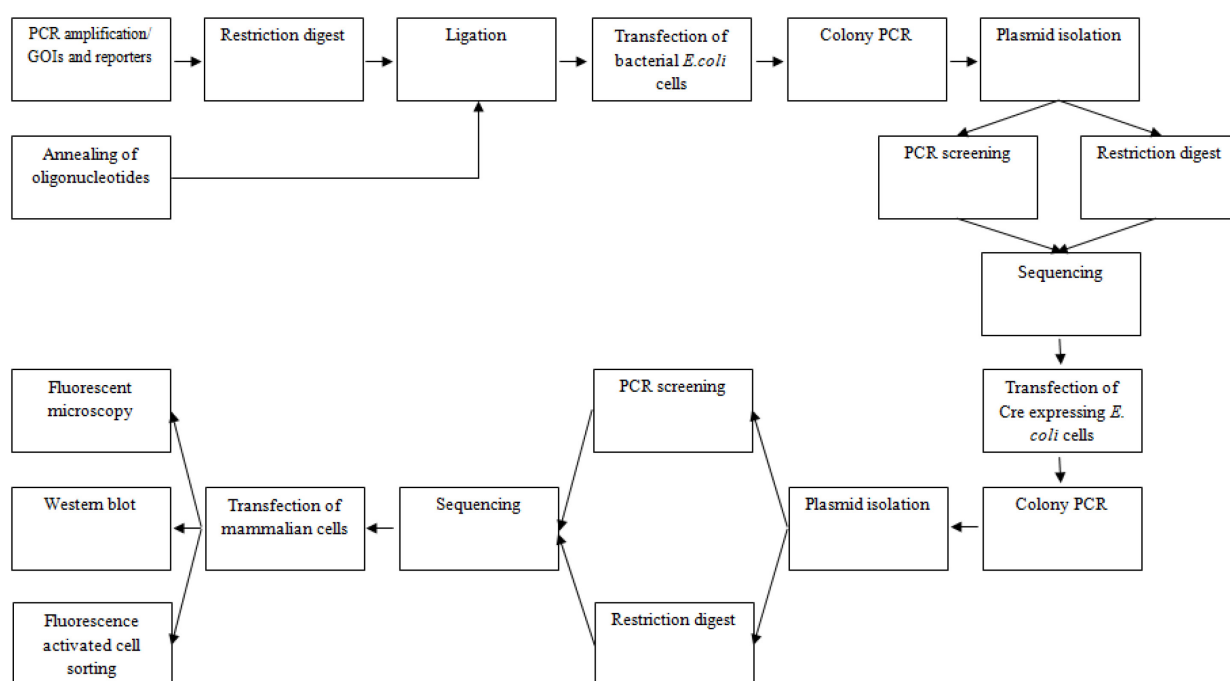
Figure 12: Multi-Hit vector system; cloning strategy.
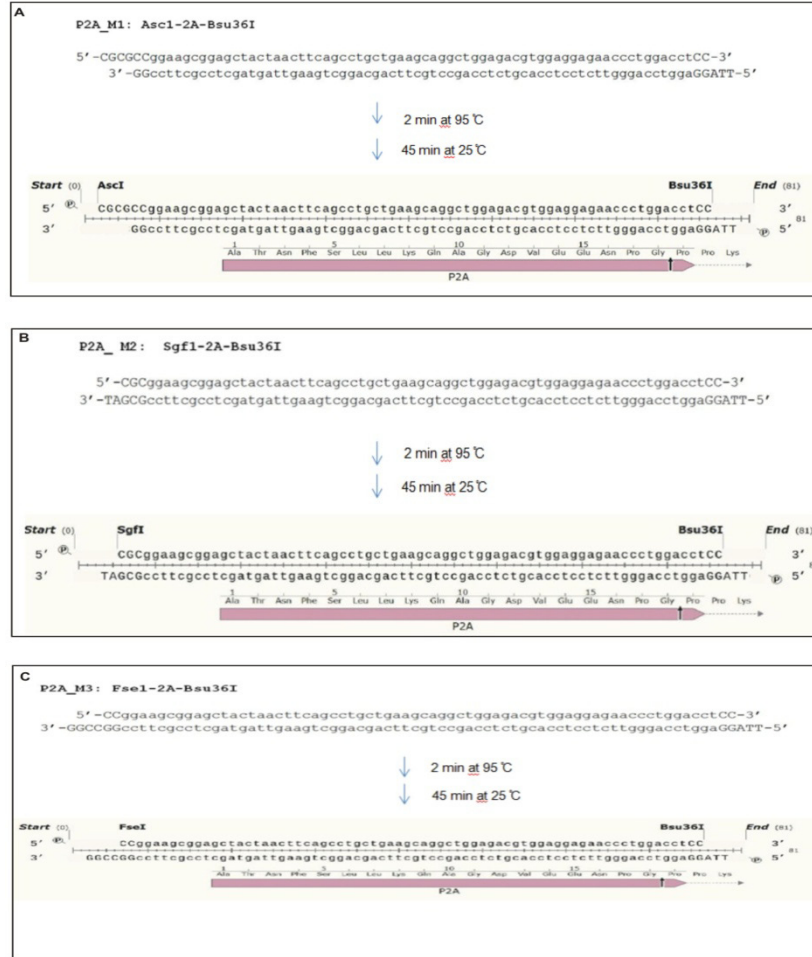
Figure 13: Generation of P2A double-stranded linkers. **(A)** The scheme shows the linker inserted in pL1-M1. AscI und Bsu36I restriction enzymes were used to cut out the 5xGtx and to replace it for P2A. **(B)** Replacement of the 5xGtx for P2A in pL2-M2 was performed with restriction enzymes SgfI and Bsu36I. **(C)** Replacement of the 5xGtx for P2A in pBS-M3 was performed with restriction enzymes FseI and Bsu36I.

Each cloning step was verified by screening of bacterial colonies by colony PCR (see 6.3.7.). PCR-positive colonies were used for plasmid isolation and proper cloning was verified by restriction digest (see 6.3.8.), PCR (see 6.3.6.) and sequencing (see 6.5.). After successful modification of expression modules in the *off* vectors, they were inverted by transformation into competent bacterial cells harboring a temperature-sensitive Cre recombinase (see 6.4.). To confirm inversion, we employed colony PCR, plasmid isolation, restriction digest and sequencing.

We verified correct cloning of V1*off*, V2*off*, V3*off* and inversion of modules in V1*on*, V2*on*, and V3*on* by restriction analysis. The sizes of the expected bands are shown in Table 1. The agarose gels in Figure 14 demonstrate that all constructs are correct and inversion worked accurately.

1.

Enzymes / Vector	Expected size of the fragments					
	HindIII	**SacII**	**XmaI**	**PmeI**	**SfiI**	**BstEII**
V1on	6449, 1575	3672, 4352	380, 7633	5076, 2948	linearization	6306, 1718,
V1off	6449, 1575	6967, 1057	3009, 5013	5076, 2948	linearization	6306, 1718

2.

Enzymes / Vector	Expected size of the fragments				
	HindIII	**XmaI**	**PmeI**	**SfiI**	**BamHI**
V2on	linearization	8831, 371	linearization	6254, 2948	linearization
V2off	linearization	5019, 4183	linearization	6254, 2948	linearization

3.

Enzymes / Vector	Expected size of the fragments			
	AscI + Bsu36I	**HindIII**	**PmeI**	**AscI**
V2on	2469, 2502, 5661	1203, 1203, 4884, 3345	linearization	8166, 2469
V2off	2469, 2842, 5324	1203, 1203, 4884, 3345	linearization	8166, 2469

Table 1: Multiple restriction digest – expected size and number of the bands. The enzymes used are ordered in columns. The rows give information about the size of the fragments obtained after the restriction digest. For the calculations we used Clone Manager Professional Suite software (Version 6).
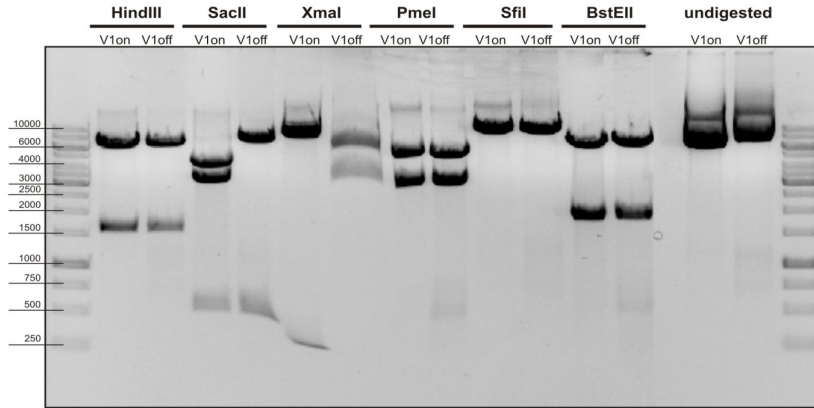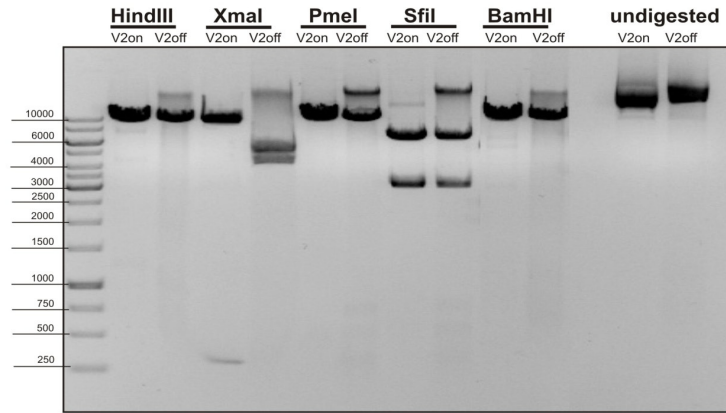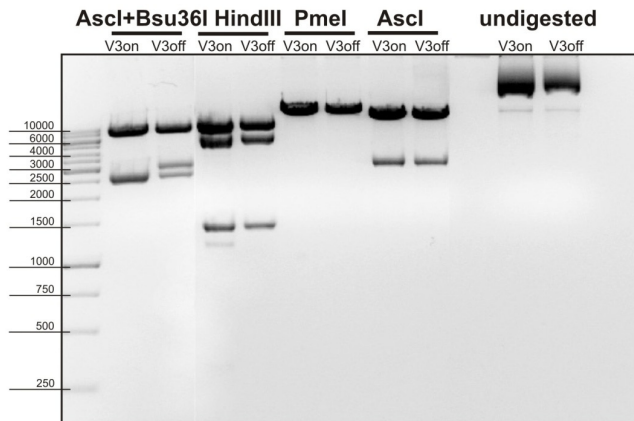
Figure 14: Restriction analysis of vectors in *off*- and *on*-orientations. (A) V1*on* and V1*off*; (B) V2*on* and V2*off*; (C) V3*on* and V3*off*. The vectors were digested with several enzymes and loaded on a 1% agarose gel, stained with SYBR Green. As size marker 1 kb Gene Ruler was loaded. The undigested vectors in *on*- and *off*-orientation of the expression module served as controls.

5.2. In vitro testing of GOI and reporter gene expression by cell transfection

We transfected HEK293T cells (6.2.4.) with V1*on*, V1*off*, V2*on*, V2*off*, V3*on* or V3*off* to test if GOIs and reporter genes can be expressed in mammalian cells in vitro. The vectors with *off*-orientation were used as negative control and Hoechst staining was used to detect the cell nuclei. The transfected cells were examined under a fluorescence microscope for reporter gene activity. As anticipated, we did not detect fluorescence of cells, transfected with V1*off*, V2*off* and V3*off* (data not shown). In contrast, transfection with V1*on,* V2*on* and V3*on* resulted in memEYFP, mCherry and nucECFP positive cell, respectively. As evident from Figure 15, memEYFP was localized to the membrane, mCherry was present in the cytosol and nucECFP was localized to the cell nuclei (evident by the colocalization of the blue Hoechst stain for nuclei and nucECFP).

In summary, the experimental results demonstrate that reporters are expressed in transfected cells and localized to the expected cellular compartment. They also demonstrate that the vectors with modules in *off*-orientation did not give fluorescent background signals.
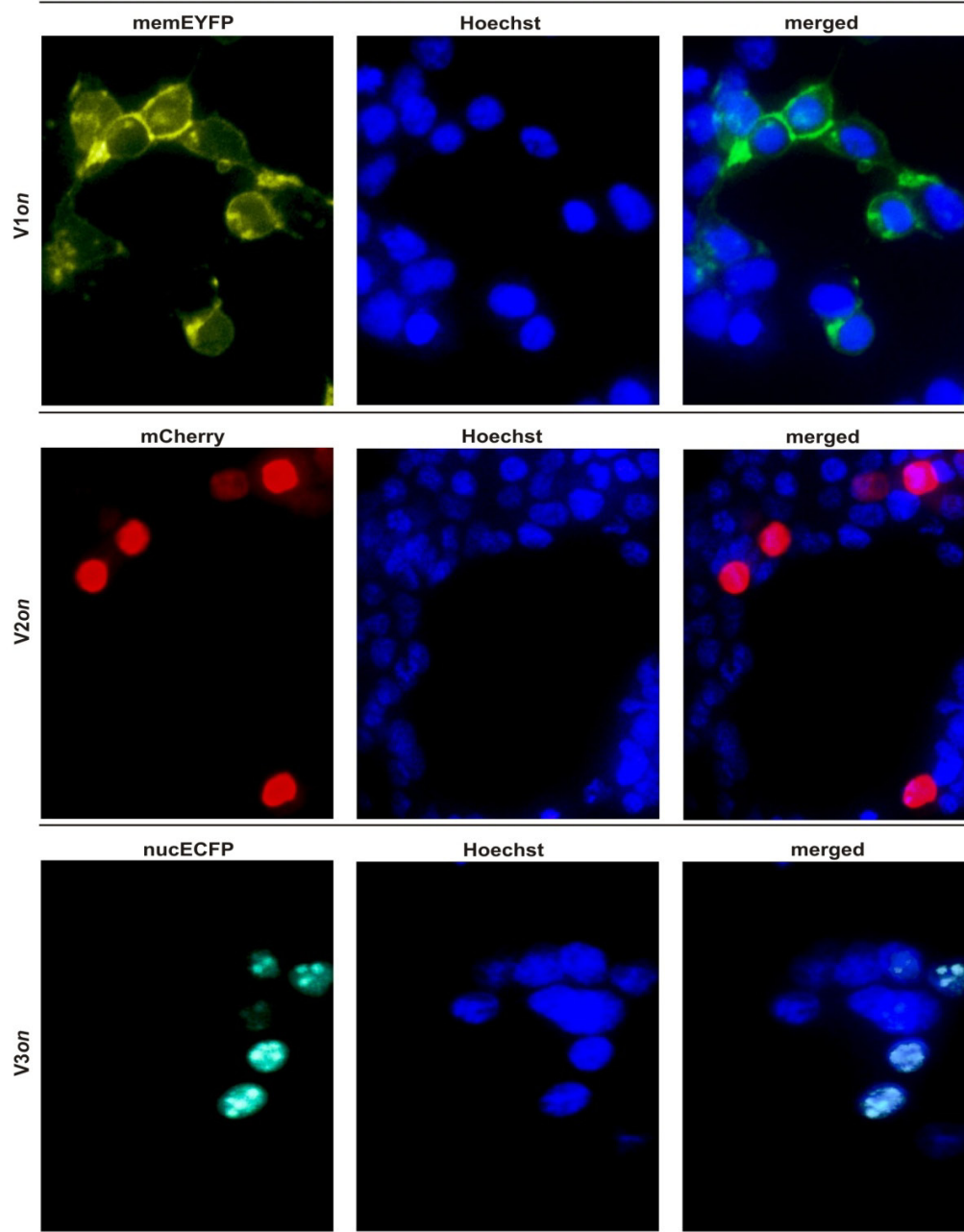
Figure 15: Monitoring of memEYFP, mCherry and nucECFP fluorescence emission. HEK293T cells were transfected with V1*on*, V2*on* and V3*on*. 14 hours post transfection, the cells were observed under the fluorescence microscope. The nuclei were stained blue with Hoechst dye.

5.3. Expression of M1, M2 and M3 in cotransfected cells

Multi-Hit vectors are used for stochastic activation of genes and assessment of activation patterns by reporter genes requires identification of cells with multiple fluorescent colors.

We performed cotransfection experiments of mammalian cells to investigate if we can detect several reporter gene activities in single cells.

First, we assessed the transfection efficiency of single vectors by FACS (Figure 16). The results revealed that the transfection efficientcy of V1*on* was approximately 23% whereas transfections efficiencies of V2*on* and V3*on* was approximately 8%.

Then, HEK293T cells were cotransfected with all three vectors in *on*-orientation and analysed by FACS. We employed two strategies to determine the percentage of cells which expressed all three reporters. Firstly, we gated on memEYFP/mCherry double-positive cells and determined the percentage of nucECFP positive cells among the gated population. As can be seen from Figure 17D, 79.4% of double-positive cells also expressed nucECFP. Secondly, we gated on mCherry/nucECFP double-positive cells and determined the percentage of memEYFP positive cells among the gated population. Most cells were triple-positive (Figure 17F).
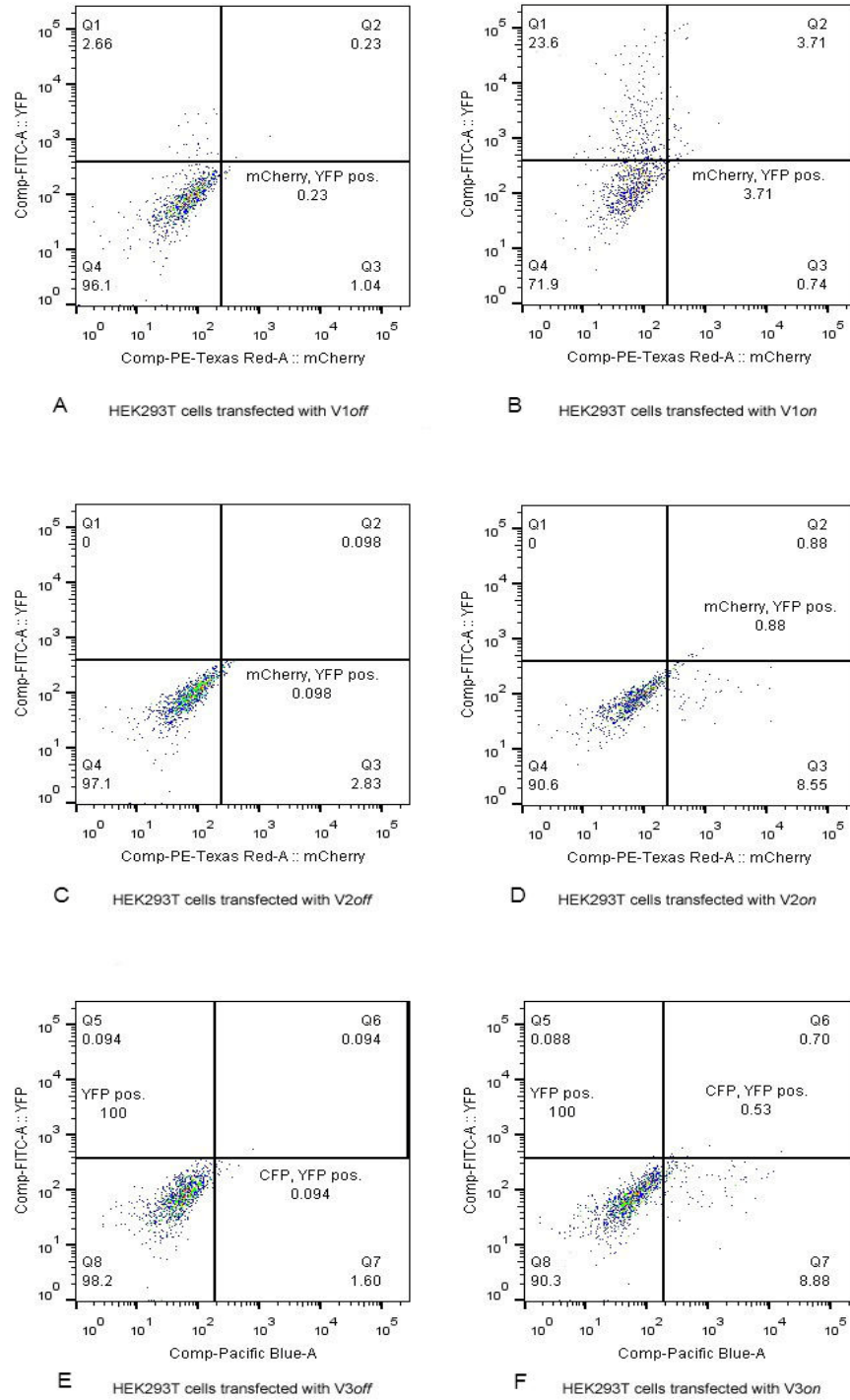
Figure 16: Flow cytometry analysis of HEK293T cells transfected with single vectors. Cells were transfected with the three vectors with *off*- (**(A)** V1*off*, **(C)** V2*off* and **(E)** V3*off*) and *on*- (**(B)**V1*on*, **(D)**V2*on* and **(F)**V3*on*) orientation of the expression modules and flow cytometry was performed
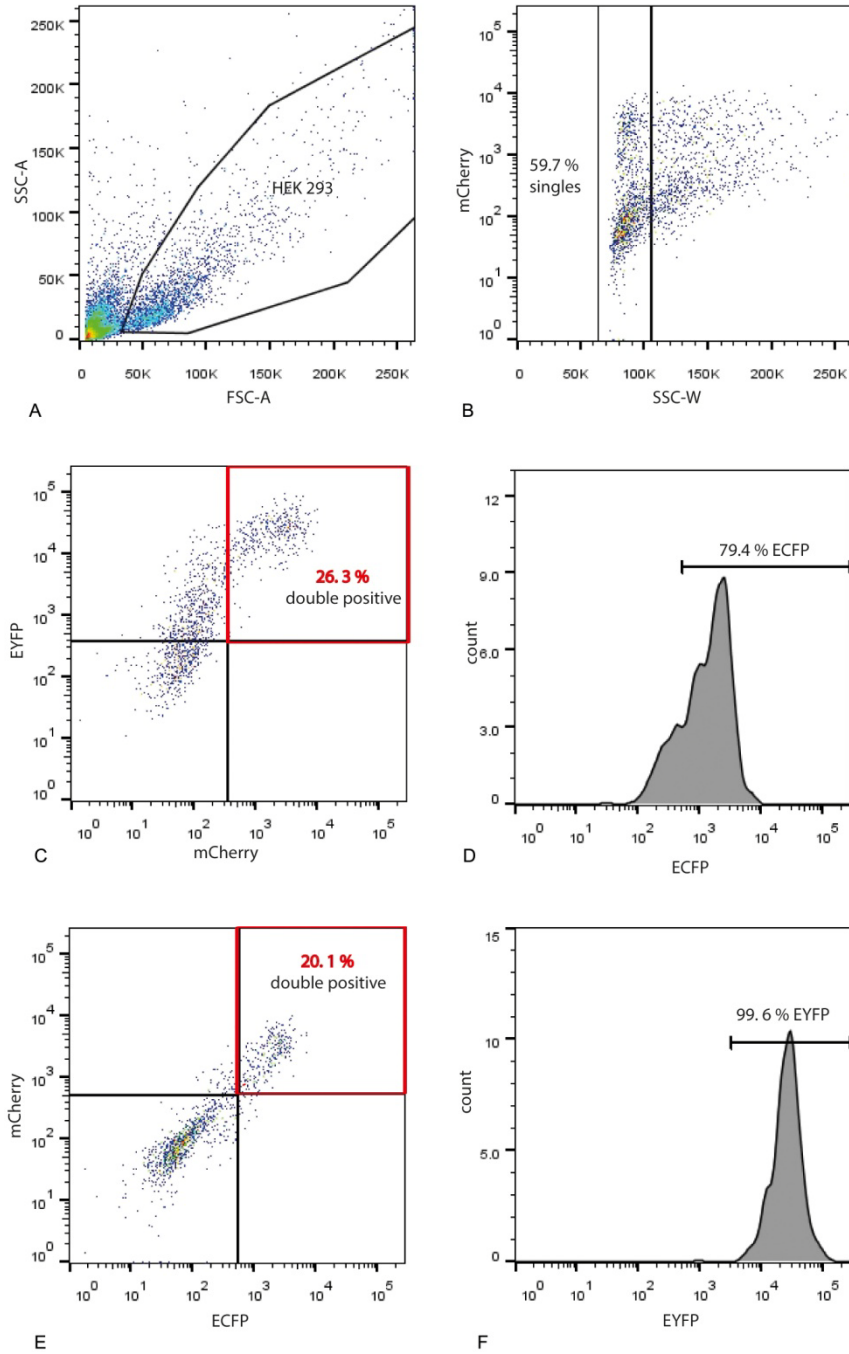
Figure 17: Determination of the percentage of memEYFP/mCherry/nucECFP triple-positive cells by FACS. HEK293T cells were cotransfected with V1*on*, V2*on* and V3*on* and analyzed by FACS. **(A)** Living cells were gated from dead cells based on cell size (FSC) and morphology (SSC). **(B)** Another gate was used to separate single cells from cell clumps. **(C)** Among single cells, the amount of memEYFP/mCherry double-positive cells was determined. **(D)** The histogram gives information about the amount of memEYFP/mCherry double-positive cells expressing also nucECFP. A second approach was used to determine the percentage of triple-positive cells. **(E)** nucECFP/mCherry double-positive cells were gated. **(F)** The percentage of memEYFP positive cells was determined in this population.

Finally, cotransfected cells were observed under the fluorescent microscope (Figure 18). We could identify triple-positive cells which were counted with ImageJ 1.48v software. Approximately 22% of transfected cells were triple-positive.
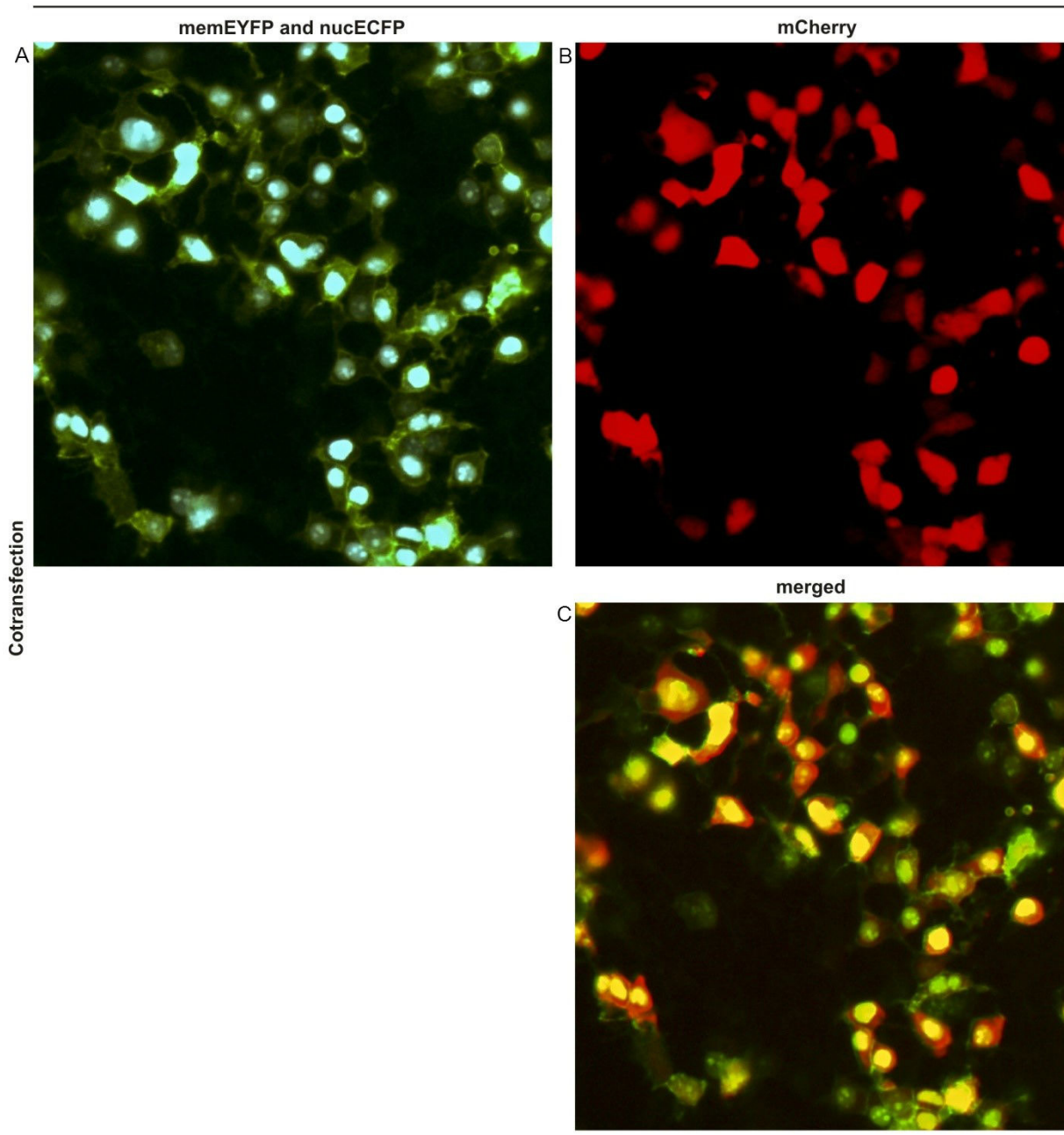
Figure 18: Fluorescence microscopy of HEK293T cells cotransfected with V1*on*, V2*on* and V3*on*. (A) memEYFP and nucECFP expression was detected using a FITC filter at 20x magnification. **(B)** mCherry positive cells were visualized with a Cy3 filter**. (C)** The merged images demonstrate coexpression and proper cellular localization of all reporter proteins.

5.4. Expression of GOIs and reporters was confirmed by Western blot analysis

The FACS and fluorescence microscopy experiments did not show whether the P2A sequence is functional because the fluorescent signals could also be due to expression of fusion proteins consisting of GOIs and reporters.

Therefore, we extracted protein from transfected cells and performed Western blot experiments to estimate the amount of fusion proteins and the stoichiometric amounts of individual GOI and reporter proteins. Prior to Western blotting, we calculated the molecular weight of GOIs, reporter proteins and fusion proteins using the following formula:

(1)   Fragment ((number of bases) / 3) x 110

Vector	Protein	Length	Size
V1*on*	memEYFP + P2A	849 bps	31.1 kDa
V1*on*	memEYFP	778 bps	28.5 kDa
V1*on*	C-RafCAAX	2004 bps	74 kDa
V2*on*	mCherry	720 bps	26.4 kDa
V2*on*	p110myr/H1047R+P2A	3326 bps	122 kDa
V3*on*	nucECFP	831 bps	30.47 kDa
V3*on*	RalA^{G23V}+P2A	691 bps	25 kDa
V1*on* fusion protein	memEYFP-P2A-C-RafCAAX	3233 bps	107.7 kDa
V2*on* fusion protein	p110myr/H1047R-P2A-mCherry	4414 bps	147.1 kDa
V3*on* fusion protein	RalA^{G23V}-P2A-nucECFP	1760 bps	58.7 kDa

Table 2: Size and length of the proteins expressed after translation of M1, M2 and M3 in HEK293T cells.

memEYFP, p110myr/H1047R and RalA^{G23V} are located upstream of P2A in M1, M2 and M3, respectively, which leads to addition of P2A-encoded extra amino acids at the C-terminal end.

5.4.1. Expression of C-RafCAAX and memEYFP

First, we performed Western blots with protein extracts from V1*on*-transfected cells. We probed with an anti-Raf1 antibody and detected a band of approximately 70 kDa (Figure 19A). Interestingly, the band intensity was similar in all samples including negative and positive controls. We used a monoclonal anti-Raf1 antibody (*ab32025*) which detects mouse, rat and human antigens. Therefore, the antibody also detects the endogenous human C-Raf1in transfected HEK293T cells which might be expressed at a high level that cannot be topped by ectopic expression with V1*on*.

According to our expectations, memEYFP is synthesized only in the positive control and cells transfected with V1*on* (Figure 19B). A difference in the size of both EYFP proteins was observed. As described above, this is due to the residual part of the P2A sequence which encodes for a short polypeptide that elongates the memEYFP. No fusion protein was detected indicating that the P2A sequence worked with 100% efficiency.
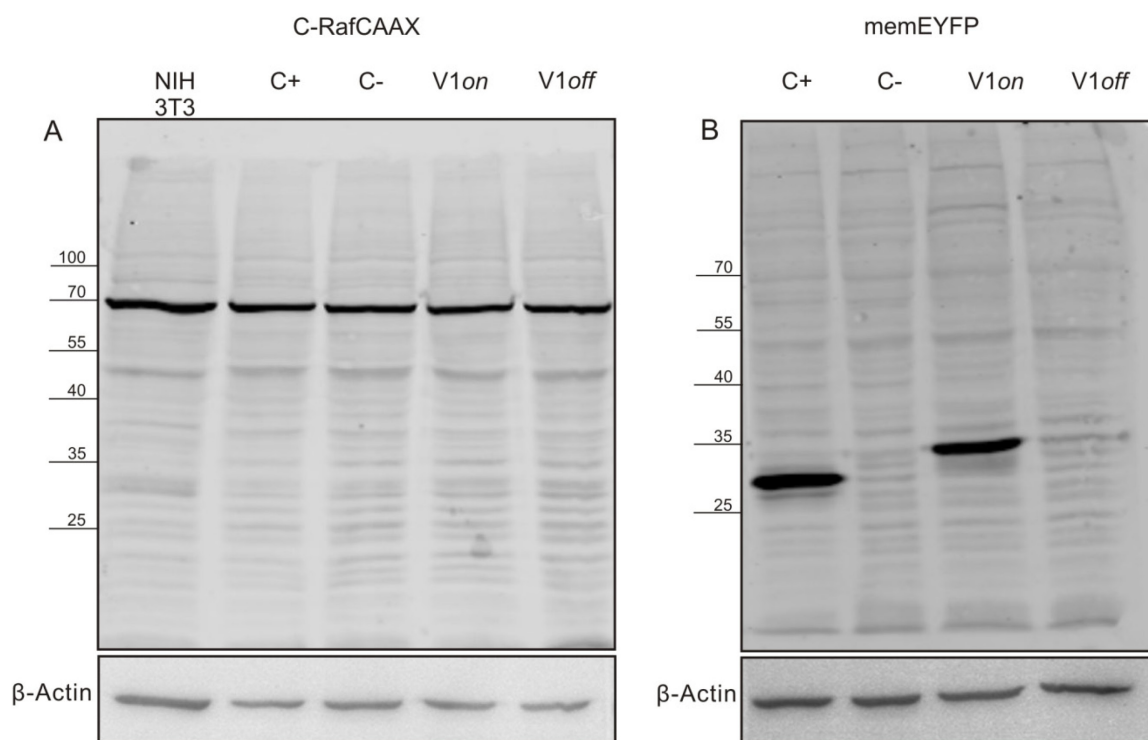
Figure 19: Expression of C-RafCAAX and memEYFP was confirmed by Western blot. (A) Western blot for Raf1. NIH 3T3: mouse fibroblasts asantibody positive control; C+: HEK293T cells transfected with C-RafCAAX expression plasmid (positive control); C-: untransfected HEK293T cells (negative control). **(B)** Western blot for GFP. C+: HEK293T cells transfected with mEYFP expression plasmid (positive control); C-: untransfected HEK293T cells. β-Actin expression served as a loading control.

5.4.2. Expression of p110myr/H1047R and mCherry

As evident from Figure 20A, p110 expression was only detected in V2*on*-transfected cells. The two positive controls (Jurkat cells and HEK293T cells transfected with an alternative p110 expressing vector) showed lower expression levels (lane 1 and lane 2). The bands were detected at 120 kDa, which is the expected size of p110myr/H1047R protein.

Expression of mCherry was detected in V2*on*-transfected cells but not in negative controls (Figure 20B). Expression of a fusion protein was not detected.
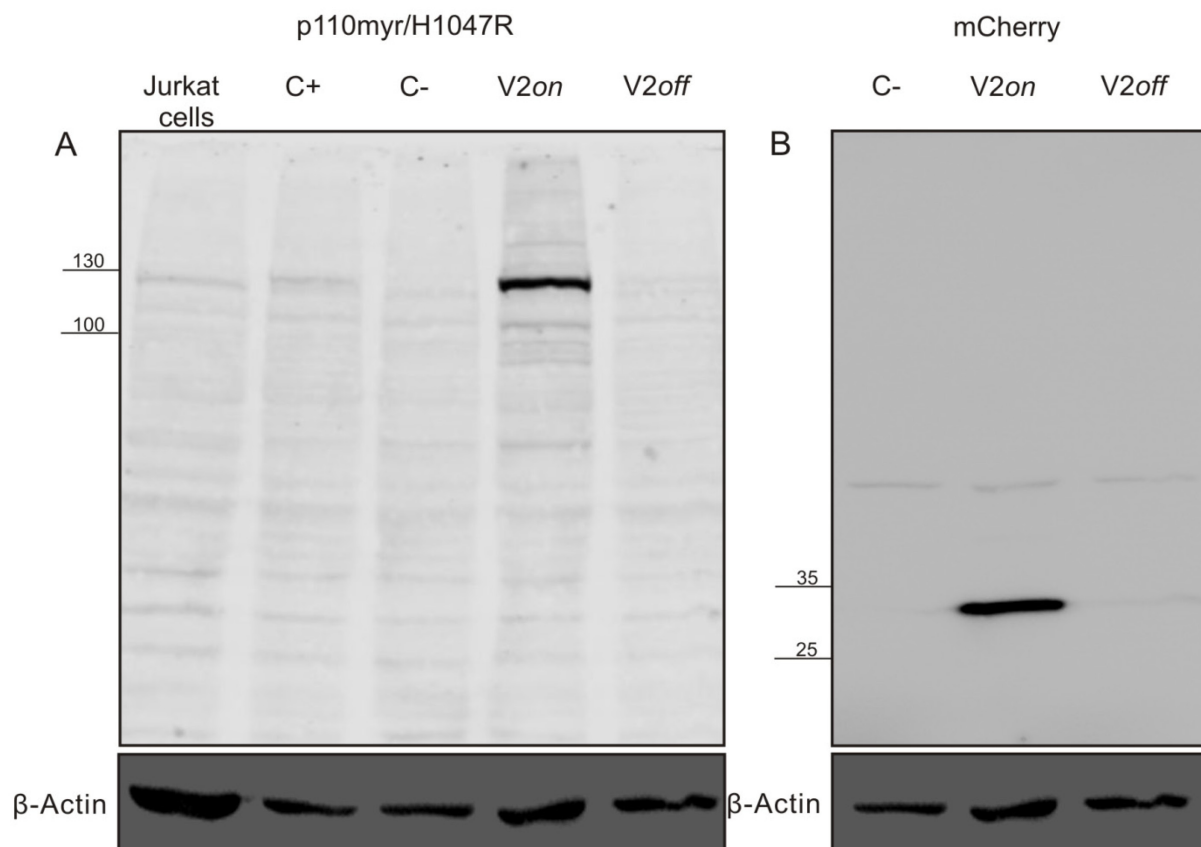
Figure 20: Expression of p110myr/H1047R and mCherry was confirmed by Western blot. (A) Western blot for p110myr/H1047R using a rabbit-derived anti-p110 antibody. **(B)** Detection of mCherry expression using a mouse-derived antibody. C-: untransfected HEK293T cells (negative control); C+: HEK293T cells transfected with a p110myr/H1047R expression plasmid (positive control); Jurkat cells: antibody positive control. β-Actin expression served as a loading control.

5.4.3. Expression of RalA^{G23V} and nucECFP

Western blotting with anti-RalA antibody resulted in the detection of a protein with a size of 25 kDa in V3*on*-transfected HEK293T cells (Figure 21A). Similar to the Western blot results for C-Raf (Figure 19A) RalA was expressed ubiquitously in all samples. We used an anti-RalA antibody *(ab126627)* that recognizes human RalA. Thus, the equal amounts of RalA in all samples including negative controls could be due to high endogenous expression in the human HEK293T cells.

In contrast, the nucECFP reporter protein was expressed only in the positive control and in V3*on*-transfected cells. As anticipated, the size of the reporter was 30 kDa. Expression of a fusion protein was not detected.
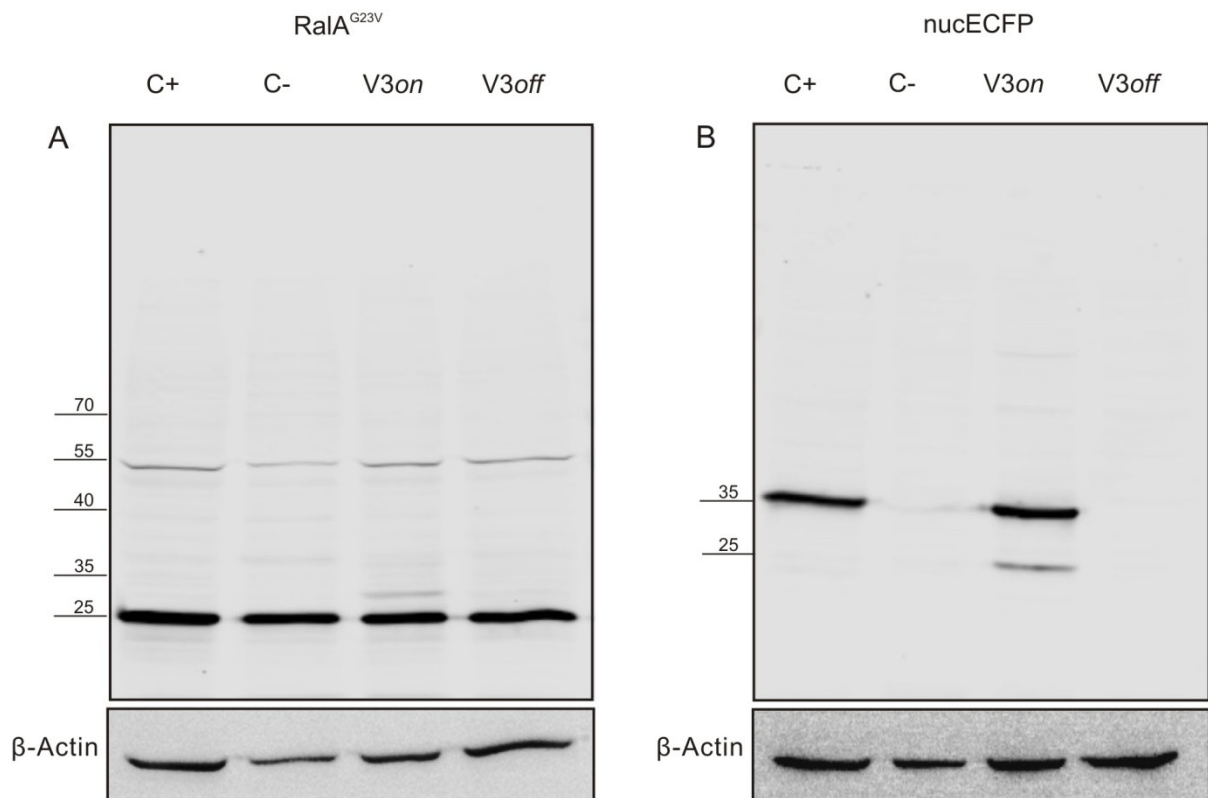
Figure 21: Expression of RalA^{G23V} and nucECFP was confirmed by Western blot. (A) Western blot for RalA and **(B)** GFP (which also detects nucECFP). C-: untransfected HEK293T cells (negative control); C+: HEK293T cells transfected with a RalA^{G23V} expression plasmid (positive control). β-Actin expression served as a loading control.

In summary, the *in vitro* experiments demonstrated that the modified Multi-Hit vectors are functional. We conclude that the P2A sequence provides reporter gene expression without production of fusion proteins.

6. Conclusion and outlook

We generated three bicistronic expression modules that will be used for stochastic activation of candidate genes in Multi-Hit vectors. In our original study, we used three expression modules for Ras effector mutants (genes of interest) which were separated from reporter genes via a 5xGtx IRES sequence. Stochastic activation of Ras effectors led to formation of lung tumors but the 5xGtx IRES did not provide reporter gene expression to identify activation patterns in vivo. Therefore, we decided to replace the 5xGtx for P2A sequences and the original reporter genes (EYFP, dsRed and hCD2t) for memEYFP, mCherry and nucECFP. Our experiments demonstrated that the P2A sequence provides reporter gene activity after cell transfection *in vitro*. The Ras effector mutants in our original study were used for selective activation of MAPK, RalGEF and PI3K signaling pathways. It was a major criticism of reviewers that these mutants do not selectively induce the mentioned pathways. Therefore we also replaced the original Ras effector mutants for more selective alleles. In detail, C-RafCAAX, RalA^{G23V} and p110myr/H1047R mutant alleles were used for constitutive activation of MAPK, RalGEF and PI3K signaling pathways, respectively. The expression modules containing the new genes of interest will be used for generation of a BAC Multi-Hit construct that will be injected into fertilized mouse oocytes for generation of Multi-Hit transgenic mice. Transient ectopic Cre expression will be induced in these mice to verify stochastic expression of genes of interest and reporters in tumors in vivo. As a long term goal, we have planned to use the new vectors as backbones for assembly of novel Multi-Hit constructs for induction of tumor metastasis in Multi-Hit transgenic mice.

7. Abbreviations

AB	Antibody
AG	Golgi apparatus
AP	Alkaline phosphatase
BAC	Bacterial artificial chromosome
B	Buffer
bp	Base pairs
BSA	Bovine serum albumin
CreER	Cre recombinase enzyme
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
EtBr	Etidium bromide
FACS	Fluorescence activated cell sorting
FCS	Fetal bovine serum
FSC	Forward-scattered light
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GF	Growth Factors
GOI	Genes of interest
GPCRs	G protein-coupled receptor
Gtx2	Type IRES sequence
HBS	Hank's balanced salt solution
HRP	Horseradish peroxidase
IRES	Internal Ribosome entry site
LB	Lysogeny broth

LD	Loading dye
M1	Expression module 1
M2	Expression module 2
M3	Expression module 3
mA	Milliampere
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
NLS signal	Nuclear localization signal
NOR	Nucleolar organizing regions
o/n	Overnight
OD	Optical density
ORF	Open reading frame
PAGE	Polyacryleamide gel electrophoresis
PCR	Polimerase chain reaction
PIP$_3$	Phosphatidylinositol triphosphate
PI	Phosphatidylinosytol
PI Buffer	Protein isolation buffer
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethanesulphonyl fluoride
pRB	Retinoblastoma protein
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and tensin homolog
RalBP1	Ral Binding protein 1
RBD	Ras-binding domain
RIPA Buffer	Radio Immunoprecipitation Assay buffer
RKIP	Raf kinase inhibitory protein
rpm	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
S.O.C	Super optimal broth with catabolite repression
SDS	Sodium dodecyl sulfate

src	Rous sarcoma
SSC	Side-scattered light
TBS	Tris-buffered saline
TBST	Tris-buffered saline plus Tween 20
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
U	Unit
WB	Western blot

8. List of Figures

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10. Supplemental Figures

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V    161 CAAGCAACACTATCCGTGTTTTCTTGCCGAACAAGCAAAGAACAGTGGTCAATGTGCGAAATGGAATGAGCTTGCATCAC
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GB   1281 CAGCCTGTACAAACACCTGCATGTCCAGGAGACCAAGTTTCAGATGTTCCAGCTAATTGACATTGCCCGGCAGACGGCTC

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GB   1921 ACGTCCCCGAGGCTGCCTGTCTTCAAGATGAGCAAGGACGGCAAGAAGAAGAAGAAGAGCAAGACAAAGTGCGTGAT
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                    ....|..
V    2001 CATGTAA
GB   2001 CATGTAA
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A)

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GB   81 GCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGG

              170       180       190       200       210       220       230       240
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V    161 GCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC
GB   161 GCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC
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                250       260       270       280       290       300       310       320
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                410       420       430       440       450       460       470       480
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   401  AGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCAC
GB  401  AGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCAC

                490       500       510       520       530       540       550       560
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   481  AAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAA
GB  481  AAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAA

                570       580       590       600       610       620       630       640
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   561  GATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG
GB  561  GATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG

                650       660       670       680       690       700       710       720
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   641  TGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTC
GB  641  TGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTC

                   730       740       750       760       770       780
            ....|....|....|....|....|....|....|....|....|....|....|....|
V   721     CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGT
GB  721     CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA
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B)

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                 10        20        30        40        50        60        70        80
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1  ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGT
GB    1  ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGT

                 90       100       110       120       130       140       150       160
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V    81  GAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGA
GB   81  GAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGA

                170       180       190       200       210       220       230       240
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   161  CCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCAC
GB  161  CCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCAC

                250       260       270       280       290       300       310       320
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   241  CCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGG
GB  241  CCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGG

                330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   321  CGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACT
GB  321  CGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACT

                410       420       430       440       450       460       470       480
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   401  TCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGC
GB  401  TCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGC

                490       500       510       520       530       540       550       560
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   481  GCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAA
GB  481  GCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAA

                570       580       590       600       610       620       630       640
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   561  GGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACA
GB  561  GGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACA
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              650       660       670       680       690       700       710
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   641  CCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACCAGCTGTACAAGTAA
GB  641  CCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACCAGCTGTACAAGTAA


              10        20        30        40        50        60        70        80
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1  ATGGGGAGCAGCAAGAGCAAGCCCAAGGACCCCAGCCAGCGCGGGGGAACTAGTCCTCCAAGACCATCATCAGGTGAACT
GB    1  ATGGGGAGCAGCAAGAGCAAGCCCAAGGACCCCAGCCAGCGCGGGGGAACTAGTCCTCCAAGACCATCATCAGGTGAACT

              90        100       110       120       130       140       150       160
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V    81  GTGGGGCATCCACTTGATGCCCCCAAGAATCCTAGTAGAATGTTTACTACCAAATGGAATGATAGTGACTTTAGAATGCC
GB   81  GTGGGGCATCCACTTGATGCCCCCAAGAATCCTAGTAGAATGTTTACTACCAAATGGAATGATAGTGACTTTAGAATGCC

              170       180       190       200       210       220       230       240
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   161  TCCGTGAGGCTACATTAATAACCATAAAGCATGAACTATTTAAAGAAGCAAGAAAATACCCCCTCCATCAACTTCTTCAA
GB  161  TCCGTGAGGCTACATTAATAACCATAAAGCATGAACTATTTAAAGAAGCAAGAAAATACCCCCTCCATCAACTTCTTCAA

              250       260       270       280       290       300       310       320
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   241  GATGAATCTTCTTACATTTTCGTAAGTGTTACCCAAGAAGCAGAAAGGGAAGAATTTTTTGATGAAACAAGACGACTTTG
GB  241  GATGAATCTTCTTACATTTTCGTAAGTGTTACCCAAGAAGCAGAAAGGGAAGAATTTTTTGATGAAACAAGACGACTTTG

              330       340       350       360       370       380       390       400
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   321  TGACCTTCGGCTTTTTCAACCCTTTTTAAAAGTAATTGAACCAGTAGGCAACCGTGAAGAAAAGATCCTCAATCGAGAAA
GB  321  TGACCTTCGGCTTTTTCAACCCTTTTTAAAAGTAATTGAACCAGTAGGCAACCGTGAAGAAAAGATCCTCAATCGAGAAA

              410       420       430       440       450       460       470       480
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   401  TTGGTTTTGCTATCGGCATGCCAGTGTGTGAATTTGATATGGTTAAAGATCCAGAAGTACAGGACTTCCGAAGAAATATT
GB  401  TTGGTTTTGCTATCGGCATGCCAGTGTGTGAATTTGATATGGTTAAAGATCCAGAAGTACAGGACTTCCGAAGAAATATT

              490       500       510       520       530       540       550       560
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   481  CTGAACGTTTGTAAAGAAGCTGTGGATCTTAGGGACCTCAATTCACCTCATAGTAGAGCAATGTATGTCTATCCTCCAAA
GB  481  CTGAACGTTTGTAAAGAAGCTGTGGATCTTAGGGACCTCAATTCACCTCATAGTAGAGCAATGTATGTCTATCCTCCAAA

              570       580       590       600       610       620       630       640
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   561  TGTAGAATCTTCACCAGAATTGCCAAAGCACATATATAATAAATTAGATAAAGGGCAAATAATAGTGGTGATCTGGGTAA
GB  561  TGTAGAATCTTCACCAGAATTGCCAAAGCACATATATAATAAATTAGATAAAGGGCAAATAATAGTGGTGATCTGGGTAA

              650       660       670       680       690       700       710       720
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   641  TAGTTTCTCCAAATAATGACAAGCAGAAGTATACTCTGAAAATCAACCATGACTGTGTACCAGAACAAGTAATTGCTGAA
GB  641  TAGTTTCTCCAAATAATGACAAGCAGAAGTATACTCTGAAAATCAACCATGACTGTGTACCAGAACAAGTAATTGCTGAA

              730       740       750       760       770       780       790       800
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   721  GCAATCAGGAAAAAAACTCGAAGTATGTTGCTATCCTCTGAACAACTAAAACTCTGTGTTTTAGAATATCAGGGCAAGTA
GB  721  GCAATCAGGAAAAAAACTCGAAGTATGTTGCTATCCTCTGAACAACTAAAACTCTGTGTTTTAGAATATCAGGGCAAGTA

              810       820       830       840       850       860       870       880
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   801  TATTTTAAAAGTGTGTGGATGTGATGAATACTTCCTAGAAAAATATCCTCTGAGTCAGTATAAGTATATAAGAAGCTGTA
GB  801  TATTTTAAAAGTGTGTGGATGTGATGAATACTTCCTAGAAAAATATCCTCTGAGTCAGTATAAGTATATAAGAAGCTGTA

              890       900       910       920       930       940       950       960
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   881  TAATGCTTGGGAGGATGCCCAATTTGATGTTGATGGCTAAAGAAAGCCTCTATTCTCAACTGCCAATGGACTGTTTTACA
GB  881  TAATGCTTGGGAGGATGCCCAATTTGATGTTGATGGCTAAAGAAAGCCTCTATTCTCAACTGCCAATGGACTGTTTTACA

              970       980       990       1000      1010      1020      1030      1040
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   961  ATGCCATCTTATTCCAGACGCATTTCCACAGCTACACCATATATGAATGGAGAAACATCTACAAATCCCTTTGGGTTAT
GB  961  ATGCCATCTTATTCCAGACGCATTTCCACAGCTACACCATATATGAATGGAGAAACATCTACAAATCCCTTTGGGTTAT

              1050      1060      1070      1080      1090      1100      1110      1120
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  1041  AAATAGTGCACTCAGAATAAAAATTCTTTGTGCAACCTACGTGAATGTAAATATTCGAGACATTGATAAGATCTATGTTC
GB 1041  AAATAGTGCACTCAGAATAAAAATTCTTTGTGCAACCTACGTGAATGTAAATATTCGAGACATTGATAAGATCTATGTTC
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                1130      1140      1150      1160      1170      1180      1190      1200
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1121    GAACAGGTATCTACCATGGAGGAGAACCCTTATGTGACAATGTGAACACTCAAAGAGTACCTTGTTCCAATCCCAGGTGG
GB    1121    GAACAGGTATCTACCATGGAGGAGAACCCTTATGTGACAATGTGAACACTCAAAGAGTACCTTGTTCCAATCCCAGGTGG

                1210      1220      1230      1240      1250      1260      1270      1280
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1201    AATGAATGGCTGAATTATGATATATACATTCCTGATCTTCCTCGTGCTGCTCGACTTTGCCTTTCCATTTGCTCTGTTAA
GB    1201    AATGAATGGCTGAATTATGATATATACATTCCTGATCTTCCTCGTGCTGCTCGACTTTGCCTTTCCATTTGCTCTGTTAA

                1290      1300      1310      1320      1330      1340      1350      1360
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1281    AGGCCGAAAGGGTGCTAAAGAGGAACACTGTCCATTGGCATGGGGAAATATAAACTTGTTTGATTACACAGACACTCTAG
GB    1281    AGGCCGAAAGGGTGCTAAAGAGGAACACTGTCCATTGGCATGGGGAAATATAAACTTGTTTGATTACACAGACACTCTAG

                1370      1380      1390      1400      1410      1420      1430      1440
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1361    TATCTGGAAAAATGGCTTTGAATCTTTGGCCAGTACCTCATGGACTAGAAGATTTGCTGAACCCTATTGGTGTTACTGGA
GB    1361    TATCTGGAAAAATGGCTTTGAATCTTTGGCCAGTACCTCATGGACTAGAAGATTTGCTGAACCCTATTGGTGTTACTGGA

                1450      1460      1470      1480      1490      1500      1510      1520
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1441    TCAAATCCAAATAAAGAAACTCCATGCTTAGAGTTGGAGTTTGACTGGTTCAGCAGTGTGGTAAAGTTCCCAGATATGTC
GB    1441    TCAAATCCAAATAAAGAAACTCCATGCTTAGAGTTGGAGTTTGACTGGTTCAGCAGTGTGGTAAAGTTCCCAGATATGTC

                1530      1540      1550      1560      1570      1580      1590      1600
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1521    AGTGATTGAAGAGCATGCCAATTGGTCTGTATCCCGAGAAGCAGGATTTAGCTATTCCCACGCAGGACTGAGTAACAGAC
GB    1521    AGTGATTGAAGAGCATGCCAATTGGTCTGTATCCCGAGAAGCAGGATTTAGCTATTCCCACGCAGGACTGAGTAACAGAC

                1610      1620      1630      1640      1650      1660      1670      1680
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1601    TAGCTAGAGACAATGAATTAAGGGAAAATGACAAAGAACAGCTCAAAGCAATTTCTACACGAGATCCTCTCTCTGAAATC
GB    1601    TAGCTAGAGACAATGAATTAAGGGAAAATGACAAAGAACAGCTCAAAGCAATTTCTACACGAGATCCTCTCTCTGAAATC

                1690      1700      1710      1720      1730      1740      1750      1760
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1681    ACTGAGCAGGAGAAAGATTTTCTATGGAGTCACAGACACTATTGTGTAACTATCCCCGAAATTCTACCCAAATTGCTTCT
GB    1681    ACTGAGCAGGAGAAAGATTTTCTATGGAGTCACAGACACTATTGTGTAACTATCCCCGAAATTCTACCCAAATTGCTTCT

                1770      1780      1790      1800      1810      1820      1830      1840
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1761    GTCTGTTAAATGGAATTCTAGAGATGAAGTAGCCCAGATGTATTGCTTGGTAAAAGATTGGCCTCCAATCAAACCTGAAC
GB    1761    GTCTGTTAAATGGAATTCTAGAGATGAAGTAGCCCAGATGTATTGCTTGGTAAAAGATTGGCCTCCAATCAAACCTGAAC

                1850      1860      1870      1880      1890      1900      1910      1920
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1841    AGGCTATGGAACTTCTGGACTGTAATTACCCAGATCCTATGGTTCGAGGTTTTGCTGTTCGGTGCTTGGAAAAATATTTA
GB    1841    AGGCTATGGAACTTCTGGACTGTAATTACCCAGATCCTATGGTTCGAGGTTTTGCTGTTCGGTGCTTGGAAAAATATTTA

                1930      1940      1950      1960      1970      1980      1990      2000
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     1921    ACAGATGACAAACTTTCTCAGTATTTAATTCAGCTAGTACAGGTCCTAAAATATGAACAATATTTGGATAACTTGCTTGT
GB    1921    ACAGATGACAAACTTTCTCAGTATTTAATTCAGCTAGTACAGGTCCTAAAATATGAACAATATTTGGATAACTTGCTTGT

                2010      2020      2030      2040      2050      2060      2070      2080
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     2001    GAGATTTTTACTGAAGAAAGCATTGACTAATCAAAGGATTGGGCACTTTTTCTTTTGGCATTTAAAATCTGAGATGCACA
GB    2001    GAGATTTTTACTGAAGAAAGCATTGACTAATCAAAGGATTGGGCACTTTTTCTTTTGGCATTTAAAATCTGAGATGCACA

                2090      2100      2110      2120      2130      2140      2150      2160
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     2081    ATAAAACAGTTAGCCAGAGGTTTGGCCTGCTTTTGGAGTCCTATTGTCGTGCATGTGGGATGTATTTGAAGCACCTGAAT
GB    2081    ATAAAACAGTTAGCCAGAGGTTTGGCCTGCTTTTGGAGTCCTATTGTCGTGCATGTGGGATGTATTTGAAGCACCTGAAT

                2170      2180      2190      2200      2210      2220      2230      2240
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     2161    AGGCAAGTCGAGGCAATGGAAAAGCTCATTAACTTAACTGACATTCTCAAACAGGAGAAGAAGGATGAAACACAAAGGT
GB    2161    AGGCAAGTCGAGGCAATGGAAAAGCTCATTAACTTAACTGACATTCTCAAACAGGAGAAGAAGGATGAAACACAAAGGT

                2250      2260      2270      2280      2290      2300      2310      2320
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V     2241    ACAGATGAAGTTTTTAGTTGAGCAAATGAGGCGACCAGATTTCATGGATGCTCTACAGGGCTTTCTGTCTCCTCTAAACC
GB    2241    ACAGATGAAGTTTTTAGTTGAGCAAATGAGGCGACCAGATTTCATGGATGCTCTACAGGGCTTTCTGTCTCCTCTAAACC
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              170       180       190       200       210       220       230       240
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  161   TGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCGTACCCCGACCACATGAAG
GB 161   TGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCGTACCCCGACCACATGAAG

              250       260       270       280       290       300       310       320
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  241   CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTA
GB 241   CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTA

              330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  321   CAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG
GB 321   CAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG

              410       420       430       440       450       460       470       480
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  401   ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAAC
GB 401   ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAAC

              490       500       510       520       530       540       550       560
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  481   GGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACAC
GB 481   GGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACAC

              570       580       590       600       610       620       630       640
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  561   CCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACG
GB 561   CCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACG

              650       660       670       680       690       700       710       720
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  641   AGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCC
GB 641   AGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCC

              730       740       750       760       770       780       790       800
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  721   GGACTCAGATCTCGAGCTGATCCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAA
GB 721   GGACTCAGATCTCGAGCTGATCCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAA

                                         810       820       830
                                    ....|....|....|....|....|....|....
V  801                               GAGAAAGGTAGGATCCACCGGATCGTAGATAA
GB 801                               GAGAAAGGTAGGATCCACCGGATCGTAGATAA
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E)

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              10        20        30        40        50        60        70        80
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  1     ATGGCTGCAAATAAGCCCAAGGGTCAAAATTCTTTGGCCTTACACAAAGTCATCATGGTGGGCAGTGTTGGTGTGGGCAA
GB 1     ATGGCTGCAAATAAGCCCAAGGGTCAAAATTCTTTGGCCTTACACAAAGTCATCATGGTGGGCAGTGTTGGTGTGGGCAA

              90        100       110       120       130       140       150       160
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  81    GTCAGCTCTGACTCTACAGTTCATGTACGATGAGTTTGTGGAGGACTATGAGCCTACCAAAGCAGACAGCTACCGGAAGA
GB 81    GTCAGCTCTGACTCTACAGTTCATGTACGATGAGTTTGTGGAGGACTATGAGCCTACCAAAGCAGACAGCTACCGGAAGA

              170       180       190       200       210       220       230       240
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  161   AGGTAGTGCTGGATGGGGAGGAAGTACAGATTGATATCTTAGATACAGCCTGGGCAGGAGGACTATGCTGCAATTAGAGAC
GB 161   AGGTAGTGCTGGATGGGGAGGAAGTACAGATTGATATCTTAGATACAGCCTGGGCAGGAGGACTATGCTGCAATTAGAGAC

              250       260       270       280       290       300       310       320
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  241   AACTACTTCCGAAGCGGGGAGGGCTTCCTCTGTGTCTTCTCTATTACAGAAATGGAATCCTTTGCAGCTACAGCTGACTT
GB 241   AACTACTTCCGAAGCGGGGAGGGCTTCCTCTGTGTCTTCTCTATTACAGAAATGGAATCCTTTGCAGCTACAGCTGACTT

              330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  321   CAGGGAGCAGATTTTTAAGAGTAAAAGAAGACGAGAATGTTCCATTTCTACTGGTTGGTAACAAATCAGATTTAGAAGATA
GB 321   CAGGGAGCAGATTTTTAAGAGTAAAAGAAGACGAGAATGTTCCATTTCTACTGGTTGGTAACAAATCAGATTTAGAAGATA

              410       420       430       440       450       460       470       480
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V  401   AAAGGCAGGTTTCTGTAGAAGAGGCAAAAAACAGAGCTGACCAGTGGAATGTTAACTACGTGGAAACGTCTGCTAAAACA
GB 401   AAAGGCAGGTTTCTGTAGAAGAGGCAAAAAACAGAGCTGACCAGTGGAATGTTAACTACGTGGAAACGTCTGCTAAAACA
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              490       500       510       520       530       540       550       560
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V   481 CGAGCTAATGTTGACAAGGTATTTTTTGATTTAATGAGAGAAATTCGAGCAAGAAAGATGGAAGACAGCAAAGAAAAGAA
GB  481 CGAGCTAATGTTGACAAGGTATTTTTTGATTTAATGAGAGAAATTCGAGCAAGAAAGATGGAAGACAGCAAAGAAAAGAA

              570       580       590       600       610       620
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
V   561 TGGAAAAAAGAAGAGGAAAAGTTTAGCCAAGAGAATCAGAGAAAGATGCTGCATTTTAT
GB  561 TGGAAAAAAGAAGAGGAAAAGTTTAGCCAAGAGAATCAGAGAAAGATGCTGCATTTTATA
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F)

Supplemental Figure 1: Sequence alignment of C-RafCAAX, memEYFP, mCherry, p110/H1047R, RalA[G23V] and nucECFP. The successfully cloning of the six GOIs was followed by sequencing. These sequences in V1, V2 and V3 (V) were compared with the corresponding sequences of the original genes used as a template for GOI amplification (GB). Bio-Edit software was used for the alignment of the sequences. Identical residues are highlighted in blue (C: cytosine), green (A: adenine), black (G: guanine), red (T: thymine). No mutations were observed in the cloned fragments. By **(A)** C-RafCAAX; **(C)** mCherry; and **(E)** nucECFP – the two sequences have 100% identical residues. **(B) (F)** The original memEYFP and RalA[G23V] have 2 bp more than the cloned sequence (AA). (**D**) The original p110/H1047R gene has two additional bases at the 3'-end. As explained above, the STOP codon of the genes inserted upstream of P2A in the *off*-orientated module was modified.
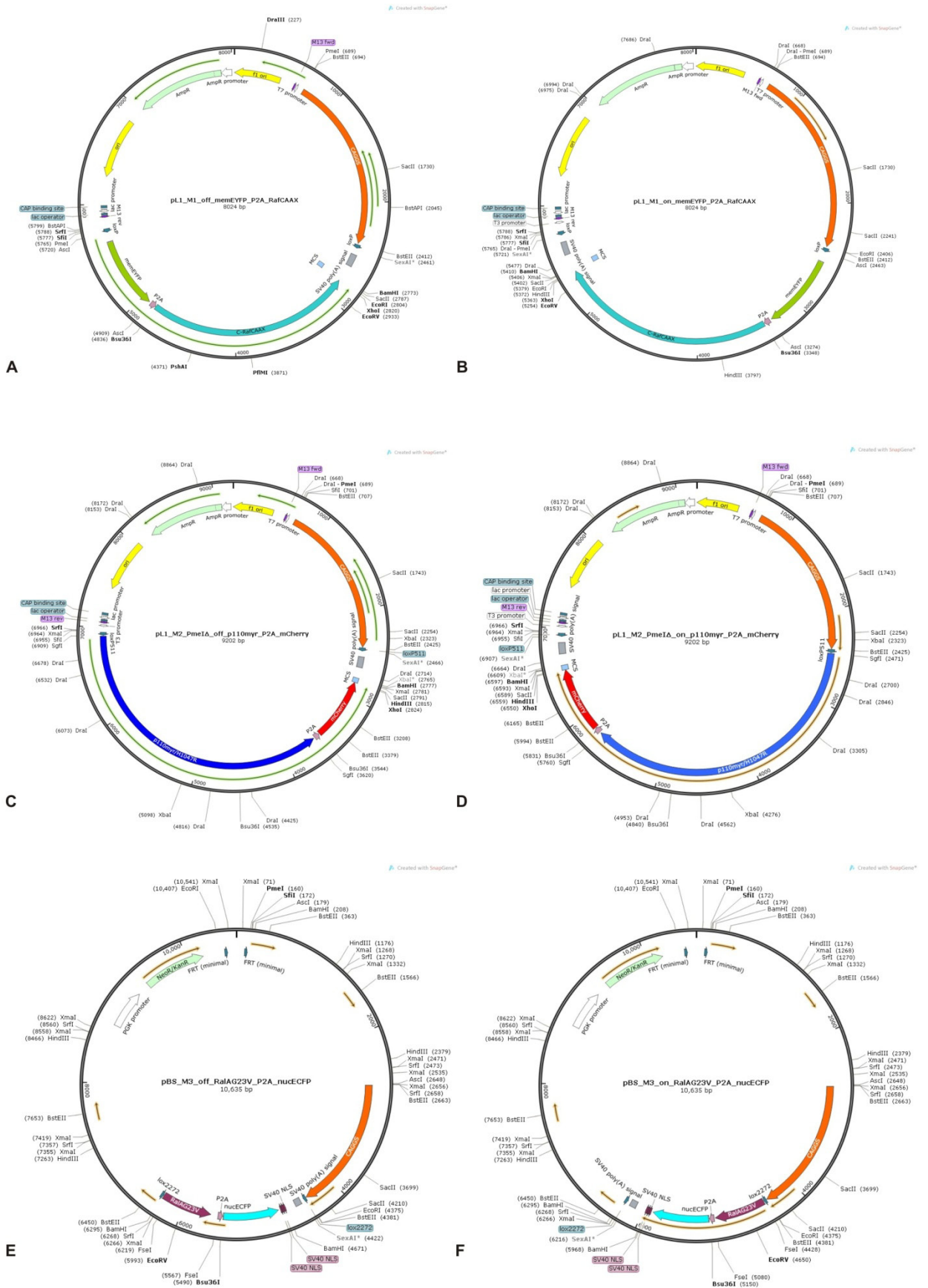
Supplemental Figure 2: Vector maps. Maps of the modified vectors, containing the expression modules M1, M2 and M3 either with *on-* or *off*-orientation were generated with Snap Gene software. The graphs show the most commonly used restriction sites and the orientation of GOIs within the expression modules.

(A) pL1_M1_off_memEYFP_P2A_RafCAAX;

(B) pL1_M1_on_memEYFP_P2A_RafCAAX;

(C) pL1_M2_PmeIΔ_off_p110myr_P2A_mCherry;

(D) pL1_M2_PmeIΔ_on_p110myr_P2A_mCherry;

(E) pBS_M3_off_RalA^{G23V}_P2A_nucECFP;

(F) pBS_M3_on_RalA^{G23V}_P2A_nucECFP.

11. Abstract

Cancer formation is a multistep process that develops over a long period of time and requires accumulation of several mutations (hits) in oncogenes and tumor suppressor genes. Recently, we have developed a Multi-Hit mouse model to investigate the multi-step development of tumors.

The model is based on Cre-mediated stochastic activation of three candidate oncogenes. Stochastic activation results in a mosaic pattern of different activation combinations in a given tissue. Cooperating activation patterns are positively selected and give rise to cancer. Multi-Hit constructs consist of three expression modules that are flanked by loxP sites. Each module is cloned in inverted (*off*) orientation and comprises a candidate gene, an internal ribosomal entry site (IRES) and a reporter gene to monitor activation of modules. The modules are flanked by antiparallel heterotypic loxP sites and expression is controlled by a strong CAGGS promoter. Therefore, activation of Cre recombinase in a Multi-Hit transgenic mouse results in flipping of modules and stochastic activation. In the original Multi-Hit construct, three mutated Ras genes, $Hras^{V12S35}$, $Hras^{V12G37}$ or $Hras^{V12C40}$, have been used as candidate genes. These Ras mutants activate different Ras downstream effector pathways (MAPK, RalGEF and PI3K). They were separated by a 5xGtx IRES sequence from EYFP, dsRed or hCD2t reporter genes.

The aim of this master project was the technological improvement of the Multi-Hit vector system.

The 5xGtx IRES in the original construct failed to provide expression of reporter genes after stochastic activation of Multi-Hit modules *in vivo*. Therefore, we replaced the 5xGtx IRES for a self-cleaving viral P2A sequence. Moreover, the original reporter genes were exchanged for memEYFP, nucECFP, mCherry and the original Ras effector mutants were replaced by more commonly used mutant genes for activation of Ras downstream effector pathways (C-RafCAAX, p110myr/H1047R, Ral^{G23V}).

The new vectors were tested for expression of candidate and reporter proteins *in vitro*. Immunofluorescence of transfected HEK293T cells verified synthesis of reporter proteins which emitted light at different wavelengths. The capability of P2A to induce 5'cap independent translation was further confirmed by Western blot analysis. Thus the viral P2A sequence has an

advantage over the originally used 5xGtx IRES and leads to efficient expression of reporter genes.

12. Zusammenfassung

Die Entstehung von Krebs ist ein langwieriger und vielstufiger Prozess und basiert auf der Anhäufung von mehreren Mutationen (Hits) in Onkogenen und Tumorsuppressorgenen. Wir haben ein Multi-Hit Mausmodell für das Untersuchen dieses vielstufigen Prozesses entwickelt. Das Modell basiert auf einer Cre-vermittelten stochastischen Aktivierung von drei Kandidaten Onkogenen. Stochastische Aktivierung führt zu einem mosaikartigen Expressionsmuster in Mausgeweben. Aus Zellen mit synergistischen Aktivierungsmustern entstehen in Folge Tumore. Die Multi-Hit Konstrukte für die Herstellung von Multi-Hit transgenen Mäusen bestehen aus drei Expressionsmodulen die von loxP Sequenzen flankiert sind. Jedes Modul wird in verkehrter Orientierung (*off*) kloniert und besteht aus einem Kanditatengen, einer internen Ribosomenbindestelle (IRES) und einem Reportergen. Die Module sind von heterotypischen loxP Sequenzen flankiert und die Expression wird von einem starken CAGGS Promoter reguliert. Aktivierung von Cre Recombinase in Mult-Hit transgenen Mäusen führt zu Inversion (flipping) und stochastischer Aktivierung der Module. In unserem originalen Multi-Hit Konstrukt haben wir drei mutierte Ras Gene, Hras^{V12S35}, Hras^{V12G37} und Hras^{V12C40}, verwendet. Diese Ras Mutanten aktivieren verschiedene Ras Effektor Signalwege (MAPK, RalGEF und PI3K). Die Mutanten wurden von den EYFP, dsRed oder hCD2t Reportergenen durch eine 5xGtx IRES Sequenz separiert.

Das Ziel dieser Masterarbeit war die technologische Weiterentwicklung des Multi-Hit Vektor Systems. Die 5xGtx IRES Sequenz im Originalkonstrukt hat nach stochastischer Aktivierung der Ras Kanditatengene *in vivo* zu keiner detektierbaren Reporteraktivität geführt. Deshalb wurde die 5xGtx IRES Sequenz durch eine virale P2A Sequenz ersetzt. Außerdem wurden die originalen Reportergene gegen memEYFP, nucECFP, mCherry und die originalen Ras Effektor Mutanten gegen gebräuchlichere Mutanten für die Aktivierung von Ras Stromabwärtssignalwegen (C-RafCAAX, p110myr/H1047R, Ral^{G23V}) ausgetauscht.

Die neuen Konstrukte wurden auf Expression von Kanditatengenen und Reportergenen *in vitro* getestet. Immunfluoreszenz von transfizierten HEK293T Zellen bestätigte Reporteraktivität. Die Fähigkeit der P2A Sequenz 5'cap unabhängige Translation zu initiieren wurde über Western

blot Experimente bestätigt. Die virale P2A Sequenz hat sich als vorteilhaft gegenüber der 5xGtx IRES Sequenz erwiesen und führte zu einer effizienten Expression der Reportergene.

Curriculum Vitae
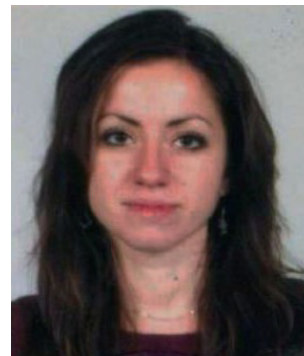
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Education

2012 - present   **University of Vienna**
                            MSc Molecular Biology

2007 - 2011   **University of Sofia**
                            Bachelor of Biotechnology

2002 - 2007   **First Language School Varna**

Work experience

October 2013 – February 2015	Institute of Cancer Research at the Medical University of Vienna **Master Thesis:** "Development of a bicistronic expression system for generation of Multi-Hit transgenic mice" **Supervisor:** Ass. Prof. Dr. Robert Eferl
April 2014 - June 2014	Internship at the Institute of Cancer Research at the Medical University of Vienna "Characterization of the stromal composition of HCCs developing in Mdr2-deficient mice" **Supervisor:** Ass. Prof. Dr. Robert Eferl
October 2013 - November 2013	Internship at the MFPL at the University of Vienna "Advanced Genetic Engineering and Proteomics in Yeast" **Supervisors:** Univ.Prof. Dr. Franz Klein and Dr. Wolfgang Ludwig Reiter
June 2011 - September 2012	Hewlett Packard & Adecco Bulgaria Technical Support Analyst
October 2010 - June 2011	Member of the study group of Assoc. Prof. Dr. M. Pesheva / Genetics laboratory by Faculty of Biology, University "Analysis of the Antioxidant Properties of Bulgarian Propolis "

Personal skills

Languages:

Bulgarian:	Mother tongue
German:	Very good spoken and written German (Test DaF)
English:	Very good spoken and written English

Computer skills:	Internet, Intranet, VPN, MS Office, Adobe Photo Shop, CorelDRAW Graphics Suite, GraphPad Prism, Bio Edith, Gene Clone, SnapGene