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MASTERARBEIT

Titel der Masterarbeit

Establishment of a Split-LUX Assay to Determine Protein Interaction of the BMP-Receptor mAlk2^{R206H} (FOP Mutant) with the Rapamycin-Associated Protein FKBP12

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III. ACKNOWLEDGEMENT

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Thank you, Song-Hi, for everything. Having you next to me made everything so much easier. Thanks for helping me out whenever I had questions in the lab and for knocking on my door every day so we could write next to each other.

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IV. SUMMARY

Fibrodysplasia Ossificans Progressiva (FOP) is a devastating disease caused by various mutations in the BMP receptor mAlk2, which leads to heterotopic ossification. The mutation this thesis is focusing on is an arginine to histidine substitution on position 206 in the GS (glycine-serine-motif) domain of the mAlk2 receptor. Due to this mutation, the receptors inhibition is drastically altered, leading to constitutive activity and permanently activated BMP (bone morphogenic protein) signaling, which consequently leads to pathological bone cell differentiation. Finding a cure for this disease or a way to decelerate its progression, while gaining an understanding of its cause, is crucial.

In this thesis, a protein complementation system (PCA) based on split-luciferase reassembling is established. To develop and further optimize a split-luciferase assay, a rapamycin inducible hetero-dimerization approach namely FRB (FKBP-rapamycin binding domain of FRAP) and FKBP12 (FK506 binding protein 12), fused to N- and C-terminal fragments of a luciferase, respectively, is used. Based on this method the interaction of the FOP mutant receptor ($mAlk2^{R206H}$) with it´s wild type inhibitor FKBP12 and interactions of wild type mAlk2 as well as wild type mAlk3 with FKBP12 are examined.

This work suggests, that $mAlk2^{R206H}$ inhibition by FKBP12 is weakened whereas the interaction of $mAlk2^{wt}$ as well as $mAlk3^{wt}$ can be observed by reassembling of the luciferase fragments fused to the receptors and inhibitor.

These results provide novel insight into an approach that is cheap and easy to apply to determine protein-protein interactions and highlight the need of further investigation of the interaction of the FOP mutant $mAlk2^{R206H}$ and its interaction partner FKBP12.

V. ZUSAMMENFASSUNG

Fibrodysplasia Ossificans Progressiva (FOP) ist eine verheerende und bisher unheilbare Krankheit, die durch verschiedene Mutationen, lokalisiert in dem BMP (bone morphogenic protein) Rezeptor mAlk2, verursacht wird. Diese Arbeit fokussiert auf eine Arginin/Histidin Substitution an Position 206 in der GS (Glycin-Serin-Motif) Domäne des mAlk2 Rezeptors, welche zu heterotropher Ossifikation führt. Aufgrund dieser Mutation ist die Hemmung des Rezeptors drastisch beeinträchtig. Dies führt im Folgenden zu einer konstitutiven Aktivität des mAlk2 Rezeptors und im Weiteren zu einer dauerhaften BMP Signalweiterleitung. Um eine Heilung, oder realistischer, eine Verlangsamung des Krankheitsverlaufs zu erzielen, ist ein tiefgehendes Verständnis der Ursache, welche den Rezeptor in einen konstant aktiven Zustand versetzt, essentiell.

In der vorliegenden Arbeit wird die Etablierung eines Proteinkomplemetationssystems (PCA), basierend auf der Reassemblierung von Luciferasefragmenten, beschrieben. Die Entwicklung, und die darauffolgende Optimierung, wurde anhand eines Rapamycin-induzierbaren Heterodimerisationsansatzes, im Speziellen FRB (FKBP-rapamycin binding domain of FRAP) und FKBP12 (FK506 binding protein 12), durchgeführt, in dem jeweils das N- beziehungsweise C-terminale Fragment einer Luziferase an das entsprechende Protein fusioniert wurde. Auf dieser Methode basierend, wurde die Wechselwirkung der FOP Mutante ($mAlk2^{R206H}$) und dessen Wildtypinhibitor FKBP12, als auch die Interaktion von wildtyp mAlk2 sowie wildtyp mAlk3 mit FKBP12 untersucht.

Die Ergebnisse dieser Arbeit lassen vermuten, dass die hemmende Wirkung von FKBP12 auf $mAlk2^{R206H}$ trotz nachzuweisender Interaktion verringert ist. Im Vergleich dazu ist eine stärkere Interaktion von $mAlk2^{wt}$ und $mAlk3^{wt}$ mit FKBP12, wie erwartet, nachzuweisen, und lässt daher auf eine stärkere Inhibierung schließen. Dies konnte durch die Reassemblierung der Luziferasefragmente gezeigt werden.

Die hier aufgezeigten Resultate liefern neue Erkenntnisse, Proteininteraktionen kostengünstig und relativ einfach nachzuweisen. Im Übrigen wird veranschaulicht,

dass dringende Notwendigkeit besteht, weitere Untersuchungen bezüglich der Wechselwirkung der FOP Mutante mAlk2^{R206H} mit dessen Interaktionspartner FKBP12 durchzuführen.

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Abbreviation	Meaning
Å	Armstrong
°C	Degree Celsius
µg	Microgram
µl	Microliter
aa	Amino acid
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
C	Carbon
CO_2	Carbon dioxide
Coelenterazine	Coelenterate luciferin
COOH	Carboxylic acid
cps	Counts per second
ddNTP	Dideoxy nucleoside triphosphates
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate-buffered saline
E. coli	*Escherichia Coli*
EDTA	Ethylenediaminetetraacetic acid
f	Forward primer

FBS	Fetal bovine serum
FK506	Tacrolimus
FKBP	FK506 binding protein
FRAP	FKBP-rapamycin associated protein
FRB	FKBP-rapamycin binding domain of FRAP
GFP	Green fluorescent protein
Glu	Glutamic acid
Gly	Glycine
G. princeps	*Gaussia princeps*
GLuc	*Gaussia princeps*
RLuc	*Renilla reniformis*
kDa	Kilo Dalton
LB	Lysogeny broth
L-Gln	L-Glutamine
M	Molar
min	Minute
ml	Milliliter
mM	Millimolar
mTOR	Mammalian target of rapamycin
N	Nitrogen
n.a.	Not available
NaCl	Sodium chloride
nm	Nanometer
o/n	Over night

PCA	Protein complementation assay
PCR	Polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PLB	Passive lysis buffer
pM	Picomolar
PPBT	Potassium phosphate buffer
Sec	Second
r	Reverse primer
R. reniformis	*Renilla reniformis*
Rpm	Revolutions per minute
TGF-β	Transforming growth factor β
U	Unit

1 BACKGROUND

1.1 SPLIT LUCIFERASE PROTEIN COMPLEMENTATION ASSAY

Biochemical pathways are systems of dynamically assembling and disassembling protein complexes. Thus, much of biological research nowadays is dealing with how, when, and where proteins interact with other proteins involved in biochemical processes. In the PCA (protein complementation assay) strategy protein-protein interaction can be measured by fusing each of the proteins of interest to two fragments of a reporter protein that has been rationally dissected into two fragments. If the proteins interact, the reporter fragments are brought into close proximity, fold into their native structure, and the PCA reporter activity is reconstituted (Ingrid Remy & Michnick, 2006). PCAs can be based on a variety of reporters giving different readouts as fluorescence (BIFC), luminescence colorimetric signals (LUX, Split-LUX PCA), as well as simple survival selection PCA (Ingrid Remy & Michnick, 2006). So far PCAs are performed using fluorescent proteins like green fluorescent protein (GFP) because a signal is provided by the intrinsic fluorophore. Nonetheless, fluorescent proteins have to be expressed at very high levels to assure signal is above background cellular fluorescence (Michnick, Ear, Manderson, Remy, & Stefan, 2007). Further, protein complementations assayed using fluorescence are non-dynamic. Moreover, luciferase reporters are actually more sensitive than fluorescence reporters because they obviate the need for exogenous illumination. External light often bleaches the fluorescence to some extent, yields a higher background fluorescence, perturbs physiology in light-sensitive tissues, and causes phototoxic damage to analyzed cells (T. F. Massoud & Gambhir, 2003). Using luminescence proteins as humanized *Renilla* luciferase (RLuc) or humanized *Gaussia* luciferase (GLuc) on the other hand allows detection of protein dynamics. Reporter gene-based approaches using full-length luciferases, allow verification of effect on transcriptional activity (Heining, Schwappacher, Horbelt, Huber, & Knaus, 2009).

1.1.1 PCA PRINCIPLE

PCAs are becoming a common method for studying the dynamics of protein-protein interactions in cells. In this strategy, two proteins of interest are fused to complementary fragments of a reporter protein (Figure 1). If the proteins interact, the reporter fragments are brought into close proximity, fold into the native structure, and the PCA reporter activity is reconstituted (I Remy & Michnick, 2001). As the name implicates, Split-LUX is based on luminescence reporter genes. For this project the N- and C-terminal fragments of *Gaussia princeps* and *Renilla reniformis* were fused to a rapamycin inducible heterodimerization approach, namely FRB (FKBP-rapamycin binding domain of FRAP) and FKBP12 (FK506 binding protein 12), respectively, in order to establish and further optimize Split-LUX PCA. The established Split-LUX assay was further used to examine protein interactions of BMP (Bone Morphogenic Protein) receptors, especially activin receptor like kinase 2 (mAlk2) carrying a point mutation on position 206 (Arg --> His), which is involved in Fibrodysplasia Ossificans Progressiva with its inhibitor FKBP12.

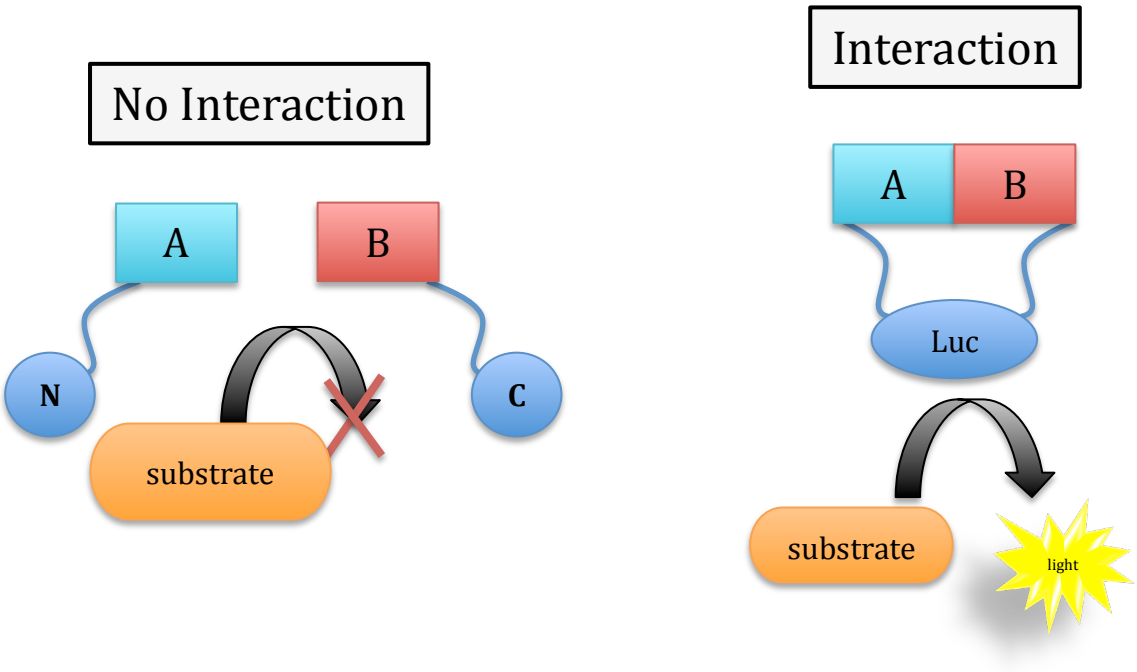


Figure 1: Basic principle of the Split-LUX PCA. The basic strategy is the fusion of two proteins of interest to two complementary fragments of a reporter gen. On the left side no interaction of the proteins of interest is taking place. Therefore, the reporter gene fragments do not come in close proximity and cannot reassemble (N and C represent N- and C-terminal luciferase fragments). Thus, no substrate conversion can be observed. On the right side the proteins and, subsequently, the reporter gene fragments do interact. This leads to substrate conversion into light, which can be measured.

1.1.2 Luciferases as Reporter Proteins

Luciferases are a diverse class of enzymes characterized by the emission of photons on substrate turnover (Wilson & Hastings, 1998). In the presence of their corresponding substrates, the bioluminescence signal generated can be quantified using a bioluminometer, indicating the expression levels of the transgenic luciferase reporter gene copies (Hampf & Gossen, 2006). The luciferases isolated from *Photinus pyralis* (FLuc) (Wet & Wood, 1987) and *Renilla reniformis* (RLuc) (W W Lorenz et al., 1996) raised interest due to their combined features ideally expected from reporter enzymes as high sensitivity, lack of background signal in most cellular systems, and ease and cost-effectiveness of the assay system (Hampf & Gossen, 2006). FLuc and RLuc use luciferin or coelenterazine as their substrates, respectively. In addition, *Gaussia princeps* luciferase (GLuc) was used to establish Split-LUX due to its smaller size (185 aa) and 100-fold higher bioluminescent

signal intensity (Ingrid Remy & Michnick, 2006) compared to FLuc and RLuc. The firefly vector pGL3-SV40 was used for normalization as it converts the substrate luciferin whereas GLuc uses coelenterazine.

1.1.2.1 *Gaussia Princeps* Luciferase (GLuc)

Gaussia princeps luciferase, which got isolated from the marine copepod G. princeps, is a monomeric protein of 185 amino acids (19,9 kDa). It catalyzes the oxidation of the substrate coelenterate luciferin (coelenterazine) in a reaction that emits blue light (480 nm) as *Renilla reniformis* luciferase does.

The substrate can permeate cell membranes and diffuse into all cellular compartments, allowing quantitative analysis in live cells. The humanized form (codon optimized for expression in cultured mammalian cells) of *Gaussia princeps* luciferase (hGLuc - referred to as GLuc for simplification in the following) can generate over 100-fold higher bioluminescent signals than the humanized forms of *Photinus pyralis* luciferase (firefly, FLuc) and *Renilla reniformis* luciferase (RLuc) in cell lysates, and is the smallest known coelenterazine-using luciferase, making it an ideal candidate for developing PCAs (Tannous, Kim, Fernandez, Weissleder, & Breakefield, 2005). Furthermore, GLuc constructs separated between Gly93 and Glu94 were used for fusion due to their high luminescence activity after complementation. The leucine Zipper–induced complementation of GLuc fragments results in reconstitution of GLuc activity (Ingrid Remy & Michnick, 2006). Further, the signal peptide of GLuc was deleted to avoid cytoplasmic secretion of the luciferase.

1.1.2.2 *Renilla Reniformis* Luciferase (RLuc)

Renilla reniformis luciferase is a well studied luciferase of 311 amino acids (34 kDa) and catalyzes the oxidative decarboxylation of coelenterazine in the presence of dissolved oxygen to yield oxyluciferin, CO_2, and blue light at 480 nm (W Walter Lorenz & Mccann, 1991) (Figure 2).

It is a common used reporter system for luciferase reporter assays as well as for PCAs. For this project RLuc fragments separated between amino acids 229 and 230 were used (Paulmurugan, Massoud, Huang, & Gambhir, 2004).

$$luciferase + coelenterazine + O_2 \Leftrightarrow\ luciferase + coelenteramide + CO_2 + hv$$

Figure 2: Conversion of the substrate coelenterazine by *Gaussia Princeps* luciferase (GLuc) and *Renilla Reniformis* luciferase (RLuc). RLuc and GLuc convert Coelenterazine into coelenteramide, CO_2, and light with an emission peak at 480 nm.

1.1.2.3 *PHOTINUS PYRALIS* LUCIFERASE (FLUC)

FLuc, a luciferase isolated from the North American *Photinus Pyralis,* catalyzes its substrate in a 2 step reaction (Figure 3) in the presence of ATP and O_2 to emit yellow-green light with an emission peak at 560 nm (Wet & Wood, 1987).

Reaction I:

$$luciferase\ +\ luciferin\ +\ ATP \overset{Mg_2}{\Longleftrightarrow} luciferase \cdot luciferyl - \mathrm{AMP}\ + PP_i$$

Reaction II:

$$luciferase \cdot luciferyl - AMP + O_2 \Longrightarrow luciferase + oxyluciferin + AMP + CO_2 + hv$$

Figure 3: Substrate conversion by *Photinus Pyralis* luciferase (FLuc). FLuc converts luciferin in a 2-step reaction into oxyluciferin, AMP, CO_2 and light with an emission peak at 560 nm.

1.1.3 NORMALIZATION OF SPLIT-LUX BY THE USE OF PGL3-SV40 FIREFLY VECTOR

For normalizing luminescence values obtained from RLuc and GLuc Split-LUX measurements the firefly vector pGL3-SV40 (simian virus 40) was implemented in

the system. This firefly vector was chosen due to its different substrate consumption compared to RLuc and GLuc. Whereas RLuc and GLuc use coelenterazine, FLuc activity can be measured by adding luciferin to the cell lysates, which allows an easy to perform set up where both, GLuc (or RLuc) and FLuc can be measured in the same probe.

1.1.4 Optimization of Split-LUX Using Protein Interaction of FKBP12 and FRB

In this thesis the known and already well-studied interaction of FRB and FKBP12 was used to establish and optimize the Split-Luciferase Complementation Assay.

The FKBP-rapamycin binding domain (FRB) is a 100 amino acid domain of the mammalian target of rapamycin (mTOR), also known as FK506 binding protein 12 - rapamycin associated protein 1 (FRAP 1 or FKBP12) (Banaszynski, Liu, & Wandless, 2005). FKBP12 is the 12 kDa FK506-binding protein discovered as a target of the immunosuppressant drug FK506 and elicits its effects by binding to it (Aghdasi et al., 2000). Besides the ability of FKBP12 to inhibit mTOR kinases or calcineurine phosphatases upon binding to rapamycin and FK506, respectively, it also binds to the unphosphorylated GS domain of Alk2 receptors (ACVR1) to inhibit BMP downstream signaling (Brasseura & Rotureauc, 2012). FKBP12 occurs in very high concentrations in all cells. Thus, it might be expected to regulate fundamental aspects of cell biology like regulation of the cell cycle (Aghdasi et al., 2000). Since the name implicates the very high affinity to rapamycin, rapamycin is crucial for the interaction of FRB and FKBP12 (Figure 4).

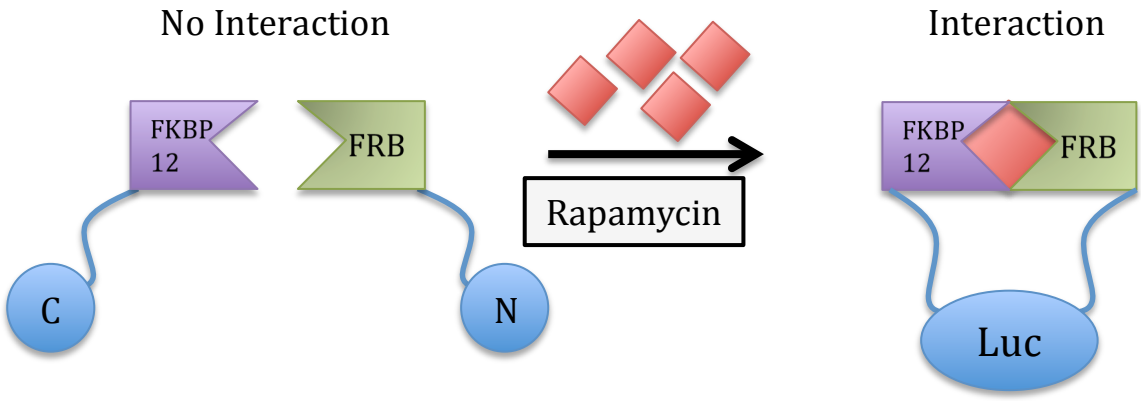


Figure 4: FKBP12/FRB interaction scheme. The C-terminal fragment of Renilla or Gaussia luciferase gets fused to FKBP12 whereas FRB is tagged with the N-terminal fragment. Addition of rapamycin is necessary to facilitate FKBP12/FRB protein complementation. When complementation of the proteins proceeds, fused luciferase fragments can complement as well and light emission, resulting from substrate conversion, can be measured.

1.1.4.1 RAPAMYCIN

Rapamycin is a 31-membered macrolide antifungal antibiotic first isolated from *Streptomyces hygroscopicus,* which shows high structural similarities to FK506. Rapamycin binds with high affinity to FKBP12 as well as to the FRB domain of mTOR. Rapamycin is an FDA-approved drug used clinically as an immunosuppressant for organ transplants. It is also important scientifically, as it is a potent and specific inhibitor of mTOR, a serine/threonine protein kinase which is a major regulator of cell growth and proliferation through the regulation of protein synthesis (Banaszynski et al., 2005).

1.1.4.2 STRUCTURE OF THE FKBP12-RAPAMYCIN COMPLEX INTERACTING WITH FRB

As already mentioned, rapamycin is a potent immunosuppressive agent that binds two proteins named FKBP12 and the FKBP-rapamycin binding domain (FRB) of mTOR. The crystal structure of the ternary complex of human FKBP12, rapamycin, and the FRB domain of FRAP at a resolution of 2.7 Å revealed the two proteins bound together as a result of the ability of rapamycin to occupy two different binding pockets simultaneously. The structure shows strong interactions between

rapamycin and both protein but hardly any interaction between these proteins without the conjunction of rapamycin (Choi & Chen, 1996).

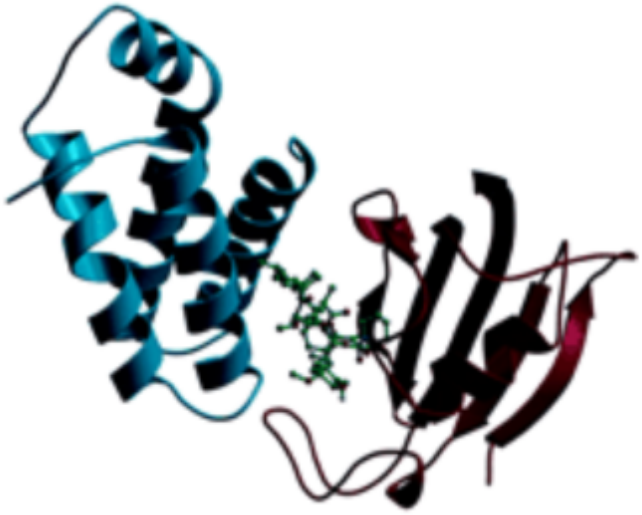


Figure 5: Structure of the FKBP12-rapamycin-mTOR complex. Structure of the ternary complex of the FKBP12-rapamycin binding domain on the FRAP/mTOR molecule (FRB) (blue), rapamycin (green), and FKBP12 (red) (Cardenas et al., 1999).

To show that rapamycin is crucial for the interaction of FKBP12 and FRB all experiments were performed with and without addition of rapamycin.

1.1.4.3 FK506

FK506 is like rapamycin a potent immunosuppressant, which is used to treat graft rejection in transplant recipients. FK506 binds as rapamycin the active site of FKBP12 (Cruz et al., 1999). If FK506 binds FKBP12, FKBP12 no longer binds to the GS domain of Alk1 (activin receptor-like kinase 1), Alk2, or Alk3 and thereby activates downstream Smad1/5, MAPK signaling and ID1 gene regulation, which is a BMP target gene, superior to rapamycin (Spiekerkoetter et al., 2013). In this thesis rapamycin is used, as described above, for protein complementation of FKBP12 and FRB. Furthermore, rapamycin and FK506 are used to inhibit FKBP12 binding to mAlk2^{wt}, mAlk2^{R206H}, and mAlk3^{wt}. Protein interaction of FKBP12 and the BMP receptor mAlk2 is explicitly described in 1.2.3 “Interaction of FKBP12 and mAlk2^{R206H}”.

1.1.4.4 FKBP12/FRB-FUSION CONSTRUCTS

In this project luciferases of *Renilla reniformis* and *Gaussia princeps* are used as reporter proteins. By using the previously mentioned well-studied protein-protein interaction of FKBP12 and FRB, it was possible to compare these systems with each other under the same conditions. To do so, either the C- or N-terminal fragment of the luciferase was fused to FKBP12 and FRB, respectively (Figure 6).

Figure 6: FKBP12 and FRB luciferase fusion constructs. (A) Fusion proteins FKBP12-CGLuc and FRB-NGLuc (Ingrid Remy & Michnick, 2006); (B) Fusion proteins FKBP12-CRLuc and FRB-NRLuc (Paulmurugan et al., 2004).

Furthermore, luciferase fragments were fused to BMP-receptors to examine BMP-receptor and FKBP12. Detailed description can be found in 1.2.4 "BMP-Receptor-Fusion Constructs".

1.2 FIBRODYSPLASIA OSSIFICANS PROGRESSIVA (FOP)

Fibrodysplasia Ossificans Progressiva (FOP, OMIM #135100) is a rare but devastating disorder of extra-skeletal bone formation having a worldwide prevalence of approximately 1 in 2,000,000 (E M Shore, Feldman, Xu, & Kaplan, 2005). Phenotypically it is an autosomal genetic disorder characterized by two clinical features as congenital malformations of the hallux (big toe) and heterotopic ossification in characteristic anatomical patterns (Lucotte, Houzet, Hubans, Lagarde, & Lenoir, 2009) (Figure 8). In FOP connective tissue progressively metamorphoses through an endochronal process into cartilage that is replaces by bone. Heterotopic ossification is typically seen first in the dorsal, axial, cranial and proximal regions of the body and later also observed in the ventral, appendicular, caudal and distal regions (Cohen, 1993; Frederick S Kaplan, Chakkalakal, & Shore, 2012; Rocke DM, Zasloff M, Peeper J, Cohen RB, 1994).

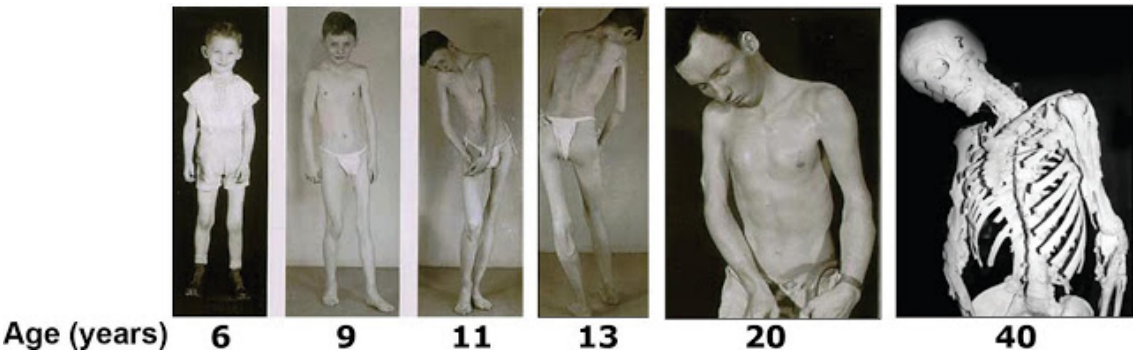


Figure 7: Progression of FOP over time.

In FOP patients, heterotopic ossification can be induced by physical or surgical stress, which limits the ability to obtain human biopsy tissue for research (Billings et al., 2008). Although skeletal muscle it the tissue most often effected by heterotopic ossification, extra-skeletal bone likewise forms in other connective tissues such as aponeuroses, fascia, ligaments and tendons. The diaphragm, tongue and extra-ocular muscles are spared from FOP. Furthermore, cardiac muscle and smooth muscle are not affected (Connor, 1982).

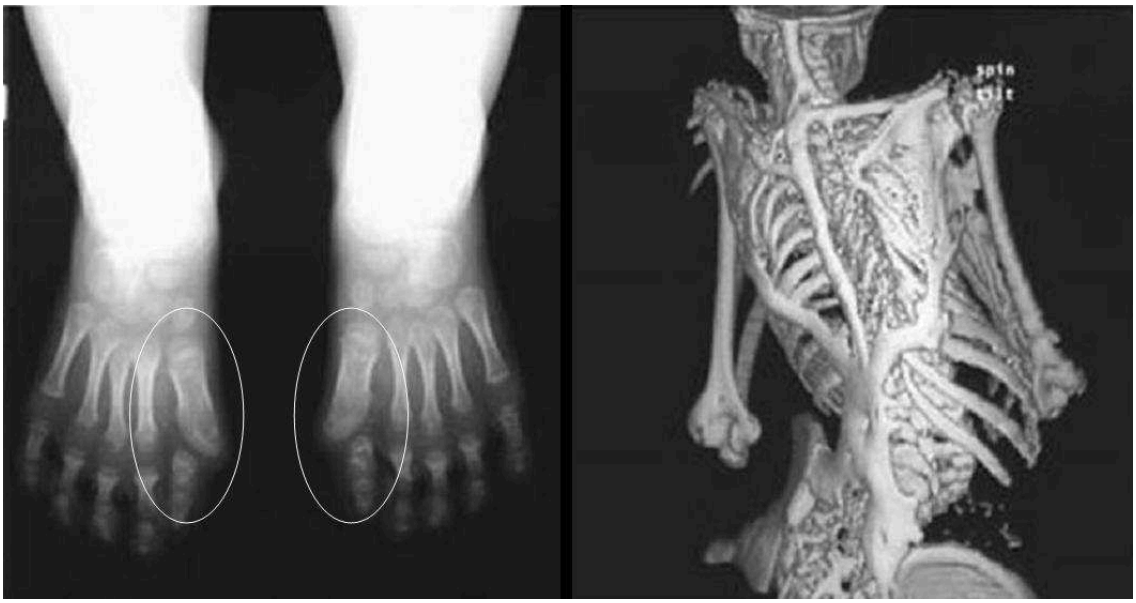


Figure 8: Characteristic bone formations in FOP. Left: antero-posterior feet radiography of a 3 year old child showing a symmetrical malformation of the great toe. Right: 3-D computer tomography of a 12 year old patients dorsum (F S Kaplan et al., 2008).

Additively, molecular mechanisms involved in FOP might offer an initial point to create new skeletal elements for regenerative medicine to treat bone fractures, failed spine fusions, traumatic bone loss or congenital agenesis of skeletal elements (Frederick S Kaplan et al., 2012). The genetic mutation, amongst others (Figure 10), causing FOP is an amino acid substitution of arginine to histidine at position 206 (R206H) on the mAlk2 receptor gene, a receptor involved in the BMP (Bone Morphogenic Protein) signaling. This mutation shifts the mAlk2 receptor, which is a BMP type 1 receptor, into an constantly active state resulting in heterotopic ossification of connective tissue.

1.2.1 Bone Morphogenic Protein (BMP) Signaling

Bone Morphogenetic Proteins (BMPs) are a class of morphogens belonging to the transforming growth factor β (TGF-β) superfamily originally discovered due to their bone inducing capacity. BMPs have the unique function of inducing cell differentiation of the osteoblastic lineage. Thus, increasing the differentiated function of osteoblasts (Canalis, 2003).

BMPs bind as dimers to their receptors, serine/threonine kinase receptors, to induce intracellular signaling. Upon ligand binding, these receptors assemble as heteromeric complexes consisting of two type I and two type II BMP-receptors

(Vrijens et al., 2013). Binding of BMP results in phosphorylation of the type I receptor by the type II receptor, which induces a conformational change of the type I receptor, whereby its stericly inhibited catalytic center becomes unblocked. Subsequently, the activated type I receptor transduces intracellular signals by phosphorylating the C-terminal domain of Smad1, 5, and 8 in the cytoplasm. Phosphorylation of these Smad molecules initiates their binding to the mediator molecule Smad4 resulting in a hetero-oligomeric complex. This complex in turn enters the nucleus in conjunction with nuclear factors and induces the expression of its specific target genes (Pasero, Giovarelli, Bucci, Gherzi, & Briata, 2012; Shi & Massague, 2003).

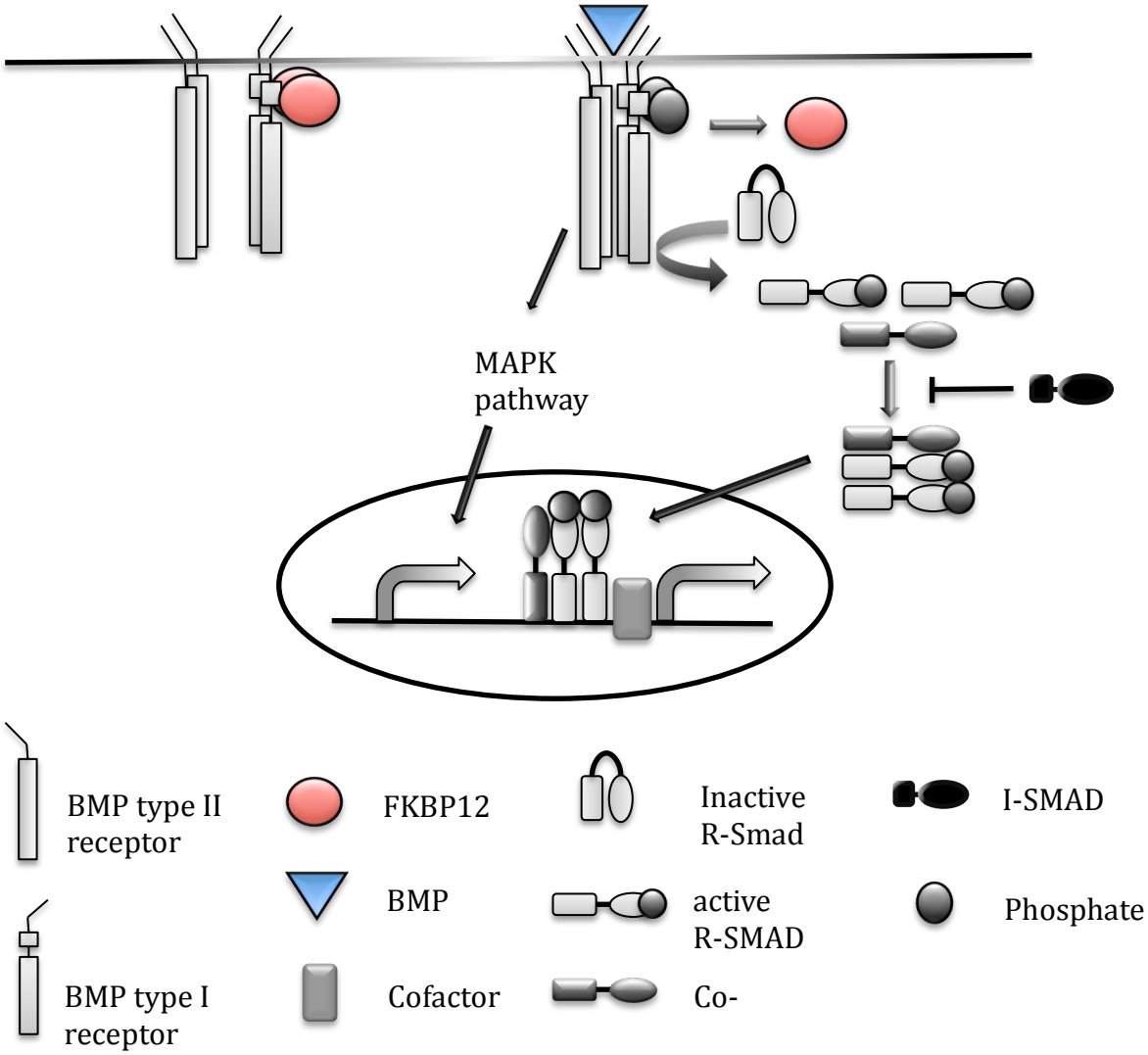


Figure 9: Canonical BMP signaling pathway. BMP canonical signaling is transduced by two type I (BMPR-IA and BMPR-IB) and two type II transmembrane serine/threonine kinase receptors, BMPR-II. BMPs bind to the heteromeric complex of type I and type II receptors. Subsequently, the type II receptor phosphorylates the type I receptor, which in turn facilitates phosphorylation of Smad1 and Smad5 (R-Smads). R-Smads directly interact with the activated type I receptor and are released upon phosphorylation. Following release from the receptor complex, R-Smads complex with Co-Smad4 and translocate into the nucleus to modulate the transcription of target genes.

1.2.2 ACVR1 R206H Mutation and its Impact on BMP Signaling

Mutations in the activin A receptor type 1 (ACVR1), also known as activin receptor like kinase 2 (Alk2), one of the bone morphogenic protein (BMP) type 1 receptors (BMPR1), have been reported in FOP patients (Song et al., 2010) and occur in highly conserved amino acids, indicating their functional importance (F S Kaplan

et al., 2009). Previous research predicts that these mutant receptors activate ACVR1 proteins and therefore increase receptor signaling (Bocciardi, Bordo, Di Duca, Di Rocco, & Ravazzolo, 2008; F S Kaplan et al., 2009; F S Kaplan, Groppe, Pignolo, & Shore, 2007; Petrie et al., 2009). Hence, signal transduction through the BMP pathway is altered in cells from individuals with FOP (Ahn, Serrano de la Pena, Shore, & Kaplan, 2003; Billings et al., 2008; de la Peña et al., 2005; Fiori, Billings, de la Peña, Kaplan, & Shore, 2006; Shafritz et al., 1996). This alteration results in increased phosphorylation of BMP pathway mediators as BMP-specific Smad proteins as well as p38 MAPK and enhanced expression of BMP transcriptional targets even when exogenous BMP ligands are absent (Frederick S Kaplan et al., 2012). Previous in vitro and in vivo analyses demonstrated that BMP signaling can be induced by the mutant ACVR1^{R206H} receptor, which activates the BMP signaling cascade without the need of BMP for initiation (Dinther et al., 2010; Fukuda et al., 2009; Shen et al., 2009; Song et al., 2010).

The ACVR1 mutation this thesis is focusing on is the p.R206H (termed mAlk^{R206H} in the following) mutation, which is an arginine to histidine substitution on codon 206 located right at the end of the glycine-serine-rich (GS) activation domain of mAlk2, schematically depicted in Figure 10 as further mutations associated with FOP.

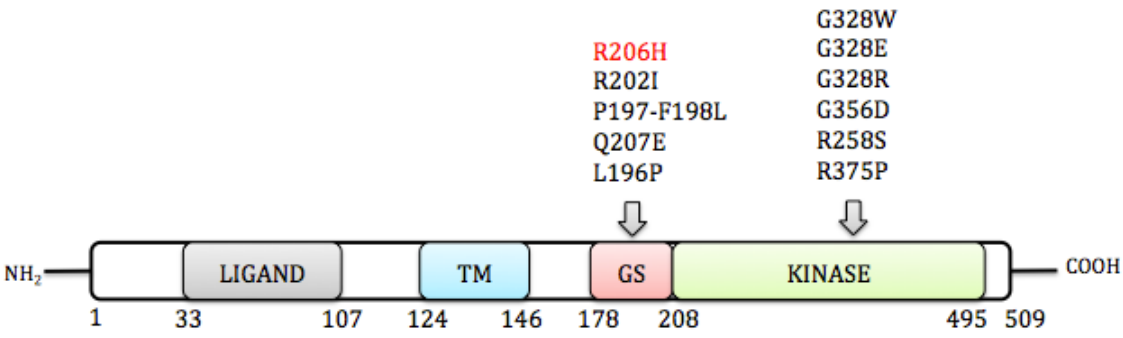


Figure 10: Schematic representation of the mAlk2 receptor and its known mutations. mAlk2 receptor scheme showing the localization of the R206H mutation in the GS domain. This arginine to histidine substitution constantly activates mAlk2 and, subsequently, BMP signaling. Besides mAlk2^{R206H} further mutations associated with FOP are depicted, which are located in the highly conserved GS or kinase domain (TM: transmembrane domain; GS: glycine-serine-rich activation domain; kinase: dual-specific kinase domain).

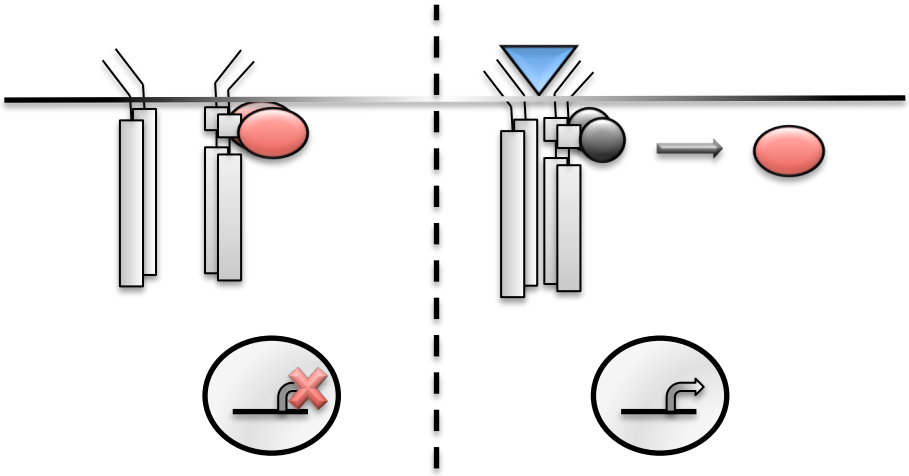
In BMP/BMP receptor (BMPR) signaling, the receptors GS domain is a very important site for transferring phosphorylation from a type II receptor to a type I receptor (Wieser, Wrana, & Massagué, 1995). Alteration of this highly conserved domain by a point mutation like R206H has a high impact on downstream signaling by constitutively activating it.

In this mechanism FKBP12 plays a role as a safeguard molecule for signal leakage by binding to the unphosphorylated GS domain (Wang, BY, & PD, 1996) . During ligand binding to the receptors, the serine moiety of the GS domain gets phosphorylated triggering a conformational change around the domain, which subsequently causes dissociation of bound FKBP12 from the GS domain. Further, R-Smads (Smad1, Smad5, and Smad8) bind to the phosphorylated GS domain (Huse, YG, Massague, & Kuriyan, 1999). It has been shown that phosphorylation levels of Smad1 or Smad5 are elevated with mAlk2^{R206H} mutation in C2C12 cells. 2010 Dinther et al. suggested that the mAlk2^{R206H} mutation might be associated with an impaired FKBP12 interaction and induction of abnormal BMP signaling (Dinther et al., 2010; Nojima et al., 2010) as Shore and Kaplan hypothesized, that FKBP12 binds less efficient to the mutated GS domain and, therefore, allows increased activation of BMP signaling (Eileen M Shore & Kaplan, 2008).

1.2.3 Interaction of FKBP12 and mAlk2^{R206H}

As it is already known from previous research, FKBP12 binds to the GS domain of the mAlk2^{wt} receptor, which leads to an inhibition of the BMP downstream signaling when the ligand is absent. In general, upon binding of the ligand BMP, BMP receptor type I and type II get into close proximity and type II gets phosphorylated by type I. Phosphorylation of the GS domain subsequently leads to the release of FKBP12 and therefore to active downstream signaling. In the present thesis the inhibition capacity of FKBP12 on the mAlk^{R206H} is examined. To do so, also the influences of the previous described rapamycin and FK506, FKBP12 inhibitors, on the interaction of FKBP12 and mAlk^{R206H} were tested.

A) ACVR1 wild-type (Arg206)



B) ACVR1 FOP Mutation (His206)

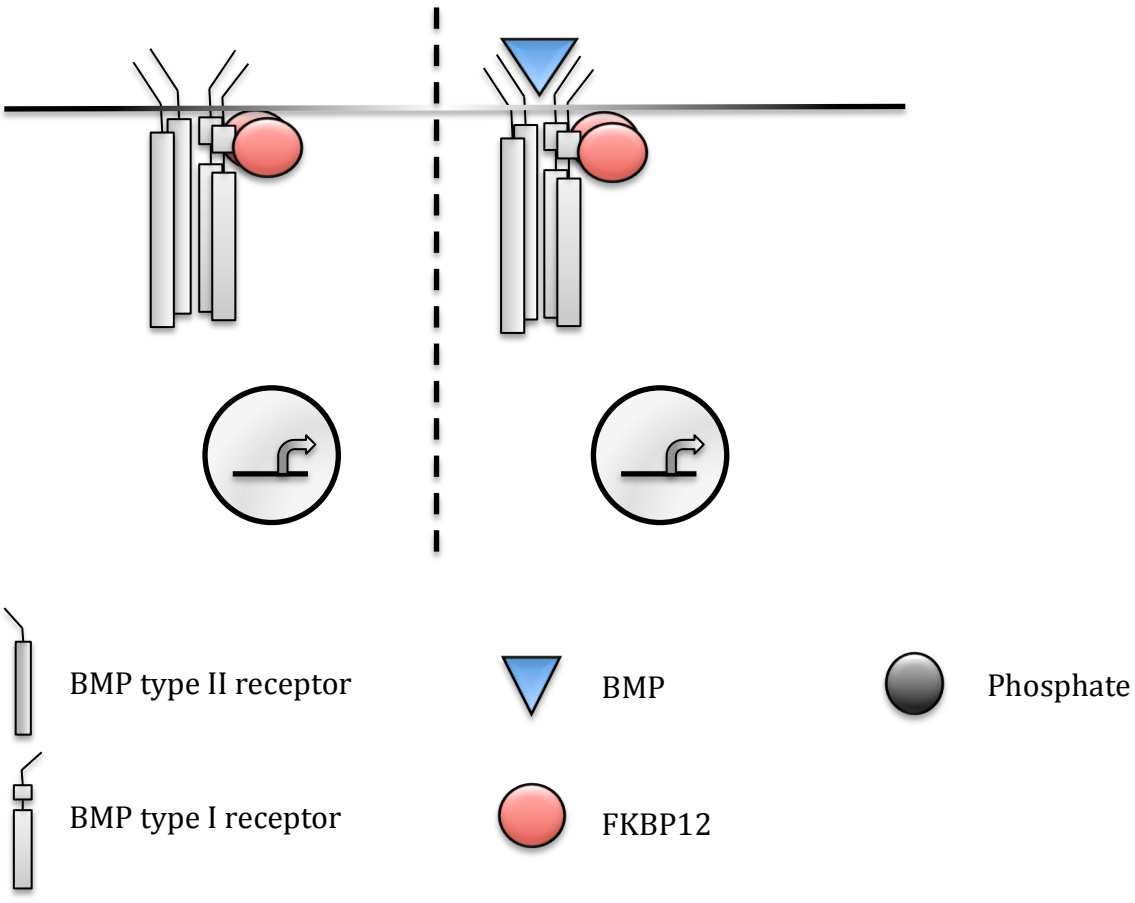


Figure 11: Hypothetical model of ligand independent activation in FOP. A) ACVR1 wild-type (Arg206): In the absence of BMP ligand, binding of FKBP12 to GS domain prevents leaky activation of downstream signaling. BMP binding triggers assembling of BMP type II and BMP type I

receptors. Further, the GS domain of BMP type II receptors get phosphorylated by type I, induces dissociation FKBP12 and BMP signaling gets activated. B) ACVR1 FOP Mutation (His206): hypothesis that FKBP12 does not correctly bind to the mutant FOP ACVR1 type I receptor (with histidine at codon 206; His206) either in the presence or the absence of BMP-receptor binding, allowing increased activation of BMP signaling (Eileen M Shore & Kaplan, 2008).

1.2.4 BMP-Receptor-Fusion Constructs

In order to examine BMP-receptor interactions of the BMP-receptors mAlk2^{wt}, mAlk2^{R206H}, and mAlk3^{wt} with FKBP12, N- and C-terminal fragments of GLuc were fused to their C-termini. Further description of the cloning strategy can be found in the Materials and Methods section.

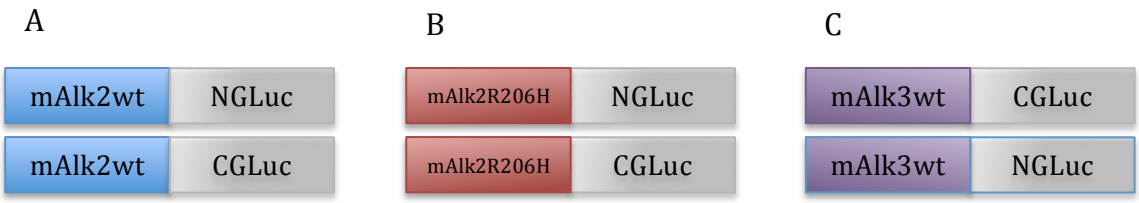


Figure 12: BMP-receptor-fusion constructs. In order to examine BMP-receptor interaction of (A) mAlk2^{wt}, (B) mAlk2^{R206H}, and (C) mAlk3^{wt} with FKBP12, N- and C-terminal fragments of GLuc were fused to BMP-receptor genes.

2 AIM

The aim of this thesis is to develop and, further, optimize a protein complementation assay (PCA) based on Split-Renilla (RLuc) and Split-Gaussia (GLuc) luciferase. To determine appropriate conditions for Split-LUX, a rapamycin inducible hetero-dimerization approach is used containing the proteins FKBP12 (rapamycin associated protein also known as RAFT) and FRB (FKBP-rapamycin binding domain) fused to C- or N-terminal luciferase fragments, respectively. FKBP12 and FRB luciferase fusion proteins were developed and validated previously by Paulmurugan et al, 2004 (Split-RLuc fusions) and Remy & Michnick, 2006 (Split-GLuc fusions). The purpose of investigating a Split-LUX assay is to determine the interaction of the BMP-receptors mAlk2^{wt}, mAlk2^{R206H} (FOP mutant), and mAlk3^{wt} with their inhibitor FKBP12 in order to examine whether FKBP12 inhibition on the FOP mutant receptor is altered compared to the inhibition capacity of FKBP12 on mAlk2^{wt}.

3 MATERIALS AND METHODS

3.1 MATERIALS

In the following, chemicals, buffer and solutions, bacteria, vectors, plasmids, primers, cells, technical devices, molecular biological reaction systems, and software and databases used are listed.

3.1.1 CHEMICALS

Used chemicals are listed in Table 1.

Table 1: Chemicals

Chemicals	Company
Coelenterazine	Biosynth
Gene ruler 100bp DNA ladder	Fermentas
Adenosintriphosphate (ATP)	Roche
Ampicillin	Roche
Agarose	SIGMA
Deoxyribonukleotidtrisphosphate (dNTPs)	Fermentas
Dulbecco´s Modified Eagle´s Medium (DMEM)	LONZA
Dulbecco´s Phosphate Buffered Saline (DPBS)	LONZA
Ethidiumbromide	Roth
Fetal calf serum (FCS)	Biochrome
LB-Agar Media	Merck
L-Glutamine (200 mM in 0,85 % NaCl-solution)	LONZA
NaCl 150 mM	
KH_2PO_4	Merck

K_2HPO_4	Roth
Orange G, Loading Dye	SIGMA
PEI 40 kDa	Polyscience, Inc
Triton-X-100 (Octylphenolpolyethylenglycol)	SIGMA

3.1.2 BUFFERS AND SOLUTIONS

Table 2: Buffers and solutions

Buffer/Solution	Composition/Company
Agarose-Gel	1 % (w/v) Agarose in SB-Puffer; 0,0025 % (v/v) EtBr
100 mM potassium phosphate buffer (PPBT)	9.2 mM KH2PO4, 91 mM K2HPO4 , 0,2 % TritonX100 in H_2O bidest
Passive lysis buffer (PLB)	Promega
SB buffer 10x	50 M $Na_2B_4O_7$ $10H_2O$ in H_2O
LB-Media	YT Mix (3,2 g Trypton, 2 g Hefeextrakt, 1 g NaCl), NaCl

3.1.3 BACTERIA

For cloning of expression plasmids, chemically competent Escherichia coli (E.coli) cells (strain TOP10) from Invitrogen were used.

3.1.4 CELLS

For transfection assays COS-1 cells (green monkey kidney cell line; source ATCC) were used.

3.1.5 VECTORS

Vectors used in this thesis are listed in Table 3 and Table 4.

Table 3: Expression vectors and plasmids

Vector	Reference
pcDNA3.1/zeo(+) FKBP12-CGLuc	(Remy and Michnick 2006)
pcDNA3.1/zeo(+) FRB-NGLuc	(Remy and Michnick 2006)
pcDNA3.1/zeo(+) zipper-CGLuc	(Remy and Michnick 2006)
pcDNA3.1/zeo(+) zipper-NGLuc	(Remy and Michnick 2006)
pCMV FKBP12-CRLuc	(Paulmurugan, Massoud et al. 2004)
pCMV FRB-NRLuc	(Paulmurugan, Massoud et al. 2004)
pCS2+	(Aberle et al., 1997)
pCS2+ mAlk2R206H	(Seemann)
pCS2+ mAlk2wt	(Seemann)
pCS2+ mAlk3	(Seemann)
BRE	(Monteiro et al. 2004)

For storage and transfer reasons the luciferase fragments were cloned into the shuttle vector pSLAX, where ClaI restriction sites flank the insert. Further, the corresponding N- or C-terminal luciferase fragment was cloned to the C-terminus of the corresponding BMP-receptor.

Table 4: Shuttle vector

Vector	Reference
pSLAX	Morgan und Fekete, 1996

Table 5: Expression plasmids carrying GLuc fusion constructs

Vector
pCS2+ mAlk2R206H-NGLuc
pCS2+ mAlk2R206H-CGLuc
pCS2+ mAlk2wt-NGLuc
pCS2+ mAlk2wt-CGLuc
pCS2+ mAlk3wt-NGLuc

3.1.6 PRIMERS

Primers were purchased from Eurofines MWG Operon. The lyophilized oligonucleotides were dissolved in water to a concentration of 100 pM and stored at -20 °C.

3.1.6.1 PRIMERS FOR GLUC FRAGMENT AMPLIFICATION

Primers for GLuc Fragment amplification were designed manually and are listed in Table 6.

Table 6: Primers for GLuc fragment amplification and sequencing

Luciferase fragment	Primer	Sequence
N-GLuc	PstI_GSlinker_f NGLuc_GSlinker_r	n.a.
C-GLuc	PstI_GSlinker_f CGLuc_GSlinker_r	n.a.

3.1.6.2 PRIMERS FOR MUTAGENESIS

Primers for mutagenesis were designed manually and are listed in Table 7. Mutagenesis was performed in order to delete the stop codon at the C-terminus of mAlk2^{wt}, mAlk2^{R206H}, and mAlk3^{wt}.

Table 7: Primers for mutagenesis

Gene	Mutation	Sequence
mAlk2^{wt}	STOP_d_f STOP_d_r	n.a.
mAlk2^{R206H}	STOP_d_f STOP_d_r	n.a.
mAlk3^{wt}	STOP_d_f STOP_d_r	n.a.

3.1.6.3 Primers for Sequencing and PCR Amplification

Primers used for sequencing are listed in Table 8.

Table 8: Primers for sequencing and PCR

Name	Sequence
CMVf	CGCAAATGGGCGGTAGGCGTG
BGHr	TAGAAGGCACAGTCGAGG
Sp6	CATTTAGGTGACACTATAG
T3	AATTAACCCTCACTAAAGGG
T7	TAATACGACTCACTATAGGG
mAlk2_seq318f	CTGCCCACTAAAGGGAAGTC
mAlk2_seq618f	CAGATAACCCTGTTGGAGTG
mAlk2_seq920f	GATTGTACTGTCCATAGCCA
mAlk2_seq1222f	GCCTTGTTCTGTGGGAAGTG
mAlk2_seq360r	GAAATTCTGTGTTCCGGGGAAGG
mAlk2_514r	TGATGAGCCCTTCAATGGTA
mAlk3_f	n.a.
mAlk3_r	n.a.

3.1.7 Molecular Biological Reaction Systems (Kits)

Kits used for DNA sequencing and DNA purification are listed in Table 9.

Table 9: Molecular biological reaction systems (kits)

Name	Use	Company
BigDye V3.1	DNA sequencing	Applied Biosystems
FastPlasmid Mini Kit	Mini DNA preparation	5Prime
Pure Yield™ Plasmid Midiprep System	Endotoxin free Midi DNA preparation	Promega
Gel-out Omni pure OLS	DNA purification	OMNI Life Science

3.1.8 Media, Buffers, and Disposables for Cell Culture Work

Disposables for cell culture work like cell culture plastics, cell culture media and additives were purchased from BD, Biochrom AG, Corning, Gibco and Lonza.

3.1.9 Technical Devices

The technical devices used are listed in Table 10 and Table 11.

Table 10: Technical devices

Name	Company
ABI 3730 capillary sequencing machine	Applied Biosystems
Biomek NXP system	Beckman Coulter
Centrifuge Allegra X15R	Beckman Coulter
Clean Bench HeraSafe KSP18	Thermo Scientific
CO_2 Incubator	Binder
Microfuge 22R	Beckman Coulter

Mithras luminescence system	Berthold
PCR cycler FlexCycler	Analytik Jena
PCR cycler Gene Amp PCR System 9700	Applied Biosystems
Tabletop Combi Spin FVL2400N	Hartenstein

Table 11: Further devices

Name	Company
Agarose gel chamber	PeqLab
CASY cell counter system	Roche Innovatis
Gel electrophoresis equipment	PeqLab
Ice machine AF30	Scotsman
Multichannel pipettes	Eppendorf
NanoDrop 1000 spectrophotometer	NanoDrop Technologies
Pipettes	Eppendorf
Rocker Duomax 1030	Heidolph
UV-Gel documentation system NightHawk	Berthold Technologies
Vortexer Vortex Genie 2	Scientific Industries
Water bath DC10	Thermo Scientific
Xstream Multipette	Eppendorf

3.1.10 SOFTWARE, DATABASES AND SERVER

Software, databases, and server used in this thesis are listed in Table 12 and Table 13.

Table 12: Software

Name	Application
DNAStar	Sequence evaluation
GraphPad prism	Data evaluation and graph generation
VectorNTI	Sequence analysis

Table 13: Databases and server

Name	Address
ClustalW2	http://www.ebi.ac.uk/Tools/clustalw2/index.html
International FOP Association (IFOPA)	http://www.ifopa.org/index.html
National Center for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/
Primer 3 plus	bin/primer3/primer3_www.cgi
PubMed	www.ncbi.nlm.nih.gov/pubmed/

3.2 MOLECULAR BIOLOGICAL METHODS

3.2.1 MUTAGENESIS PCR

For site-directed mutation of sequences an in vitro mutagenesis PCR was performed. PFU Polymerase was used, having a 3´→5´ exonuclease activity for precise DNA amplification. Mutations are generated by PCR using a pair of oligonucleotide primers designed for deletion of STOP-codons at the C-termini of BMP-receptors.

Table 14: Mutagenesis PCR pipetting scheme

Ingredients	Volume
DNA template (~ 50 ng)	1 µl
Primer 1 (10 pM)	1 µl
Primer 2 (10 pM)	1 µl
dNTPs (12,5 mM)	1 µl
Mg_2SO_4	1 µl
***Pfu* Buffer**	5 µl
***Pfu* Polymerase (4 U)**	1 µl
Water	ad 50 µl

Table 15: Mutagenesis PCR program

Step	Temperature	Time	Cycles
Initial melting	95 °C	1 min	
Melting	95 °C	30 sec	20
Primer annealing	55 °C	1 min	
Elongation	68 °C	*Pfu* 500 bases / 1 min + 30 sec	
Final elongation	68 °C	15 min	
Cooling	4 °C	∞	

3.2.2 DPN-I RESTRICTION DIGEST

The purpose of DpnI restriction digest is to eliminate parental plasmid DNA by the endonuclease DpnI for further transformation. The plasmid DNA of most of the *E. coli* stems is methylated, whereas the DNA amplified by mutagenesis PCR having the mutation is not. DpnI digests specifically methylated DNA. For digesting mutagenesis PCR products 1 µl of DpnI (10/µl) (Fermentas) was added to the 50 µl PCR product and incubated at 37 °C for 1 h. Next, transformation was performed.

3.2.3 DEPHOSPORYLATION OF DNA SEGMENTS

To avoid re-ligation of the cut DNA segments, DNA was incubated with the enzyme SAP (*Shrimp Alkaline Phosphatase*) for 1 h at RT. SAP removes free phosphate residues and hinders formation of new phosphodiesterbonds between free DNA termini.

3.2.4 AGAROSE-GEL ELECTROPHORESIS

To fractionate DNA fragments a 1 % preparative or analytical agarose gel (1 % agarose dissolved in SB buffer whilst heating) was used. The loading buffer Orange G was added to the DNA solution in a 5:1 ratio. Fractionation in a preparative gel

was performed at 100 V for 90 – 120 min. Analytical fractionation was done at 200 V for 20 – 30 min. Due to addition of ethidiumbromide DNA fragments were visualized by exposing the gel to UV light (300 nm). To determine fragment size a Gene ruler 100bp DNA ladder was used. Documentation was performed by the use of a UV-gel documentation system (NightHawk).

3.2.5 Purification of DNA Fragments

Purification of DNA fragments obtained through PCR amplification or restriction digest was performed by electrophoretic fractionation in a preparative agarose gel (1 %). The band of appropriate size was cut out under UV light and purificated by using Gel-out Omni pure kit (OMNI Life Science) according to the manufacturers instructions.

3.2.6 Ligation

Target plasmids were cut by the use of appropriate restriction enzymes. The obtained DNA sequences were incorporated into the plasmid by the use of T4 ligase (Fermentas) while incubated at 16 °C o/n.

Table 16: Ligation pipetting scheme

Ingredients	Volume
Vector	100 ng
Insert	x ng
10x T4 ligase buffer	2 µl
T4 ligase	0,4 µl
ATP 10 mM	1 µl
Water	ad 20 µl

3.2.7 Transformation

The DNA plasmids were transformed into chemo-competent *E.coli* Top10 cells (Invitrogen). Uptake of DNA was initiated via heat shock at 42 °C for 90 sec after

incubation of 100 µl of cells with 5 µl DpnI-digested PCR product for 20 min. After heat shocking the cells were incubated again for 5 min on ice. Subsequently, cells were plated on LB plates containing 100 µg/ml ampicillin as selective marker. The cells were grown at 37 °C over night.

3.2.8 COLONY PCR AND ORIENTATION PCR

Colony PCR was performed to prove whether the colonies grown over night carry the expected plasmids. Therefore, the picked colony was incubated directly by the use of a sterile pipette tip in the colony PCR formulation (Table 17), which was pipetted beforehand into a 96-well PCR plate. By using appropriate primers, it was possible to determined whether the expected insert got incorporated into the bacteria genome and if the insert is orientated correctly.

Table 17: Colony PCR formulation

Ingredients	Volume
Primer f (10 pM)	0,4 µl
Primer r (10 pM)	0,4 µl
dNTPs (1,25 mM)	0,5 µl
***Tag* Polymerase**	0,25 µl
***Tag* Buffer 10x**	2 µl
Water	ad 20 µl

Table 18: Colony PCR program

Step	Temperature	Time	Cycles
Initial melting	96 °C	10 min	
Melting	96 °C	30 sec	35
Primer Annealing	55 °C	30 sec	
Elongation	72 °C	2 min 30 sec	
Cooling	4 °C	∞	

3.2.9 Preparation of Glycerin Stocks

Glycerin stocks were prepared for long-term storage of bacterial clones. 1 ml of fresh bacterial culture and 0,8 ml of 87 % glycerin were pipetted into a 2 ml cryo tube and stored at -80 °C.

3.2.10 Cloning of BMP-Receptor Luciferase Fusions

Applying the described methods, the GLuc fusion constructs of the murine BMP-receptors mAlk2^{wt}, mAlk2^{R206H}, and mAlk3^{wt} listed in Table 5 were cloned into the plasmid pCS2+ using corresponding enzymes.

3.2.11 Isolation of Plasmid DNA

After transformation a clone was picked from the agar plate incubated over night and cultivated in lysogeny broth (LB) media with 50 mg/ml ampicillin at 37 °C at 220 revolutions per minute (rpm) over night. 50 ml of the bacteria culture were centrifuged at 4750 rpm. The subsequent isolation of plasmid DNA was performed using Pure Yield™ Plasmid Midiprep System (Promega). According to manufacturer´s instructions an alkaline lysis was performed and plasmid DNA was isolated through column purification in 400 µl elution buffer. After plasmid DNA preparation the concentration was determined by using Nano Drop.

3.2.12 SEQUENCING PCR

The sequencing of clones was performed according to Sanger (Sanger, Nicklen et al. 1977). In contrast to a common PCR, the dNTP mix contains some ddNTPs to stop synthesis upon incorporation into the produced DNA single strand. Thereby, labeling the last position with a fluorescent marker. This procedure results in fragments of random length. Hence, the composition of the DNA section of interest can be determined by elution of these fragments over a gel bed column and subsequent fluorescent detection of the single base.

Table 19 and Table 20 show the reaction mix composition and the sequencing PCR program.

Table 19: Sequencing PCR pipetting scheme

Ingredients	Volume	Concentration
Plasmid DNA	X	50 - 200 ng
Primer 1	2 µl	10 pM
Big Dye v3.1	0,5 µl	4 U
Sequencing Buffer	2 µl	5 x
Water	ad 10 µl	

Table 20: Sequencing PCR program

Step	Temperature	Time	Cycles
Initial melting	96 °C	1 min	
Melting	96 °C	10 sec	25
Primer annealing	50 °C	5 sec	
Elongation	60 °C	4 min	

DNA precipitation and analysis of the sequencing PCRs were performed in the Institute for Medical Genetics, Charité (Berlin) with a Biomek NXP system (Beckman Coulter) and ABI 3730 capillary sequencing machine (Applied Biosystems).

3.3 CELL BIOLOGICAL METHODS

3.3.1 CULTIVATION OF CELL LINE

For cultivation of the cell line COS-1 (green monkey kidney cell line; source ATCC), DMEM containing 4.5 g/l glucose, 10 % FBS superior (Biochrom) and 2 % L-Gln were used and cells were incubated at 37 °C and 5 % CO_2. Media was exchanged every 2-3 days.

3.3.1.1 THAWING OF CELLS

For long-term storage cells were kept in liquid nitrogen. For cultivation an aliquot was thawed in a 37 °C water bath and transferred into 10 ml of pre-warmed medium. To remove the DMSO in the freezing medium, cells were pelleted at 1000 rpm for 5 min at RT. After the supernatant was discarded, cells were resuspended in fresh COS-1 medium (4.5 g/l glucose, 10 % FBS superior (Biochrom), 2 % L-Gln) and cultivated in a 75 cm^2 cell culture flask.

3.3.1.2 SPLITTING OF CELLS

As COS-1 cells are adherent cells, trypsin had to be used to detach the cells from the cell culture plastic. Growth medium was removed and the cells were washed with DPBS before addition of 0.05 % trypsin/EDTA (Lonza) and were incubated for 3 min at 37 °C. When the majority of cells were in suspension, adding fresh medium stopped the enzymatic reaction. In general, the 10^{th} part of this cell solution was mixed with new medium in a fresh cell culture flask of the same size. This process was repeated 3 times a week. When a specific cell density should be seeded, the concentration was determined using the CASY cell counter.

3.3.2 SPLIT-LUCIFERASE REPORTER ASSAY

A split-luciferase reporter assay allows detection of protein-protein interactions. In this paper the interaction of FRB and BMP-receptors with its interaction partner FKBP12 was examined by using fusion constructs of these proteins where either the N-terminal or C-terminal section of a luciferase reporter enzyme was fused to it. Quantitative assessment of refolding of the luciferases in their native structure when the fused proteins were in very close proximity was performed.

Besides the protein interaction itself, the usability of split-Renilla and split-Gaussia as a split-luciferase reporter got tested. The amount of refolded luciferase enzymes in the cell can be quantified by adding their substrate coelenterazine and measuring bioluminescence in the Mithras luminometer for normalization a pGL3-SV40 firefly vector was co-transfected and measured separately.

3.3.2.1 TRANSFECTION OF COS-1 CELLS

COS-1 cells were transfected transiently with PEI 40 (Polyethylenimine 40 kDa) 24 hours after seeding the cells in a 96 or 24 well plate at a density of $1x10^4$ or $4x10^4$ cells per well, respectively. The cells were transfected with either pCMV vectors containing FRB-NRLuc and FKBP12-CRuc, pcDNA3.1/zeo(+) vectors containing FRB-NGLuc and FKBP12-CGLuc, or pCS2+ vectors containing the BMP-receptor split-luciferase constructs. When cells seeded in a 96 well plate were transfected, a transfection mix based on NaCl 150 mM containing 0,25 µg DNA and 0,5 µg PEI 40 per sample was used. When using a 24 well plate the concentrations increased to 1 µg DNA and 2 µg PEI 40. Cells were treated with a final concentration of 40 nM rapamycin for 3 hours or left untreated prior to luminescence analysis after 24 h transfection. To investigate the impact of FK506, cells were treated with a final concentration of 1 µM FK506 or left untreated.

3.3.2.2 LUCIFERASE ACTIVITY MEASUREMENT

After 24 h of incubation, the transfected cells were lysed. In order to find the best conditions for measuring Split-RLuc and Split-GLuc activity, different conditions needed to be tested. Therefore, cells were lysed in 40 µl (96 well plate) or 250 µl (24 well plate) 0.1 M potassium phosphate buffer (9,2 mM KH_2PO_4, 91 ml K_2HPO_4, 0,2 % Triton X-100) and incubated while shaking for at least 5 min until cells detached from the surface. For the second approach 1x Passive Lysis Buffer (Promega) was used in same volumes for lysis and incubated 15 min while shaking. The resulting cell lysates were homogenized using a multichannel pipette and 25 µl got transferred to a black 96 well plate (Nunc, Carlsbad) optimized for luciferase measurements. 0,4 mM native coelenterazine (Biosynth, Hertfordshire) in methanol was diluted in DMEM (Biochrom) to a final concentration of 20 µM and 25 ml were added per sample. Renilla or Gaussia luciferase activity was determined 10 min after addition of coelenterazine or luciferin for Firefly.

Alternatively, luciferase activity was measured immediately after substrate addition. To do so, substrate was added by using the Mithras injection device. Every split-luciferase condition was transfected in 3 replicates and each assay was performed 3 times independently.

4 RESULTS

4.1 OPTIMIZATION OF SPLIT-LUX PCAS

A Split-LUX assay has to meet the requirements to allow firm protein complementation. For Split-RLuc and Split-GLuc assays normalized with FLuc, suitable cell lysis conditions and point in time for luminescence measurement needed to defined especially.

As previously demonstrated in the Materials and Methods section, the N- and C-terminal Renilla luciferase DNA fragments were inserted in a pCMV plasmid (Paulmurugan et al., 2004) downstream the protein of interest. Gaussia luciferase fragments were also incorporated at the C-terminus of FKBP12, FRB and GCN4 zipper fragments (positive controls) using a pcDNA3.1/zeo(+) plasmid. Transfection assays were performed in COS-1 cells, which were incubated for 24 h after transfection and treated with rapamycin 3 h prior cell lysis or left untreated. For normalization co-transfection of Firefly pGL3-SV40 was performed.

4.1.1 LYSIS CONDITIONS INFLUENCE SPLIT-LUX COMPLEMENTATIONS

To allow stable protein complementation, the Split-LUX assay necessitates the formulation of a suitable lysis solution. In order to establish a Split-LUX assay applicable for RLuc and GLuc fusion proteins, the lysis solutions PPBT (Potassium Phosphate Buffer (Hampf & Gossen, 2006)) and PLB (Passive Lysis Buffer (Promega)) were tested. To examine suitable lysis conditions, the protein interaction of FKBP12 and FRB was used. The FKBP12/FRB protein interaction was chosen as a test system, due to the interactions prerequisite needing rapamycin for protein complementation. Therefore, cells were treated with rapamycin or left untreated. For avoiding false positive examination of unspecific interaction, FKBP12-CRLuc and FRB-NRLuc were co-transfected with vectors containing either NRLuc or CRLuc, respectively.

4.1.1.1 PASSIVE LYSIS BUFFER ALLOWS RLUC COMPLEMENTATION

First, lysis conditions for Split-Rluc needed to be examined. In order to allow stable interaction of the proteins of interest and Renilla luciferase fragment

complementation, suitable lysis conditions are crucial. Consequently, COS-1 cells were co-transfected with FKBP12-CRLuc/FRB-NRLuc and the Firefly vector pGL3-SV40 for normalization to determine a lysis buffer, which chemically allows protein complementation. The transfection assays were performed in duplicates. Therefore, cells were lysed 24 h after transfection in either PPBT or PLB for comparison. Next, luminescence activity was measured using the Mithras luminescence system.

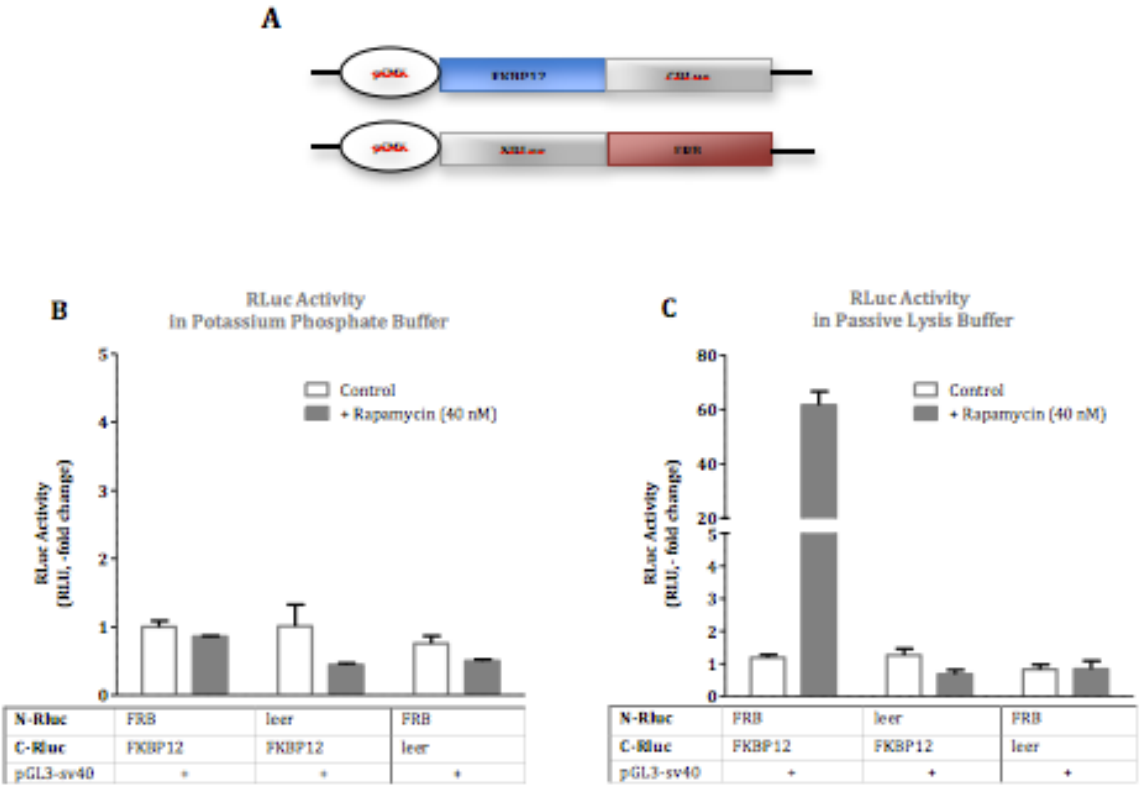


Figure 13: Comparison of RLuc protein complementation in potassium phosphate buffer and passive lysis buffer cell lysates. COS-1 cells were co-transfected with the indicated RLuc PCA fusions (FKBP12-CRLuc, FRB-NRLuc) shown in (A). After 24 h cells were lysed in potassium phosphate buffer (PPBT) or passive lysis buffer (PLB). Cells were treated with 40 nM rapamycin (dark gray bars) 3 h before luminometric analysis or left untreated (white bars). Bars represent mean ±SEM calculated from triplicates. For normalization the firefly vector pGL3-SV40 was co-transfected. Data are presented as fold change compared to FRB-NRLuc/FKBP12-CRLuc rapamycin untreated. (B) RLuc Split-LUX in PPBT cell lysates. (C) RLuc Split-LUX in PLB cell lysates.

As Figure 13 shows, luminescence activity varies drastically based on the lysis conditions chosen. When cells were lysed in PPBT no protein interaction could be observed (Figure 13 B). Neither the interacting pair FKBP12/FRB treated with the connector rapamycin, nor the controls show any activation. In contrast, when cell lysis was performed in PLB (Figure 13 C), luciferase complementation could be measured. Therefore, Renilla luminescence, just like Firefly luminescence, was clearly detectable in FKBP12/FRB (+rapamycin) cell lysates. Furthermore, neither of the non-interacting protein pairs FKBP12/FRB (-rapamycin), NGLuc/FKBP12-CGLuc, or FRB-NGLuc/CGLuc resulted in detectable luminescence confirming that the PCA reporter fragments do not spontaneously assemble. In order to determine lysis conditions suitable for Renilla, Firefly, and Gaussia Split-LUX assays, the experiment was performed again in equal measure but using GLuc as the reporter system (Figure 14).

4.1.1.2 GLuc Complementation is Detectable in PLB and PPBT Lysates

After lysis conditions for Split-LUX using Renilla luciferase as a reporter were determined, it needed to be tested whether the usage of PLB is suitable for Gaussia luciferase as well. To do so, COS-1 cells were co-transfected with FRB-NGLuc/FKBP12-CGLuc and the corresponding controls. As a positive control the constitutive interaction between GCN4 leucine zipper fusions was used (Zipper-NGLuc/Zipper-CGLuc) (Ingrid Remy & Michnick, 2006). The non-interacting fusion protein pairs FRB-NGLuc/Zipper-CGLuc and Zipper-NGLuc/FKBP12-CGLuc were used as negative controls. To allow normalization of GLuc measurement values pGL3-SV40 Firefly was co-transfected. As the experiment was executed for Renilla luciferase, the Split-GLuc assay was performed in duplicates to allow comparison of PPBT and PLB (Figure 14). Rapamycin was added 3 h prior cell lysis, which was performed 24 h after transfection.

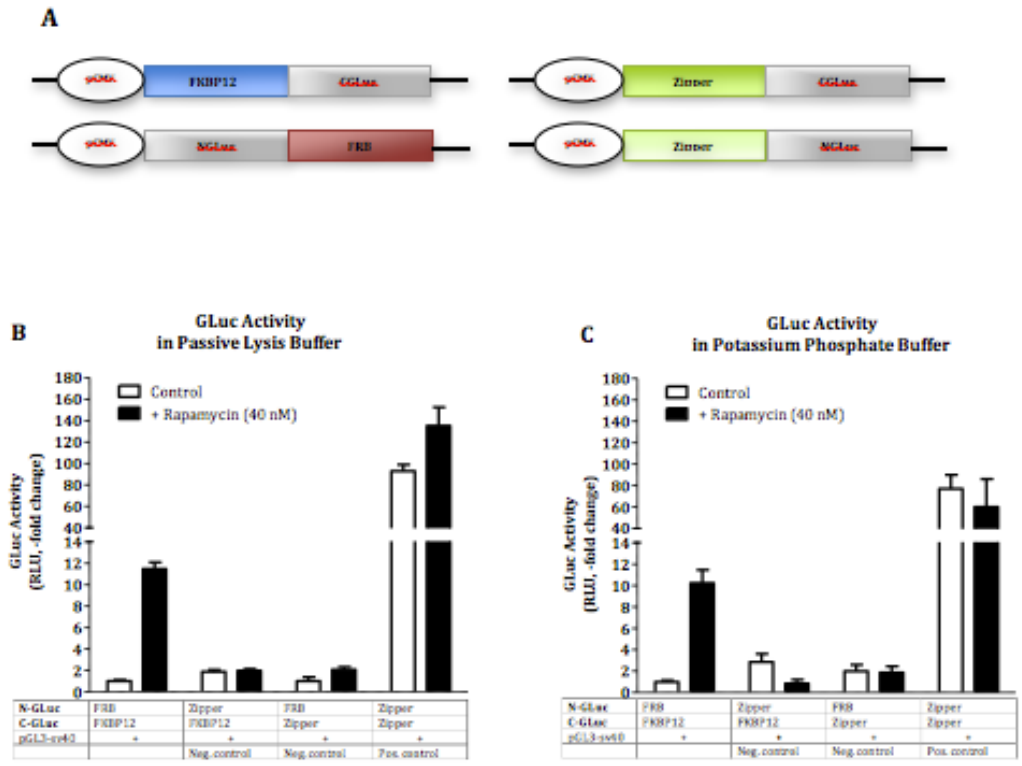


Figure 14: Comparison of GLuc protein complementation in passive lysis buffer and potassium phosphate buffer cell lysates. COS-1 cells were co-transfected with the indicated GLuc PCA fusion pairs (FRB-NGLuc/FKBP12-CGLuc; Zipper-NGLuc/Zipper-CGLuc; (shown in (A)) and the negative controls FRB-NGLuc/Zipper-CGLuc; Zipper-NGLuc/FKBP12-CGLuc). After 24 h cells were lysed in potassium phosphate buffer (PPBT) or passive lysis buffer (PLB). Cells were treated with 40 nM rapamycin (black bars) 3 h before luminometric analysis or left untreated (white bars). Bars represent mean ±SEM calculated from triplicates. For normalization the firefly vector pGL3-SV40 was co-transfected. Data are presented as fold change compared to FRB-NGLuc/FKBP12 rapamycin untreated. (B) GLuc Split-LUX in PLB cell lysates. (C) GLuc Split-LUX in PPBT cell lysates.

The data displayed in Figure 14 indicate that the GLuc activity was measurable in FKBP12/FRB (+ rapamycin) cells lysed in PLB and PPBT. Furthermore, neither of the non-interacting protein pairs Zipper/FKBP12 and FRB/Zipper, resulted in detectable luminescence confirming that the GLuc PCA reporter fragments do not spontaneously assemble. In PLB cell lysates (Figure 14 (B)) the FKBP12/FRB (+rapamycin) protein pair a 12 fold higher activation was detectable than in FKBP12/FRB (-rapamycin) samples. In addition, GCN4 leucine Zipper positive controls with added rapamycin, show almost 10 fold higher activation than FKBP12/FRB (+rapamycin) in PLB cell lysates and rapamycin untreated Zipper

pairs show even a 12 fold higher activation (Figure 14 (B)). In cell samples lysed in PPBT (Figure 14 (C)) the luminescence activity detected in FRB/FKBP12 (+rapamycin) is 10 fold higher than the activity of rapamycin untreated FRB/FKBP12 interaction. The GCN4 leucine Zipper interaction shows 7 fold higher activation than FRB/FKBP12 (+rapamycin) in PPBT. Equally to the non-interacting pairs in PLB lysates (Figure 14 (B)), Zipper/FKBP12 and FRB/Zipper show no detectable interaction.

Due to the outcomes based on measurements performed in COS-1 cell extracts from the same transfected population prepared in parallel for either Renilla or Gaussia in the respective lysis buffer (PLB/RLuc, PPBT/RLuc, PLB/GLuc, PPBT/GLuc), GLuc and PLB were selected for further experiments. Gaussia luciferase showed higher activation than Renilla luciferase did. Furthermore, GLuc fragment complementation was detectable in PLB and PPBT, which leads to the conclusion that Split-GLuc interaction might be more stable and less sensitive to lysis buffer conditions. Moreover, GLuc might interfere less with the interaction of the proteins of interest due to its much smaller size compared to RLuc. Therefore, Gaussia luciferase seems to be more suitable for Split-LUX and, therefore, the reporter system of choice for further experiments. In addition, further cell lysis was performed in PLB, because it seems to interfere less with protein interaction.

4.1.1.3 Luminescence Activity of Gaussia Luciferase Starts to Decrease Shortly After Substrate Addition

To determine the most suitable point in time for luminescence measurement, cell lysates positive for FKBP12-CGLuc/FRB-NGLuc and Zipper-CGLuc/Zipper-NGLuc complementation were used for kinetic measurements. The cell extracts used were from a transfected cell population prepared in PLB. In order to detect unspecific protein interactions, cell lysates containing non-interacting pairs Zipper-NGLuc/FKBP12-CGLuc, FRB-NGLuc/Zipper-CGLuc were measured in parallel. Furthermore, to investigate if PLB triggers auto-luminescence of coelenterazine, pure PLB was measured. Luciferase activity was measured every 5 seconds over a time period of 15 min using a Mithras luminescence system. Substrate addition was performed by the Mithras´ dispenser application.

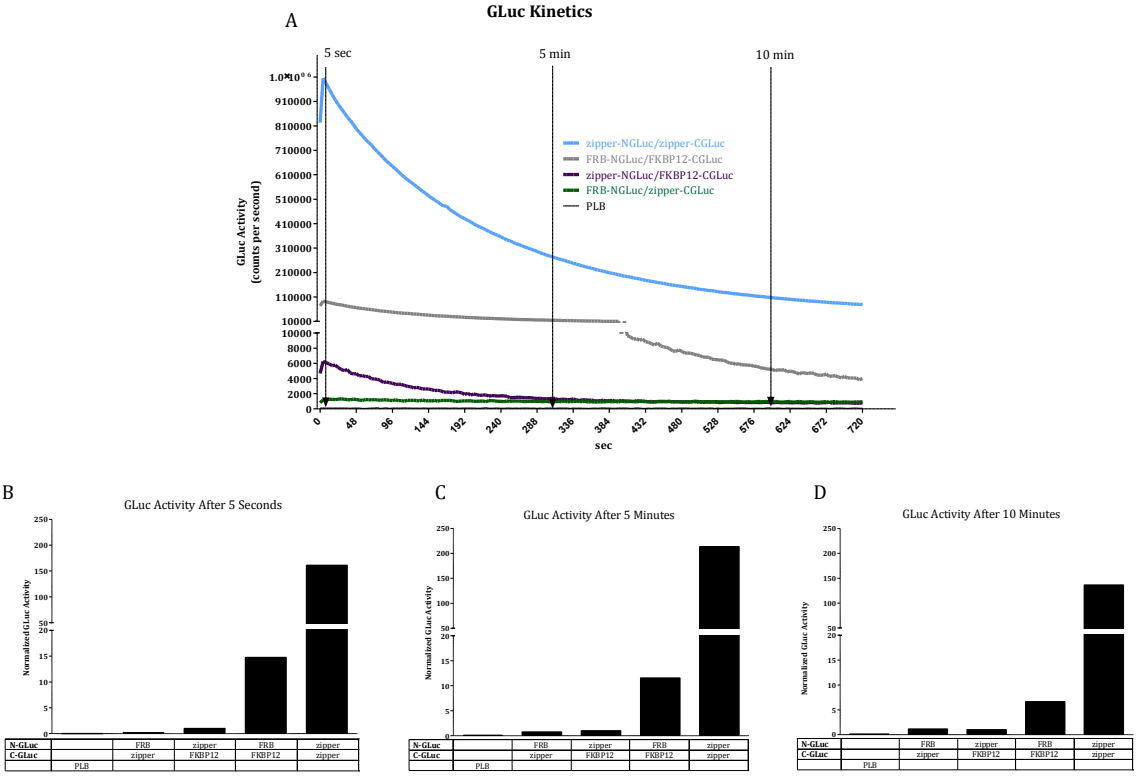


Figure 15: Luminescence activity of GLuc is most intense 5 seconds after substrate addition. COS-1 cells expressing FKBP12-CGLuc/FRB-NGLuc, GCN4 Zipper fusions (Zipper-NGLuc/Zipper-CGLuc), and negative controls (FKBP12-CGLuc/Zipper-NGLuc, FRB-NGLuc/Zipper-CGLuc) were lysed in PLB and measured to determine progression of GLuc activity. Cells expressing FKBP12-CGLuc/FRB-NGLuc were treated with 40 nM rapamycin 3 h prior analysis. (A) To identify the kinetics of GLuc, substrate addition was performed using the dispenser application of a Mithras luminescence system. Luciferase activity was measured every 5 seconds over a time period of 15 min. (B, C, D) GLuc activity after 5 sec, 5 min, and 10 min is depicted. Auto-luminescence of the substrate coelenterazine was determined by injecting substrate solution to a PLB blank sample. Mean value of FKBP12-CGLuc/Zipper-NGLuc was used for normalization.

Bioluminescence activity of the re-complemented GLuc fragments was most intense 5 seconds after substrate addition and decreased rapidly with time as it is shown in Figure 15 (A). The luminescence activity of the interacting pair Zipper-NGLuc/Zipper-CGLuc showed a maximum value of $1x10^6$ counts per second (cps), whereas for FKBP12-CGLuc/FRB-NGLuc a value of 110.000 cps was measured. Furthermore, the non-interacting pair Zipper-NGLuc/FKBP12-CGLuc showed a value of 6000 cps, which might be due to unspecific binding just measurable when luminescence activity peaks. As the graph (Figure 15 A) shows, detectable

bioluminescence of the non-interacting pair vanished 5 min after substrate addition. Measurement values of the blank PLB sample and FRB-NGLuc/Zipper-CLuc show no activation. In Figure 15 (B, C, D) the normalized luminescence values after 5 sec, 5 min, and 10 min are depicted showing a progressive decline of LUX activity. 5 sec after substrate addition the positive control Zipper-NGLuc/Zipper-CGLuc shows a 10 times more intense signal than FKBP12/FRB. After 10 min the zipper complementation exposes even a 30-fold intensity compared to FKBP12/FRB. Due to this findings, further experiments were conducted by measuring samples 5 seconds after substrate addition to increase the probability to detect even weakly interacting proteins. The drawback of immediate Gaussia luminescence measurement on the other hand is the detection of unspecific protein interaction or interaction of just the luciferase fragments itself, which might interfere with reliability of the measurement results. These outcomes have to be taken into consideration for analysis of further luciferase assays using GLuc as the reporter system.

4.1.1.4 GLuc Activity of FKBP12/FRB Complementation is 10 Fold Higher 5 Seconds After Substrate Addition Than After 10 Minutes

To depict the improved precision of measurements done 5 seconds after substrate addition compared to those performed after 10 minutes, a GLuc Split-LUX assay using the protein pair FKBP12/FRB for co-transfection of COS-1 cells was applied. Therefore, COS-1 cells were co-transfected with GCN4 Zipper fusions for positive controls, the negative controls Zipper/FKBP12 and FRB/Zipper, and FRB/FKBP12 fused to corresponding GLuc fragments. Each condition was performed in duplicates, which were treated 3 h prior analysis with rapamycin or left untreated. Measurement was carried out using the Mithras luminescence system.

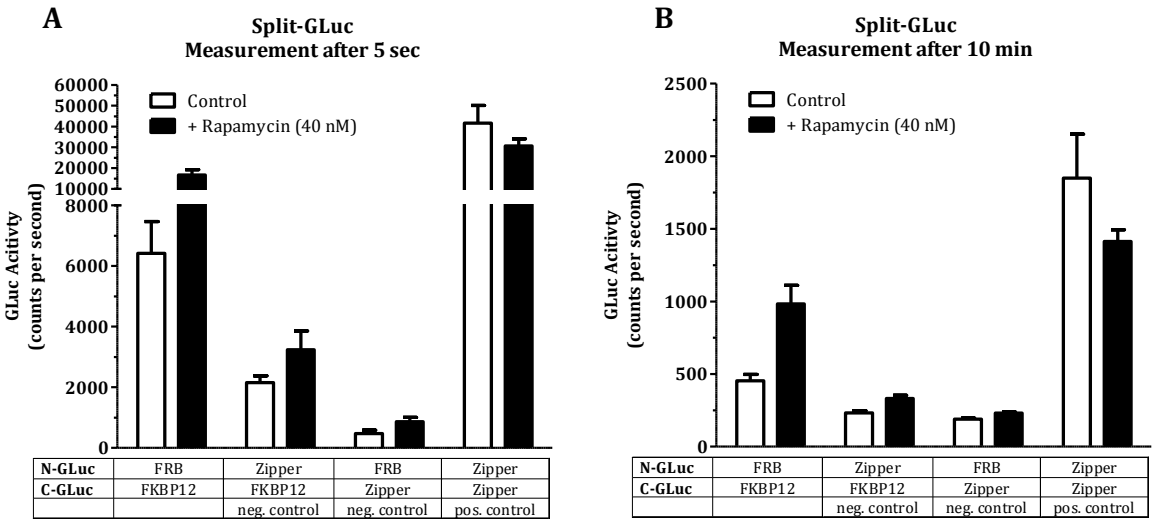


Figure 16: Measurement of GLuc activity of FKBP12/FRB complementation after 5 sec and 10 min. COS-1 cells were transfected with the indicated GCN4 leucine Zipper fusions (Zipper-NGLuc/Zipper-CGLuc), FKBP12-CGLuc/FRB-NGLuc, FKBP12-CGLuc/Zipper-NGLuc and Zipper-CGLuc/FRB-NGLuc to determine GLuc activity after 5 sec and 10 min. Cells were treated with 40 nM rapamycin 3 h before bioluminometric analysis or left untreated. 24 h after transfection cells were lysed in PLB. Values are presented in counts per second. (A) To identify GLuc Activity after 5 seconds after substrate addition substrate was added using the dispenser application of a Mithras luminescence system. (B) After 10 min samples were measured again.

As the data in Figure 16 shows luminescence values are 20 times higher when measured 5 seconds after substrate addition compared to those after 10 minutes. To depict actual measurement values for GLuc, normalization was excluded for this experimental set up. The FRB/FKBP12 cell lysates treated with rapamycin show luminescence activity of 20.000 cps (Figure 16 b, black bars) when measured 5 sec after substrate addition, whereas the activity declines to 1000 cps when the measurement was performed 10 min after. In addition, the constitutive interacting Zipper pair shows far higher values when measurement was performed right after addition of the substrate. As it is depicted in Figure 16 B, even the very strong interaction of the GCN4 Zipper pair shows a very low luminescence activity of less than 2000 cps, which is not a reliable value for protein complementation. In contrast, the non-interacting pair Zipper/FKBP12 (+rapamycin) shows even higher activation with a value of 3000 cps when measured after 5 seconds. This might be due to weak interaction of the luciferase fragments even though the proteins of interest do not actually complement. Due to

this findings further Split-LUX assays were measured 5 sec after substrate addition. This might also allow detection of weaker proteins interaction, which would not be detectable when measured 10 min after substrate addition. Nevertheless, immediate evaluation of luminescence activity might show weak unspecific interaction, which would not be detectable when measurement is performed after 10 min.

4.2 Interaction of BMP-Receptors with Their Inhibitor FKBP12

Due to the finding depicted in "4.1 Optimization of Split-LUX PCAs" Split-LUX assays for verifying BMP-receptor interactions with their inhibitor the FK506 binding protein (FKBP12) were performed using GLuc fragments for receptor tagging, PLB for cell lysis after transfection, and luminescence activity was measured 5 seconds after substrate addition.

The BMP-receptor/FKBP12 Split-LUX assay was performed to test whether FKBP12 inhibition, due to the binding of FKBP12 to the GS domain of BMP receptors, is weakened in mAlk2 having the FOP mutation R206H (mAlk2^{R206H}) compared to mAlk2^{wt} and mAlk3^{wt}. To do so, GLuc fragments were fused to the C-termini of these BMP-receptors (detailed information is depicted in the Material and Method section). As expression vector pCMV was used.

4.2.1 C-Terminal Tagging of BMßP-Receptors Allows Cytoplasmic Complementation of Split-GLuc

Previous research suggests, that the BMP-receptor inhibitor FKBP12 might interact weaker with the GS domain of the FOP mutant mAlk2^{R206H} than it does with mAlk2^{wt} (Eileen M Shore & Kaplan, 2008). Therefore, downstream BMP signaling is active even in the absence of a ligand.

To determine the interaction of BMP-receptor mAlk2^{wt}, mAlk2^{R206H}, and mAlk3^{wt} with FKBP12 these receptors were fused at their C-termini to the N-terminal fragment of Gaussia luciferase. The FKBP12 fusion construct is designed having the C-terminal fragment of GLuc at the C-terminus. Therefore, C-terminal tagging of full-length BMP-receptors was performed using the N-terminal fragment of GLuc to be able to examine cytoplasmic complementation of mAlk2^{wt}, mAlk2^{R206H}, and mAlk3^{wt} with FKBP12.

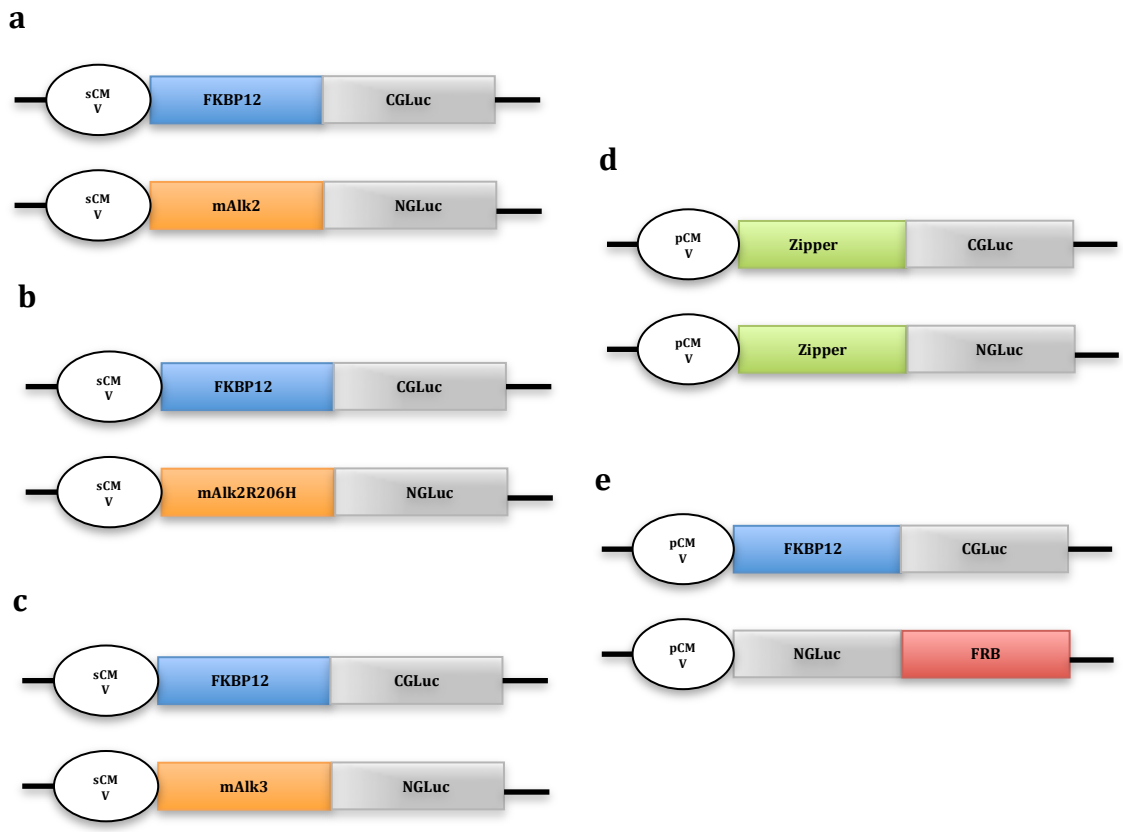
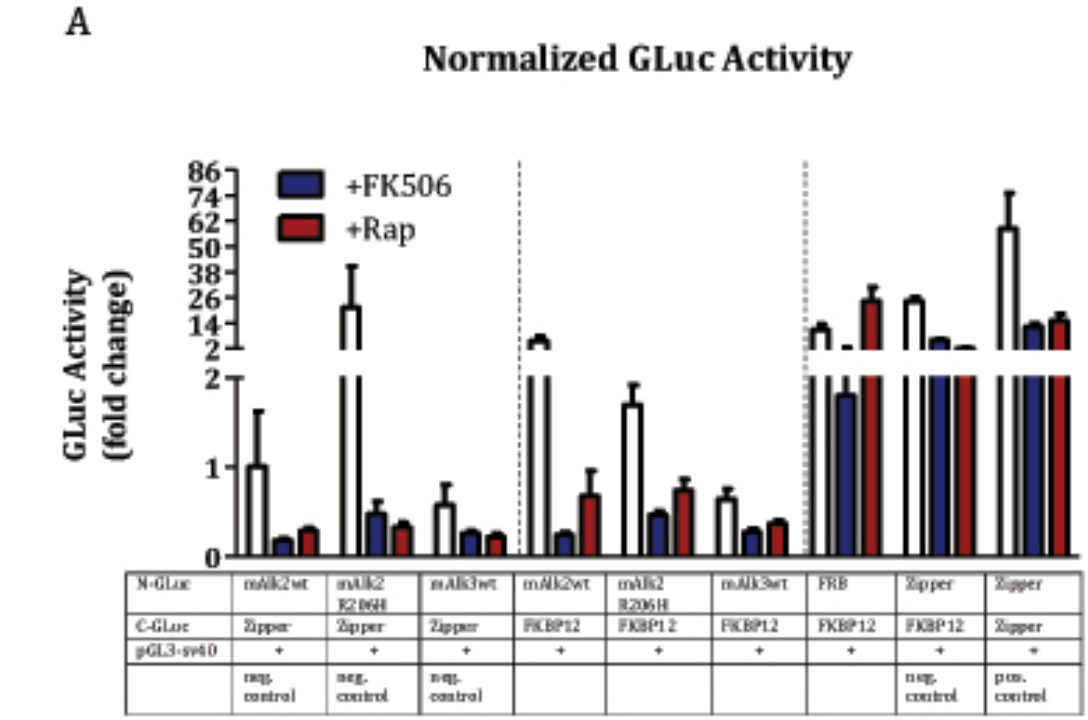


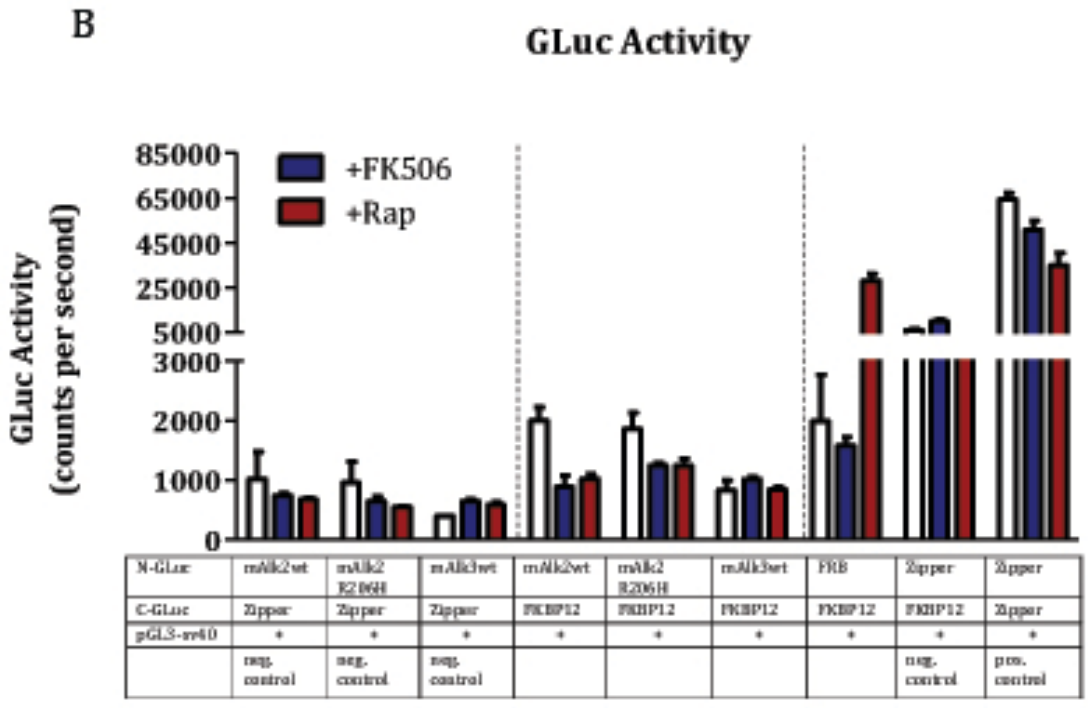
Figure 17: BMP-receptor fusion constructs tagged with N-terminal GLuc fragments. Scheme representing GLuc fusion combinations used for co-transfection of COS-1 cells to examine BMP-receptor interaction with their inhibitor FKBP12. (Data shown in **Figure 18**) (a) FKBP12-CGLuc/mAlk2wt-NGLuc (b) FKBP12-CGLuc/mAlk2R206H-NGLuc (c) FKBP12-CGLuc/mAlk3wt-NGLuc (d) negative control FKBP12-CGLuc/NGLuc-FRB (e) positive control GCN4 Zipper fusions Zipper-CGLuc/Zipper-NGLuc.

4.2.2 Immunosuppressive Agents FK506 and Rapamycin Inhibit Protein Complementation of BMP-Receptors and Their Inhibitor FKBP12

In order to determine whether FK506 and/or rapamycin inhibit the protein complementation of BMP-receptors and FKBP12, COS-1 cells were co-transfected with the GLuc fusion combinations shown in Figure 18. In addition, COS-1 cells were co-transfected mAlk2^{wt}, mAlk2^{R206H}, and mAlk3^{wt} fusion constructs in combination with the corresponding leucin Zipper fusions for negative controls. Cells were incubated for 24 h and 3 h prior analysis either FK506 or FKBP12 were added to the cell extracts or cells were left untreated.

FK506 binds like rapamycin the active site of FKBP12 (Cruz et al., 1999) with which FKBP12 also binds to the GS domain of BMP-receptors. Thus, it is expected that addition of FK506 or rapamycin might block BMP-receptor/FKBP12 interaction due to the occupation of the active binding site of FKBP12 by either FK506 or rapamycin.





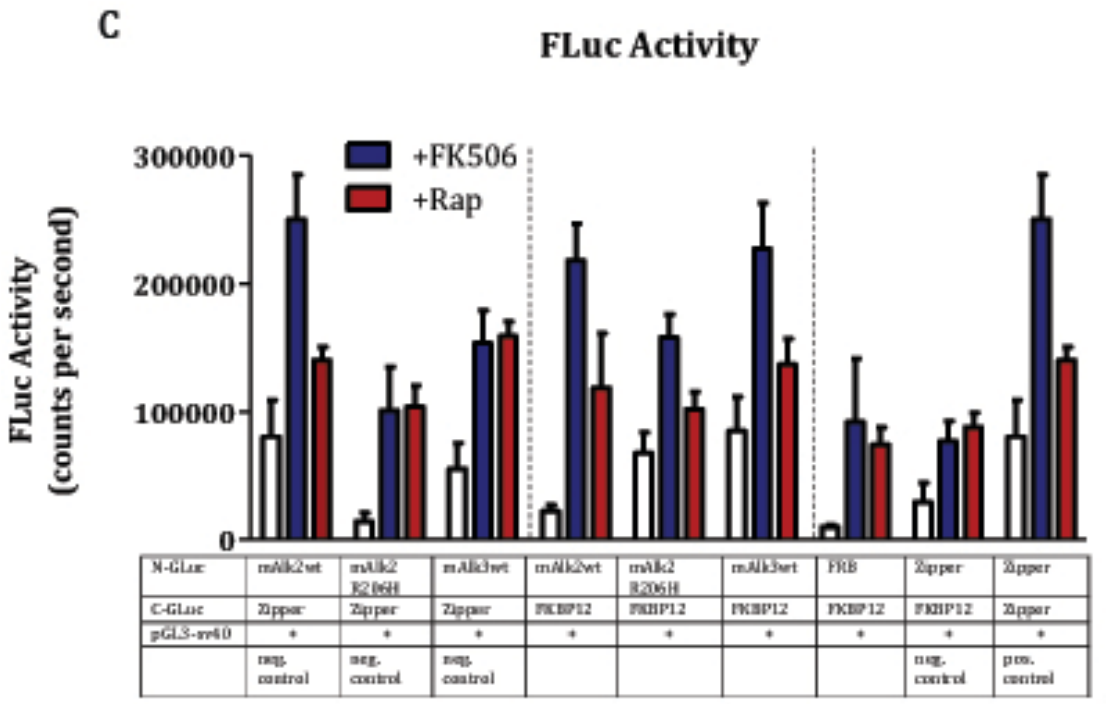


Figure 18: Split-LUX assay showing the interaction of BMP-receptors and FKBP12 and the influence of rapamycin and FK506. COS-1 cells were treated with 40 nM rapamycin or 1 µM FK506 3 h prior analysis or left untreated. After lysis in PLB luminescence was measured. Each bar represents the mean ± SEM of triplicates. (A) Fold change GLuc activity: Each indicated sample was co-transfected with the pGL3-SV40 firefly for normalization. Gaussia luciferase values (B) were normalized to firefly luciferase values (C) and fold inductions relative to mAlk2wt-NGLuc/Zipper-CGLuc (rapamycin and FK506 untreated) were determined.

As it is depicted in the middle section of Figure 18 A (GLuc activity fold induction values relative to mAlk2^{wt}-NGLuc/Zipper-CGLuc rapamycin and FK506 untreated) protein complementation of FKBP12/mAlk2^{wt} and FKBP12/mAlk2^{R206H} (white bars) is disturbed by addition of rapamycin (red bars) and FK506 (blue bars) as expected. Moreover, there is no interaction observed of mAlk3^{wt} and FKBP12. Further, the normalized values (Figure 18 A) show that BMP-receptors seem to complement with the co-transfected Zipper constructs. In contrast, raw GLuc data (Figure 18 B) depict that there is no protein complementation of BMP-receptor/Zipper pairs. Even though it seems like rapamycin or FK506 do weaken the Zipper interaction slightly, the last section of Figure 18 B shows that GCN4 leucin Zipper pairs do complement whether rapamycin or FK506 are added or not. Furthermore, the FKBP12/FRB interaction can just be observed in the presence of rapamycin. Nevertheless, the negative control FKBP12/Zipper shows GLuc activity

high enough to conclude that this protein pair does interact as is was already observed when GLuc kinetic was analyzed (Figure 15).

On the other hand, FLuc activity (Figure 15 C) seems to be inconsistent and fluctuating drastically, which falsifies GLuc activity if data is normalized as Figure 15 A shows.

4.2.3 mAlk2^{R206H}-GLuc Fusions Show No Smad-Signaling

In order to determine if Gaussia luciferase tagging interferes with the receptors ability to activate BMP downstream signaling, a BRE (BMP responsive element) assay was performed. Using a BRE-Luc construct, Smad signaling dependent induction of BMP-receptors can be examined. Therefore, COS-1 cells were transiently co-transfected with either the untagged receptors mAlk2^{R206H}, mAlk3^{wt} or their tagged counterparts (mAlk2^{R206H}-NGLuc, mAlk3^{wt}-NGLuc), BRE and TK Renilla for normalization.

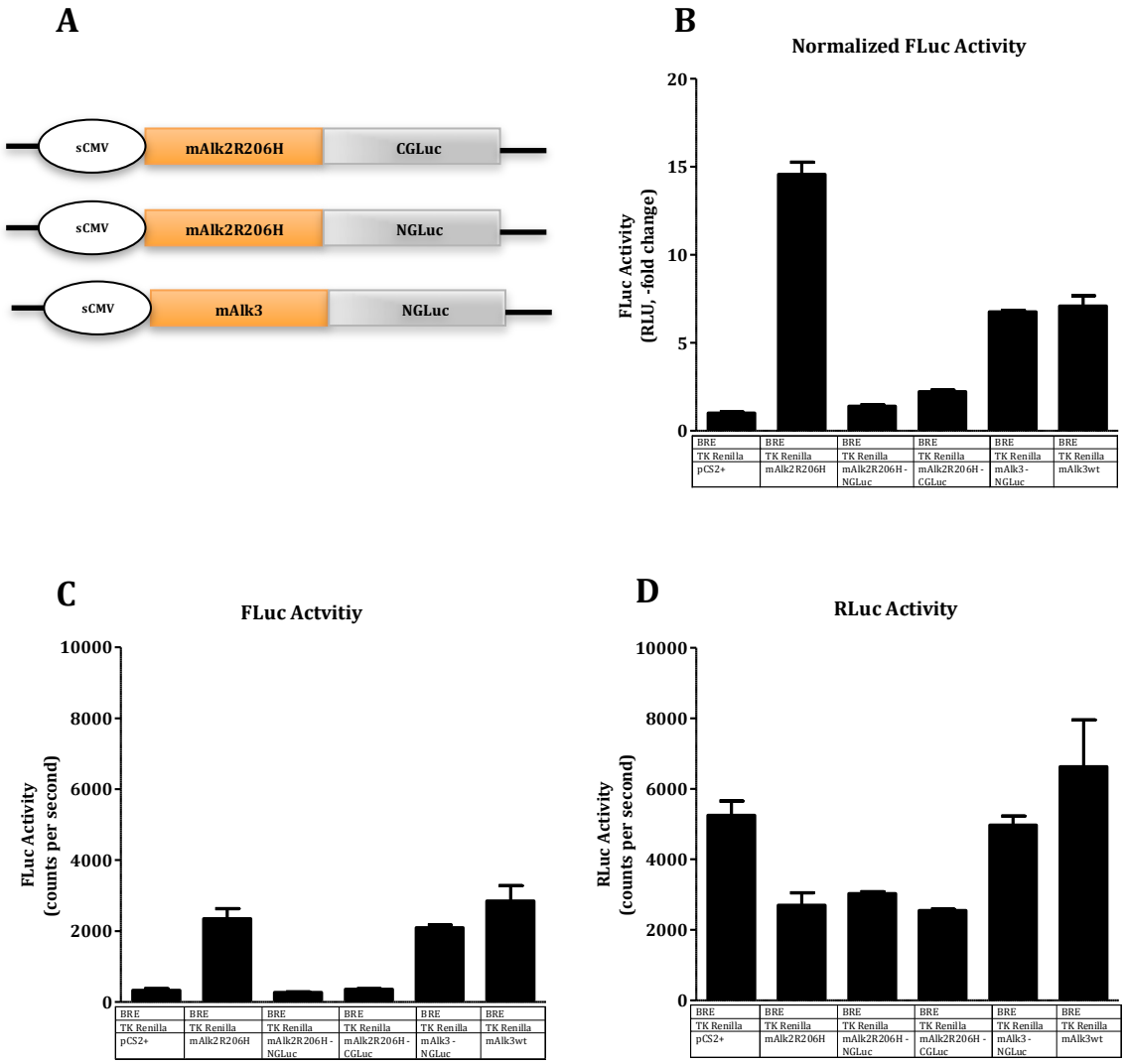


Figure 19: Smad-signaling dependent induction of BMP-receptors of BRE-Luc activity in COS-1 cells. COS-1 cells were co-transfected with the indicated BMP-receptor fusions (mAlk2R206H-NGLuc, mAlk2R206H-CGLuc, mAlk3wt-NGLuc) and the corresponding untagged controls mAlk2R206H, mAlk3wt and with pCS2+ as a negative control to determine the Smad-signaling ability of BMP-receptors fused to GLuc fragments. 24 h after transfection Firefly luciferase activity was assessed by luminometric analysis. (A) Schematic representation of BMP-receptor fusion constructs (C) Firefly luciferase activities were normalized to (D) Renilla luciferase values and (B) fold inductions relative to pCS2+ were determined. Each bar represents the mean ± SEM of triplicates. The assay was performed using a BMP responsive element (BRE) fused to a Firefly luciferase reporter gene.

As Figure 19 B shows, expression of untagged mAlk2^{R206H} and mAlk3^{wt} leads to activation of Smad-signaling, which induces the expression of Firefly luciferase. In contrast, mAlk2^{R206H}-NGLuc and mAlk2^{R206H}-CGLuc show no or minimal signaling

activity, whereas mAlk3^{wt}-NGLuc seems to persists to induce downstream signaling as untagged mAlk3^{wt} does.

5 DISCUSSION

The present thesis reports about the development and optimization of a protein complementation assay (PCA) based on Split-Renilla (RLuc) and further on Split-Gaussia (GLuc) luciferase. To determine appropriate conditions for Split-LUX a rapamycin inducible hetero-dimerization approach was used containing the proteins FKBP12 (rapamycin associated protein also known as RAFT) and FRB (FKBP-rapamycin binding domain) fused to C- or N-terminal luciferase fragments, respectively. FKBP12 and FRB luciferase fusion proteins were developed and have been previously validated by Paulmurugan et al, 2004 (Split-RLuc fusions) and Remy & Michnick, 2006 (Split-GLuc fusions). The purpose of investigating a Split-LUX assay was to determine the interaction of the BMP-receptors mAlk2^{wt}, mAlk2^{R206H} (FOP mutant), and mAlk3^{wt} with their inhibitor FKBP12. Further, the influences of the FKBP12 inhibitors FK506 and rapamycin on the BMP-receptor/FKBP12 interaction were examined.

5.1 SPLIT-LUX ASSAYS REQUIRE SPECIFIC LYSIS CONDITIONS

To establish a Split-LUX assay, the N-terminal as well as the C-terminal domain of Renilla or Gaussia luciferase is connected to the proteins of interest (FKBP12 and FRB) via a flexible linker, which allows folding of the luciferase fragments once protein complementation occurs. Folding of the fragments reactivates the luciferase when its substrate is present. Hence, luciferase activity can be measured. The findings show that appropriate lysis conditions are crucial to avoid disruption of luciferase folding. When using PPBT (potassium phosphate buffer), RLuc bioluminescence could not be observed (Figure 13) while GLuc activity could (Figure 14). At the same time, after lysing those cells containing RLuc fusions in PLB (passive lysis buffer) (Figure 13), RLuc enzyme activity was detectable at even higher levels than the activity of GLuc in similar treated cells. GLuc complementation on the other hand could be detected in PPBT and PLB lysates (Figure 14). PLB seems to provide appropriate conditions for RLuc as well as for GLuc in order to fold in their native structures and be active. PPBT seems to disrupt the assembling of NRLuc and CRLuc. The detectable signal of GLuc in cell lysates containing PPBT might be explained by its ability to generate over 100-fold higher bioluminescent signals than RLuc does (Ingrid Remy & Michnick, 2006).

Moreover, Gaussia luciferase is much smaller than Renilla luciferase leading to the assumption that GLuc might be more stable. RLuc on the other hand needs specific environmental conditions for complementation. Therefore, lysis conditions need to be optimized. Further, measurements should be performed in phenolred-free media, which might decrease background signal intensity. Another approach to further optimize specifically FKBP12-FRB complementation would be a variation in rapamycin addition. It was suggested that FKBP12 and FRB show the highest luminescence activity 60 seconds after rapamycin addition (Ingrid Remy & Michnick, 2006). Thus, when measurements are applied 3 hours after rapamycin addition, as was done in this thesis, FKBP12 and FRB might be partly dissociated leading to weakened luciferase intensity. Therefore, the measurement time best suitable should be determined.

5.2 Gaussia Luciferase Activity Declines Drastically Shortly After Substrate Addition

As it can be seen in Figure 15, GLuc activity drastically declines right after substrate addition. Therefore, contrary to measurement set ups applied in previous luciferase assays (Hampf & Gossen, 2006) in which luciferase activity was measured after 10 minutes, it can be concluded that measurements should be done 10 seconds after substrate was added. After doing this, weaker interactions could be observed, which may remain undetected otherwise. To determine whether the interaction of mAlk2^{wt} and mAlk2^{R206H} with FKBP12 (Figure 18) is specific, GLuc kinetics measurements should be performed like it has been applied for several protein complementations as it can be seen in Figure 15. If the signal given by mAlk2^{wt}/FKBP12 or mAlk2^{R206H}/FKBP12 interaction would decline to basal level, as was observed for Zipper/FKBP12, complementation might be unspecific and proteins probably dissociate after a few minutes.

5.3 FKBP12 Binds to mALK^{R206H} but its Inhibition Deposition Capacity Might be Altered Compared to those of mAlk2^{wt}

The results shown in Figure 18 suggest that mAlk2^{wt} and mAlk^{R206H} interaction with FKBP12 is not specifically different. Since it is hypothesized that mAlk^{R206H} inhibition by FKBP12 is weakened compared to those of mAlk2^{wt} (Eileen M Shore

& Kaplan, 2008), Split-LUX lacks features to determine precise protein interaction strength. By applying Split-LUX to examine these interactions on the other hand, it can be shown that FKBP12 does interact with mALK^{R206H}.

5.4 Performance of Split-LUX Assays in COS-1 Cells When Using Transfection Reagent PEI Does Not Lead to Stable Outcomes

Further, the normalization performed by co-transfecting a Firefly pGL3-SV40 vector seems to be problematic. As Figure 18 shows, GLuc values for BMP-receptor/Zipper complementations are on basal level. This leads to the conclusion that these proteins do not interact. When GLuc values are normalized on the other hand (Figure 18 A), it seems like protein interactions used as negative controls do interact. Thus, normalization distorts GLuc outcomes. One approach for optimization might be the use of a different cell line. NIH3T3 cells, for example, showed higher transfection efficiency than COS-1 cells did. This might lead to less fluctuating FLuc values and to a more trustworthy normalization. In addition, using a different transfection reagent might lead to better and more stable transfection efficiency. As previous experiments showed, transfection agent PEI seems to be less effective than, for example, lipofectamin is. Especially, lipofectamin transfected NIH3T3 cells show high transfection efficiency and results stable along numerous repetitions (data not shown).

Further, a mAlk2^{R206H}-FKBP12/mAlk2^{wt}-FKBP12 complementation assay performed in NIH3T3 cells and using lipofectamin (similar experimental set up as in the COS-1 cell approach (Figure 18)) showed significantly less interaction of mAlk2^{R206H}-FKBP12 referenced to mAlk2^{wt}-FKBP12 (data not shown due to inefficient repetitions). Another approach, which could lead to an improved Split-LUX assay, might be the performance of cell lysis in phenolred-free media. Phenolred might disturb the measurement results by giving a higher background signal.

5.5 mAlk2 Receptor Fusions show no Smad-Signaling

As the results depicted in Figure 19 show, GLuc tagging seems to disrupt Smad-signaling induced by mAlk^{R206H}, whereas mAlk3^{wt} downstream abilities do not seem to be altered when a GLuc fragment is C-terminally fused to the receptor.

mAlk2^{R206H} and mAlk3^{wt} constructs lacking GLuc fragments show constitutive activity as expected and reported previously (Dinther et al., 2010). Luciferase tagging of BMP-receptors might intrude BMP-receptor type I phosphorylation by type II due to blockage of the GS domain by the luciferase fragment.

Another possibility could be steric hindrance performed by the luciferase fragments. Meaning, that the luciferase fragments might disable BMP-receptor type II and type I to get into close proximity based on the mobility of the Luc fragments due to the flexible linker attached to it. Close receptor proximity would further allow BMP-receptor type I phosphorylation and Smad-signaling. Another possibility for Smad-signaling inhibition of mAlk2^{R206H} might be that the fused luciferase fragment blocks binding of R-Smad to the phosphate at the GS domain.

In order to determine why mAlk3^{wt}-GLuc fusions remain active while mAlk2^{R206H} loses signaling ability when fused to GLuc, further examination needs to be performed like testing the phosphorylation state of the type I receptor by, for example, immunoprecipitation. Another approach to test whether the receptor is phosphorylated could be a Split-Lux assay performed with BMP-receptor fusions and Smad fusions. If the receptor is phosphorylated, R-Smad (Smad 1) would bind with its trans-activating MH2 domain to the GS domain of the receptor, luciferase fragments would assemble due to the close proximity and luminescence activity could be measured.

5.6 INVESTIGATIONS OF IN VITRO PCA BASED ON FLUC

While this thesis investigated the applicability of protein complementation using split-luciferase fragments in cultured cells, it might be desirable to perform PCAs also *in vitro*, to know whether the interaction is direct or not, since many inhibitors, mediators, or enhancers may exist in the complex cellular milieu (Ohmuro-Matsuyama, Chung, & Ueda, 2013). To establish an *in vitro* Split-LUX assay Ohmuro et al. used, as it was demonstrated in this thesis, the rapamycin inducible protein interaction FKBP12/FRB fused to the corresponding firefly luciferase fragments.

Further, to examine whether reversibility of a protein interaction can be measured, Mdm2 and it´s interaction partner p53 were fused to N- and C- terminal firefly luciferase fragments. The Mdm2 oncoprotein is a cellular inhibitor of the

p53 tumor suppressor. It binds to the trans-activation domain of p53 to downregulate it´s ability to activate transcription. In certain cancer types, Mdm2 amplification is a common event and contributes to the inactivation of p53 (Kussie, 1996). The *in vitro* FLuc PCA was performed by mixing the probes p53-CFLuc and Mdm2-NFLuc at 100 nM each and, for initiating reversibility, the known p53-Mdm2 inhibitor Nutlin-3 was added. Ohmuro et al. clearly showed a Nutlin-3 concentration dependent luminescence inhibition (Ohmuro-Matsuyama et al., 2013). Similar to these findings, the present thesis also examined protein complementation inhibition as it is depicted in Figure 18. Here, FK506 and rapamycin were added to the protein pairs mAlk2^{wt}-NGLuc/FKBP12-CGLuc and mAlk2^{R206H}-NGLuc/FKBP12-CGLuc. These results showed inhibition of these interaction. Thus, it can be concluded that inhibition of protein complementation can be examined by Split-LUX PCA *in vivo*, like is was performed in this thesis, as well as *in vitro*.

5.7 IMAGING PROTEIN INTERACTIONS BEYOND SPLIT-LUX

Split-protein resassembly has established itself as an easy to apply and inexpensive tool to image and visualize cellular processes. Besides the luciferase approach, proteins having a fluorescence nature like GFP or YFP have been used to examine protein interactions. However, the irreversibility of a split-fluorescence protein interaction limits their ability to report on temporal information, although they are useful for trapping low affinity interactions (Nyfeler, Michnick, & Hauri, 2005). Split-luciferase reporters on the other hand have been shown to be reversible (Porter, Stains, Jester, & Ghosh, 2008). Thus, offering advantages such as low background, as no exogenous light is required. Nevertheless, increasing light output as well as substrate availability and stability inside cells or tissues is an ongoing challenge (Shekhawat & Ghosh, 2011).

In order to expand the scope of split-luminescent reporters, multicolor luciferases have been described (Hida et al., 2009) showing improved sensitivity. A multicolor approach was applied in this thesis in order to normalize luciferase measurement values.

To go beyond absorbance, fluorescence, and luminescence based modalities, Massoud et al. utilized a positron emission tomography (PET)-based split reporter

assay using herpes simplex virus type 1 thymidine kinase (TK) as the reporter system. In order to create this system, the protein interaction FKBP12/FRB was used, where the N-terminal TK fragment was fused to FRB wheras the C-terminal kinase fragment was fused to FKBP12. (T. Massoud, Paulmurugan, & Gambhir, 2010) This PET approach couples a sensitive radioactivity based imaging technique, with split protein reassembly and can potentially provide a new approach for clinical imaging of protein-protein interactions in cells as well as in live animals (Shekhawat & Ghosh, 2011).

Further, a rapid cell-fusion-based phenotypic HIV-1 tropism assay was published recently by Teeranaipong et al.. Here, Renilla luciferase and GFP were used to create a phenotypic tropism assay for HIV infection. To do so, Renilla luciferase and GFP were split into two different constructs and stably expressed in the glioma-derived NP-2 cell lines, which expressed CD4/CXCR4 (N4X4) or CD4/CCR5 (N4R5). This dual split reporter protein system was validated by the use of HIV-1 reference strains and, further, applied to test clinical samples from patients with HIV-1 infection (Teeranaipong et al., 2013).

6 CONCLUSION AND OUTLOOK

In this thesis a split-luciferase protein complementation assay was developed and its applicability on BMP-receptor/FKBP12 interaction investigated. This was accomplished by establishing a Split-LUX assay using the known and well-tested protein interaction of FKBP12 and FRB. This work shows that various conditions influence luciferase fragment complementation and are crucial to be optimized for reliable outcomes. Further, the interaction of the FOP mutant receptor mAlk2^{R206H} and its inhibitor FKBP12 was experimentally verified.

This work demonstrates that a Split-LUX approach is applicable for BMP-receptor interactions. To optimize a Split-LUX based protein complementation the experimental set up needs further investigations – including investigation of factors like improvement of transfection efficiency, most suitable cell line, cell lysis conditions, and measurement procedure.

To further investigate the interaction of mAlk2^{R206H} and FKBP12 and, moreover, the impact of the FKBP12 binding to the FOP mutant receptor several experiments should be performed. One approach would be to apply a BRE Smad assay on mAlk2^{R206H} and FKBP12 to examine if FKBP12 binding to the GS domain of the receptor alters the FOP mutants constitutively active state.

Moreover, the kinetics of Gaussia luciferase fragment complementation of the mAlk2^{R206H}/FKBP12 interaction should be examined by measuring the luminescence intensity over a time period of 15 min starting right after substrate addition. If the luminescence intensity declines down to basal level it could be concluded that this interaction is non-specific. Therefore, FKBP12 might not interact with mAlk2^{R206H}.

Despite the interaction of mAlk2^{R206H} and FKBP12, homo- and hetero-dimer interaction of BMP-receptors using Split-LUX should be investigated to get further insights to the overall BMP-receptor interactions which might lead to a deeper understanding of Fibordysplasia Ossificans Progressiva.

This thesis provides further insights into the applicability of Split-LUX on BMP-receptor interactions. Even though Split-LUX is capable of improvement, it could be shown that FKBP12 most likely does interact with mAlk2^{R206H} despite its

altered GS domain. However, the impact of modification in the GS domain of mAlk2^{R206H} and, subsequently, its altered inhibition is still unclear.

Nevertheless, this work shows that Split-LUX is a cost-efficient molecular biological method to investigate protein interactions.

7 REFERENCES

Aghdasi, B., Ye, K., Resnick, A., Huang, A., Ha, H. C., Guo, X., Dawson, T. M., et al. (2000). physiologic regulator of the cell cycle, 1–6.

Ahn, J., Serrano de la Pena, L., Shore, E. M., & Kaplan, F. S. (2003). Paresis of a Bone Morphogenetic Protein-Antagonist Response in a Genetic Disorder of Heterotopic Skeletogenesis. *The Journal of Bone & Joint Surgery*, *85*(4), 667–674. Retrieved from http://dx.doi.org/

Banaszynski, L. a, Liu, C. W., & Wandless, T. J. (2005). Characterization of the FKBP.rapamycin.FRB ternary complex. *Journal of the American Chemical Society*, *127*(13), 4715–21. doi:10.1021/ja043277y

Billings, P. C., Fiori, J. L., Bentwood, J. L., O'Connell, M. P., Jiao, X., Nussbaum, B., Caron, R. J., et al. (2008). Dysregulated BMP signaling and enhanced osteogenic differentiation of connective tissue progenitor cells from patients with fibrodysplasia ossificans progressiva (FOP). *Journal of Bone and Mineral Research*, *23*(3), 305–313. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2669159&tool=pmcentrez&rendertype=abstract

Bocciardi, R., Bordo, D., Di Duca, M., Di Rocco, M., & Ravazzolo, R. (2008). Mutational analysis of the ACVR1 gene in Italian patients affected with fibrodysplasia ossificans progressiva: confirmations and advancements. *Eur J Hum Genet*, *17*(3), 311–318. Retrieved from http://dx.doi.org/10.1038/ejhg.2008.178

Brasseura, A., & Rotureauc, B. (2012). Trypanosoma brucei FKBP12 Differentially Controls Motility and Cytokinesis in Procyclic and Bloodstream Forms. *Eukaryotic Cell*, *12*(2), 168–181.

Canalis, E. (2003). Bone Morphogenetic Proteins, Their Antagonists, and the Skeleton. *Endocrine Reviews*, *24*(2), 218–235. doi:10.1210/er.2002-0023

Cardenas, M. E., Cruz, M. C., Del Poeta, M., Chung, N., Perfect, J. R., & Heitman, J. (1999). Antifungal activities of antineoplastic agents: Saccharomyces cerevisiae as a model system to study drug action. *Clinical microbiology reviews*, *12*(4), 583–611. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=88926&tool=pmcentrez&rendertype=abstract

Choi, J., & Chen, J. (1996). Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science*, *273*(5272), 239–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8662507

Cohen, R. (1993). The natural history of heterotopic ossification in patients who have fibrodysplasia ossificans progressiva. A study of forty-four patients. *The*

Journal of Bone & Joint Surgery, *75*(2), 215–219. Retrieved from http://dx.doi.org/

Connor, J. M. E. D. (1982). Fibrodysplasia ossificans progressiva; the clinical features and natural history of 34 patients. *The Journal of Bone & Joint Surgery*, *0301-620X*(82), 1021–0076.

Cruz, M. C., Cavallo, L. M., Görlach, J. M., Cox, G., Perfect, J. R., Cardenas, M. E., & Heitman, J. (1999). Rapamycin antifungal action is mediated via conserved complexes with FKBP12 and TOR kinase homologs in Cryptococcus neoformans. *Molecular and cellular biology*, *19*(6), 4101–12. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=104369&tool=pmcentrez&rendertype=abstract

De la Peña, L. S., Billings, P. C., Fiori, J. L., Ahn, J., Kaplan, F. S., & Shore, E. M. (2005). Fibrodysplasia Ossificans Progressiva (FOP), a Disorder of Ectopic Osteogenesis, Misregulates Cell Surface Expression and Trafficking of BMPRIA. *Journal of Bone and Mineral Research*, *20*(7), 1168–1176. doi:10.1359/JBMR.050305

Dinther, M. Van, Visser, N., Gorter, D. J. J. De, Doorn, J., Boer, J. De, & Dijke, P. (2010). ALK2 R206H Mutation Linked to Fibrodysplasia, *25*(6), 1208–1215. doi:10.1002/jbmr.091110

Fiori, J. L., Billings, P. C., De la Peña, L. S., Kaplan, F. S., & Shore, E. M. (2006). Dysregulation of the BMP-p38 MAPK Signaling Pathway in Cells From Patients With Fibrodysplasia Ossificans Progressiva (FOP). *Journal of Bone and Mineral Research*, *21*(6), 902–909. doi:10.1359/jbmr.060215

Fukuda, T., Kohda, M., Kanomata, K., Nojima, J., Nakamura, A., Kamizono, J., Noguchi, Y., et al. (2009). Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. *The Journal of biological chemistry*, *284*(11), 7149–56. doi:10.1074/jbc.M801681200

Hampf, M., & Gossen, M. (2006). A protocol for combined Photinus and Renilla luciferase quantification compatible with protein assays. *Analytical biochemistry*, *356*(1), 94–9. doi:10.1016/j.ab.2006.04.046

Heining, E., Schwappacher, R., Horbelt, D., Huber, O., & Knaus, P. (2009). Crosstalk of cyclic GMP dependent kinase I and BMP signaling. *BMC Pharmacology*, *9*(Suppl 1), P23. doi:10.1186/1471-2210-9-S1-P23

Hida, N., Awais, M., Takeuchi, M., Ueno, N., Tashiro, M., Takagi, C., Singh, T., et al. (2009). High-sensitivity real-time imaging of dual protein-protein interactions in living subjects using multicolor luciferases. *PloS one*, *4*(6), e5868. doi:10.1371/journal.pone.0005868

Huse, M., YG, C., Massague, J., & Kuriyan, J. (1999). Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12.

Cell, *96*, 425–436. Retrieved from http://dx.doi.org/10.1016/S0092-8674(00)80555-3

Kaplan, F S, Groppe, J., Pignolo, R. J., & Shore, E. M. (2007). Morphogen receptor genes and metamorphogenes: skeleton keys to metamorphosis. *Ann N Y Acad Sci*, *1116*, 113–133.

Kaplan, F S, Xu, M., Glaser, D. L., Collins, F., Connor, M., Kitterman, J., Sillence, D., et al. (2008). Early diagnosis of fibrodysplasia ossificans progressiva. *Pediatrics*, *121*, e1295–e1300.

Kaplan, F S, Xu, M., Seemann, P., Connor, M., Glaser, D. L., Carroll, L., Delai, P., et al. (2009). Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. *Hum Mutat*, *30*, 379–390.

Kaplan, Frederick S, Chakkalakal, S. a, & Shore, E. M. (2012). Fibrodysplasia ossificans progressiva: mechanisms and models of skeletal metamorphosis. *Disease models & mechanisms*, *5*(6), 756–62. doi:10.1242/dmm.010280

Kussie, P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*, *8*(274(5289), 948–53.

Lorenz, W W, Cormier, M. J., O'Kane, D. J., Hua, D., Escher, A. A., & Szalay, A. A. (1996). Expression of the Renilla reniformis Luciferase Gene in Mammalian Cells. *Journal of Bioluminescence and Chemiluminescence*, *11*(1), 31–37. doi:10.1002/(SICI)1099-1271(199601)11:1<31::AID-BIO398>3.0.CO;2-M

Lorenz, W Walter, & Mccann, R. (1991). Isolation and expression of a cDNA encoding Renilla reniformis luciferase, *88*(May), 4438–4442.

Lucotte, G., Houzet, A., Hubans, C., Lagarde, J. P., & Lenoir, G. (2009). Mutations of the noggin (NOG) and of the activin A type I receptor (ACVR1) genes in a series of twenty-seven French fibrodysplasia ossificans progressiva (FOP) patients. *Genetic counseling Geneva Switzerland*, *20*(1), 53–62. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19400542

Massoud, T. F., & Gambhir, S. S. (2003). Molecular imaging in living subjects : seeing fundamental biological processes in a new light. *Genes Dev*, *17*, 545–580. doi:10.1101/gad.1047403

Massoud, T., Paulmurugan, R., & Gambhir, S. S. (2010). A molecularly engineered split reporter for imaging protein- protein interactions with positron emission tomography, *16*(8), 921–926. doi:10.1038/nm.2185.A

Michnick, S. W., Ear, P. H., Manderson, E. N., Remy, I., & Stefan, E. (2007). Universal strategies in research and drug discovery based on protein-fragment complementation assays. *Nat Rev Drug Discov*, *6*(7), 569–582. Retrieved from http://dx.doi.org/10.1038/nrd2311

Nojima, J., Kanomata, K., Takada, Y., Fukuda, T., Kokabu, S., Ohte, S., Takada, T., et al. (2010). Dual roles of smad proteins in the conversion from myoblasts to osteoblastic cells by bone morphogenetic proteins. *The Journal of biological chemistry*, *285*(20), 15577–86. doi:10.1074/jbc.M109.028019

Nyfeler, B., Michnick, S. W., & Hauri, H.-P. (2005). Capturing protein interactions in the secretory pathway of living cells. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(18), 6350–5. doi:10.1073/pnas.0501976102

Ohmuro-Matsuyama, Y., Chung, C.-I., & Ueda, H. (2013). Demonstration of protein-fragment complementation assay using purified firefly luciferase fragments. *BMC biotechnology*, *13*(1), 31. doi:10.1186/1472-6750-13-31

Pasero, M., Giovarelli, M., Bucci, G., Gherzi, R., & Briata, P. (2012, November 29). Bone Morphogenetic Protein/SMAD Signaling Orients Cell Fate Decision by Impairing KSRP-Dependent MicroRNA Maturation. *Cell Reports*. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S2211124712003750

Paulmurugan, R., Massoud, T. F., Huang, J., & Gambhir, S. S. (2004). Molecular imaging of drug-modulated protein-protein interactions in living subjects. *Cancer Research*, *64*(6), 2113–2119. doi:10.1158/0008-5472.CAN-03-2972

Petrie, K. A., Lee, W. H., Bullock, A. N., Pointon, J. J., Smith, R., Russell, R. G. G., Brown, M. A., et al. (2009). Novel Mutations in ACVR1 Result in Atypical Features in Two Fibrodysplasia Ossificans Progressiva Patients. *PLoS ONE*, *4*(3), e5005. Retrieved from http://dx.doi.org/10.1371/journal.pone.0005005

Porter, J. R., Stains, C. I., Jester, B. W., & Ghosh, I. (2008). A General and Rapid Cell-Free Approach for the Interrogation of Protein−Protein, Protein−DNA, and Protein−RNA Interactions and their Antagonists Utilizing Split-Protein Reporters. *Journal of the American Chemical Society*, *130*(20), 6488–6497. doi:10.1021/ja7114579

Remy, I, & Michnick, S. W. (2001). Visualization of biochemical networks in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(14), 7678–83. doi:10.1073/pnas.131216098

Remy, Ingrid, & Michnick, S. W. (2006). A highly sensitive protein- protein interaction assay based on Gaussia luciferase, *3*(12), 977–979. doi:10.1038/NMETH979

Rocke DM, Zasloff M, Peeper J, Cohen RB, K. F. (1994). Age- and joint-specific risk of initial heterotopic ossification in patients who have fibrodysplasia ossificans progressiva. *Clin Orthop Relat Res.*, (301), 243–8.

Shafritz, A. B., Shore, E. M., Gannon, F. H., Zasloff, M. A., Taub, R., Muenke, M., & Kaplan, F. S. (1996). Overexpression of an Osteogenic Morphogen in

Fibrodysplasia Ossificans Progressiva. *New England Journal of Medicine*, *335*(8), 555–561. doi:10.1056/NEJM199608223350804

Shekhawat, S., & Ghosh, I. (2011). Split-Protein Systems: Beyond Binary Protein-Protein Interaction, *15*(6), 789–797. doi:10.1016/j.cbpa.2011.10.014.Split-Protein

Shen, Q., Little, S. C., Xu, M., Haupt, J., Ast, C., Katagiri, T., Mundlos, S., et al. (2009). The fibrodysplasia ossificans progressiva R20H ACVR1 mutation activates BMP independent chondrogenesis and zebrafish embargo ventralization. *J Clin Invest*, *119*, 3462–3472.

Shi, Y., & Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, *113*, 685–700. Retrieved from http://dx.doi.org/10.1016/S0092-8674(03)00432-X

Shore, E M, Feldman, G. J., Xu, M., & Kaplan, F. S. (2005). The genetics of fibrodysplasia ossificans progressiva. *Clin Rev Bone Miner Metab*, *3*, 201–204.

Shore, Eileen M, & Kaplan, F. S. (2008, September 1). Insights from a rare genetic disorder of extra-skeletal bone formation, fibrodysplasia ossificans progressiva (FOP). *Bone*. Elsevier Science. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S8756328208002780?showall=true

Song, G.-A., Kim, H.-J., Woo, K.-M., Baek, J.-H., Kim, G.-S., Choi, J.-Y., & Ryoo, H.-M. (2010). Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva. *The Journal of biological chemistry*, *285*(29), 22542–53. doi:10.1074/jbc.M109.094557

Spiekerkoetter, E., Tian, X., Cai, J., Hopper, R. K., Sudheendra, D., Li, C. G., El-bizri, N., et al. (2013). rescues endothelial dysfunction , and reverses pulmonary hypertension, *123*(8). doi:10.1172/JCI65592.3600

Tannous, B. A., Kim, D.-E., Fernandez, J. L., Weissleder, R., & Breakefield, X. O. (2005). Codon-Optimized Gaussia Luciferase cDNA for Mammalian Gene Expression in Culture and in Vivo. *Mol Ther*, *11*(3), 435–443. Retrieved from http://dx.doi.org/10.1016/j.ymthe.2004.10.016

Teeranaipong, P., Hosoya, N., Kawana-Tachikawa, A., Fujii, T., Koibuchi, T., Nakamura, H., Koga, M., et al. (2013). Development of a rapid cell-fusion-based phenotypic HIV-1 tropism assay. *Journal of the International AIDS Society*, *16*(1), 18723. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3778210&tool=pmcentrez&rendertype=abstract

Vrijens, K., Lin, W., Cui, J., Farmer, D., Low, J., Pronier, E., Zeng, F., et al. (2013). Identification of Small Molecule Activators of BMP Signaling, *8*(3). doi:10.1371/journal.pone.0059045

Wang, T., BY, L., & PD, D. (1996). The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell*, *86*, 435–444. Retrieved from http://dx.doi.org/10.1016/S0092-8674(00)80116-6

Wet, J. R., & Wood, K. V. (1987). Firefly Luciferase Gene : Structure and Expression in Mammalian Cells. *Molecular and cellular biology*, *7*(2), 725–737. doi:10.1128/MCB.7.2.725.Updated

Wieser, R., Wrana, J. L., & Massagué, J. (1995). GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *The EMBO journal*, *14*(10), 2199–208. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=398326&tool=pmcentrez&rendertype=abstract

Wilson, T., & Hastings, J. W. (1998). BIOLUMINESCENCE. *Cell and Developmental Biology*, *14*(1), 197–230. doi:10.1146/annurev.cellbio.14.1.197

8 CURRICULUM VITAE

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2010 – 2013	Master Studies of Molecular Biology focusing on Molecular Medicine at the University of Vienna
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2003 – 2006	Studies of Human Medicine at the Medical University Vienna
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2010	**Bachelor Thesis** **"Oriented Immobilization of Antibodies (IgG) by Reduction of Disulfide-Bonds and by Protein G"**
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