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DEVELOPMENT OF A GRK2-INHIBITORY PEPTIDE

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1 Summary

G protein-coupled receptor (GPCR) kinases (GRKs) are most prominently known for phosphorylating agonist-occupied receptors resulting in signal attenuation.
GRK2, the most thoroughly investigated GRK isoform, is recognized as a potential drug target for disorders such as heart failure and hypertension. However, efforts to find a GRK2-inhibitor have been futile.

The ubiquitously expressed GRK2 displays a tri-domain structure with the central kinase domain harbouring the enzyme active site, flanked by an amino terminal domain mediating receptor recognition and a carboxyl terminal domain (GRK2CTD) involved in localization to the plasma membrane. More specifically, Gβγ-interaction with GRK2CTD is required for kinase translocation to the plasma membrane towards receptor substrates. Blocking of this interaction with βARKct, a Gβγ-scavenging peptide derived from the carboxyl terminal domain of GRK2, successfully inhibits GRK2 activity and prevents heart failure development in animal models. Moreover, interfering with Gβγ binding of GRK2 confers subtype-specificity, because membrane localization of other GRK isoforms employs different mechanisms. In a similar manner, compounds specifically interacting with GRK2CTD would be expected to block GRK2 function. Nevertheless, such an approach, to our knowledge, was not investigated to date.

Therefore, in this study, a peptide phage display library screen was used as an approach towards finding of GRK2 domain-specific interaction partners. Specifically, the focus was to identify peptides that interact with GRK2CTD. The peptides' inhibitory features were subsequently characterized in different *in vitro* assays and *in vivo*.

The three GRK2 domains were purified individually from bacterial cultures. In a next step, GRK2CTD was used as bait in a peptide phage display library screen, which yielded the following 7-mer peptide sequences: ASTLIVF, IRYVPQT, HGGVRLY, HYTDFRW, IVSLQTP, and HYIDFRW.

To investigate sequence homology with known proteins, a protein data base search was performed. Peptide sequence IVSLQTP is partially contained in the nuclear-factor of activated T-cells and peptide ASTLIVF shares sequence identity with the WD repeat-containing protein 76 (WDR76). WDR76 is structurally related to Gβγ, another WD repeat-containing protein and, as mentioned before, a known GRK2 interaction and activation partner. Based on WDR76, the original peptide sequence (ASTLIVF) was elongated carboxyl (WD1) and amino (WD3) terminally. WD1 and WD3 were used for further experiments.

For WD3, the interaction with GRK2CTD could be confirmed by ELISA technique, whereas WD1 did not significantly interact with GRK2CTD.

To study the effect of WD1 and WD3 on the Gβγ-GRK2CTD interaction essential for kinase activity, an inhibition ELISA with recombinant Gβ3 purified from bacterial culture and GRK2CTD was established. In this assay, WD3 successfully blocked the Gβ3-GRK2CTD protein-protein interaction. In contrast, WD1 did not interfere with the binding of GRK2CTD to Gβ3.

In addition to its role in receptor phosphorylation and subsequent desensitization, GRK2 also phosphorylates a number of cytosolic non-receptor substrates.

To investigate, whether WD1 and WD3 influence GRK2 kinase activity, an *in vitro* phosphorylation assay using a soluble substrate of GRK2 was performed. WD3 reduced GRK2-mediated phosphorylation of this non-receptor substrate, whereas WD1 did not display any inhibitory effect.

The impact of WD1 and WD3 on desensitization of the GPCR bradykinin receptor type 2 was assessed *in vitro* in HEK cells. Additionally, another peptide (WD2), which was elongated based on the original peptide sequence both amino and carboxyl terminally, was tested. Only WD3 increased bradykinin-stimulated calcium signalling, indicating reduced GRK2-mediated receptor desensitization.

To further investigate the properties of WD3 *in vivo*, a transgenic mouse model with cardiac-specific expression was established. In a pressure overload model of cardiac dysfunction, WD3 retarded signs of heart failure *in vivo*.

Taken together, modification of a phage display-derived peptide yielded a peptide prototype with GRK2-inhibitory features *in vitro* and *in vivo*. By employing a novel strategy to GRK2-inhibition, it could serve as a lead structure for further development into a candidate drug compound.

2 Zusammenfassung

G-protein-gekoppelte Rezeptorkinasen (GRKs) phosphorylieren und regulieren dadurch aktivierte G-protein-gekoppelte Rezeptoren (GPCRs), was zu einer Signaldämpfung führt. GRK2, die am besten untersuchte GRK-Isoform, gilt als ein möglicher Angriffspunkt für die Behandlung von Krankheiten wie Herzinsuffizienz und Hypertonie. Trotz grosser Bemühungen zur Entwicklung von Inhibitoren konnte bis heute keine Substanz erfolgreich in der Klinik etabliert werden.

Die ubiquitär exprimierte GRK2 besteht aus drei Domänen mit unterschiedlichen Funktionen: der zentralen Kinasedomäne, welche das aktive Zentrum beherbergt; der aminoterminalen Domäne, welche Rezeptorsubstrate erkennt und bindet; und der carboxylterminalen Domäne (GRK2CTD), durch welche der Transport der Kinase zur Plasmamembran vermittelt wird. Eine Voraussetzung für diesen Schritt sowie die Aktivierung des Enzyms ist die Interaktion mit Gβγ. Wird diese durch βARKct, einem von der GRK2CTD abgeleitetem Gβγ-Scavengerpeptid, gestört, inhibiert dies die Aktivität von GRK2 und vermag das Auftreten von Herzinsuffizienz im Tiermodell zu verhindern. Da die übrigen GRK-Isoformen mittels alternativer Mechanismen zur Plasmamembran gelangen, ermöglicht die gezielte Störung der Gβγ-GRK2CTD-Bindung eine subtyp-spezifische Inhibierung. Analog dazu wird erwartet, dass Substanzen, welche selektiv mit der GRK2CTD interagieren, die Kinaseaktivität vermindern. Ein solcher Ansatz wurde jedoch nach unserem Wissen bisher noch nicht untersucht.

Zunächst wurden die drei GRK2-Domänen einzeln aus Bakterienkulturen aufgereinigt. In einem nächsten Schritt wurde ein Phagen-Display mit einer Peptidbibliothek durchgeführt. Dabei wurde GRK2CTD als Zielmolekül verwendet. Es wurden die folgenden, jeweils sieben Aminosäuren enthaltenden Sequenzen gefunden: ASTLIVF, IRYVPQT, HGGVRLY, HYTDFRW, IVSLQTP und HYIDFRW.

Mit den gefundenen Peptidsequenzen wurde eine Proteindatenbank durchsucht, um allfällige Homologien mit bekannten Proteinen zu erkennen. Dabei wurde eine teilweise Übereinstimmung der Peptidsequenz IVSLQTP mit NFAT, dem nuclear factor of activated T-cells, gefunden. Des Weiteren ist die Peptidsequenz ASTLIVF auch in WDR76, dem WD repeat-containing protein 76, enthalten. WDR76 weist eine ähnliche Struktur wie Gβγ auf, welches ebenfalls ein WD repeat-containing protein ist. Ausserdem ist Gβγ, wie bereits erwähnt, verantwortlich für die Aktivierung von GRK2 und den Transport zur Plasmamembran in Richtung Rezeptorsubstrate. Deshalb wurde das ursprüngliche Peptid basierend auf der Proteinsequenz von WDR76 in Richtung Carboxylterminus (WD1) und Aminoterminus (WD3) verlängert. Die Peptide WD1 und WD3 wurden im Folgenden für weitere Experimente verwendet.

Für WD3 konnte die Interaktion mit der GRK2CTD mittels ELISA bestätigt werden, wo hingegen WD1 nicht signifikant mit der GRK2CTD interagierte.

Daraufhin wurde ein Inhibierungs-ELISA mit aus Bakterienkulturen aufgereinigtem Gβ3 sowie GRK2CTD durchgeführt, um den Effekt der beiden Peptide WD1 und WD3 auf die Gβγ-GRK2CTD-Interaktion zu untersuchen. Dabei konnte WD3 die Gβ3-GRK2CTD Protein-Protein-Interaktion empfindlich stören. Im Gegensatz dazu beeinflusste WD1 die Bindung von GRK2CTD zu Gβ3 nicht.

Neben der Phosphorylierung und Desensibilisierung von Rezeptoren kann GRK2 auch eine Reihe von zytosolischen Nichtrezeptorsubstraten phosphorylieren.

Ein solch lösliches Substrat wurde in einer *in vitro* Phosphorylierungsreaktion eingesetzt mit dem Ziel, den Effekt von WD1 und WD3 auf die Kinaseaktivität von GRK2 zu untersuchen. Dabei vermochte WD3 die GRK2-vermittelte Phosphorylierung dieses Nichtrezeptorsubstrates zu verhindern, während WD1 keinen Effekt zeigte.

Der Einfluss von WD1 und WD3 auf die Desensibilisierung eines G-protein-gekoppelten Rezeptors, d.h. des Bradykininrezeptors Typ 2, wurde *in vitr*o in HEK-Zellen untersucht. Zusätzlich wurde eine weiteres Peptid (WD2) getestet, welches basierend auf der ursprünglichen Peptidsequenz in Richtung Amino- und Carboxylterminus verlängert worden war. Ausschliesslich WD3 erhöhte das Bradykinin-stimulierte Kalziumsignal, was auf eine reduzierte GRK2-vermittelte Rezeptordesensibilisierung hinweist.

Um die Eigenschaften von WD3 weiter *in vivo* zu untersuchen, wurde ein transgenes Mausmodell, welches die WD3-Peptidsequenz spezifisch im Herzen exprimiert, etabliert. WD3 verzögerte im Mausmodell das Auftreten von Herzinsuffizienzanzeichen in einer durch chronische Hypertonie erzeugten kardialen Hypertrophie.

Zusammenfassend wird in dieser Arbeit eine modifizierte Version eines von einem Phagen-Display stammenden Peptids vorgestellt. Dieser Peptidprototyp zeigt *in vitro* und *in vivo* GRK2-inhibierende Eigenschaften. Dabei könnte insbesondere die Anwendung einer neuartigen Strategie, um GRK2 zu inhibieren, bedeuten, dass dieser Prototyp als Ausgangsstruktur zur Entwicklung eines Medikamentes dienen könnte.

3 Abbreviations

6xHis	hexahistidine-tag/His-tag
7TM	seven-transmembrane
A	adenine
AAC	abdominal aortic constriction
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AGC	family of protein kinases A, G and C
APS	ammonium persulfate
AT1R	angiotensin II type-1 receptor
ATP	adenosine triphosphate
B2R	bradykinin receptor B2
BBS	BES-buffered saline
BES	*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
βAR	β-adrenergic receptor
βARKct	amino acids 495-689 of GRK2
βARKnt	amino acids 1-14 of GRK2
βarr	β-arrestin
BLAST	Basic local alignment search tool
BSA	bovine serum albumin
C	cytosine (DNA) / carboxyl (amino acids)
CAMKIIδ	Ca^{2+}/calmodulin-dependent kinase IIδ
cAMP	3',5'-cyclic adenosine monophosphate
cDNA	complementary DNA
DAG	diacylglycerol
DMEM	Dulbecco's modified eagle's medium
DMF	*N,N*-dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
ds	double-stranded
ECL	enhanced chemoluminescence
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FBS	foetal bovine serum
Fura-2AM	Fura-2-acetoxymethyl ester
G	guanine

G protein	guanine nucleotide-binding protein
G418	Geneticin
GAP	GTPase-activating protein
Gα	α-subunit of the heterotrimeric G protein
Gβ	β-subunit of the heterotrimeric G protein
Gβ3	G protein subunit β-3, isoform 1
Gβ3-s	G protein subunit β-3, isoform 2
Gβγ	βγ-complex of the heterotrimeric G protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GIRK	G protein-gated inwardly rectifying potassium channel
GPCR	G protein-coupled receptor
GRAFS	glutamate, rhodopsin, adhesion, frizzled/taste2, secretin
GRK	G protein-coupled receptor kinase
GRK2CTD	carboxyl terminal domain of GRK2 (amino acids 454-689)
GRK2KD	kinase domain of GRK2 (amino acids 191-453)
GRK2NTD	amino terminal domain of GRK2 (amino acids 1-190)
GTP	guanosine triphosphate
GTPase	GTP-hydrolysing enzyme
HEK293A	cell line derived from human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IP_3	inositol-1,4,5-triphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α
lac	lactose
LB	lysogeny broth
LDS	lithium dodecyl sulphate
mAB	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MHC	myosin heavy chain
miRNA	micro ribonucleic acid
MOPS	3-(*N*-morpholino)propanesulfonic acid
N	amino
NES	nuclear export signal
Ni-NTA	nickel-nitrilotriacetic acid
NLS	nuclear localization signal
NO	nitric oxide

OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS-Tween
PCR	polymerase chain reaction
PDC	phosducin
PEBP	phosphatidylethanolamine-binding protein
PEG	polyethylene glycol
Pfu	*Pyrococcus furiosus*
pfu	plaque forming units
PH	pleckstrin homology
pI	isoelectric point
PIP$_2$	phosphatidylinositol-4,5-biphosphate
PKA	protein kinase A/cAMP-dependent protein kinase
PKC	protein kinase C
PLB	phospholamban
PLCβ	β-isoform of phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PPI	protein-protein interaction
PVDF	polyvinylidene difluoride
RAAS	renin-angiotensin-aldosterone system
RACK1	receptor for activated C kinase 1
RGS	regulator of G protein signalling
RH	regulator of G protein signalling homology
RhoGEF	Rho guanine nucleotide exchange factor
RKIP	Raf-1 kinase inhibitor protein
RS	arginine/serine
RT	room temperature (25 °C)
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Sf9	*Spodoptera frugiperda*
SIGK	SIGKAFKILGYPDYD
SIRK	SIRKALNILGYPDYD
SOB	super optimal broth
SOC	super optimal broth with catabolite repression
SRSF1	serine/arginine-rich splicing factor 1
ss	single-stranded
SSRI	selective serotonin reuptake inhibitor
T	thymine
TAE	Tris, acetic acid, EDTA

Taq	*Thermus aquaticus*
TBE	Tris, boric acid, EDTA
TBS	Tris-buffered saline
TBST	TBS-Tween
TE	Tris, EDTA
TEMED	tetramethylethylenediamine
Tris	Tris(hydroxymethyl) amino methane
TSB	transformation and storage buffer
WD	Tryptophan-Aspartic acid
WD1	ASTLIVGHWDGNMSLVDRRT
WD2	FLAEDASTLIVGHWDGNMSL
WD3	ERSSFSSFDFLAEDASTLIVF
WDR	WD repeat-containing protein
WDR76	human WD repeat-containing protein 76
x-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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4 Introduction

4.1 G protein-coupled receptor signalling

4.1.1 Characteristics of G protein-coupled receptors

The family of G protein-coupled receptors (GPCRs) comprises the largest and most diverse group of membrane receptors in the mammalian genome. Over 800 human genes encode these structurally highly conserved proteins with paramount importance in the field of pharmacotherapy (1,2).

Originally named for their interaction with heterotrimeric guanine nucleotide-binding proteins (G proteins), they are also known as seven-transmembrane (7TM) receptors owing to their unique characteristic motif of seven membrane-spanning α-helices. Other features include an N-terminal extracellular domain, a C-terminal intracellular tail, and loops on both sides of the cell membrane connecting the transmembrane helices (3,4). Understanding of GPCR functionality has tremendously increased with the availability of the first high-resolution crystal structure of rhodopsin in 2000 (5). In the last five years, the number of GPCR crystal structures has increased and more importantly, some receptors are available in multiple conformations (6).

The GRAFS classification system divides GPCRs based on sequence similarities into five families: glutamate, rhodopsin, adhesion, frizzled/taste2, secretin. The rhodopsin family is by far the largest family including around 700 receptors and can be classed into subgroups (α-δ) (7).

GPCRs activate a complex downstream signalling network

GPCRs take a prominent role in transmembrane signal transduction pathways and hence are involved in almost all physiological processes (8,9). They react to a variety of extracellular stimuli such as photons, peptides, proteins, small organic molecules, amino acids and ions (10).

According to the classical concept, the receptors elicit their diverse physiological functions upon stimulation by initiation of a signalling cascade involving heterotrimeric G proteins, second messenger molecules and a variety of effector proteins (11-13).

In recent years, growing evidence for arrestin-mediated G protein-independent signalling pathways has emerged. Classically, arrestins promote G protein-uncoupling following receptor phosphorylation by a G protein-coupled receptor kinase (homologous desensitization) (14). However, non-canonical functions for arrestins in scaffolding signal complexes initiating functions distinct from G protein-dependent processes have increasingly come into focus (15-17).

This led to a paradigm shift towards an extended model of more complex intracellular signalling networks initiated by 7TM receptors (Figure 1).
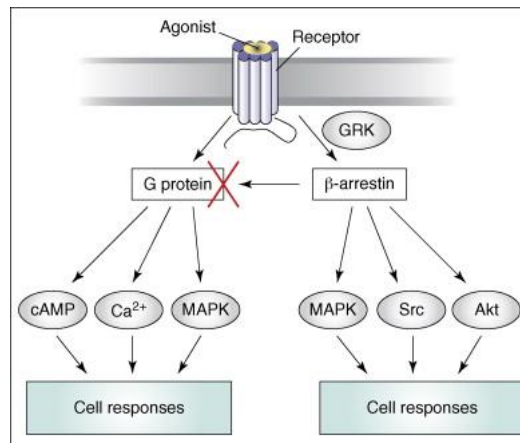
Figure 1: Agonist-binding of a GPCR initiates a downstream signalling network
Following agonist binding, GPCRs may activate G protein-dependent or β-arrestin-dependent (G protein-independent) pathways. Binding of β-arrestins to GPCRs phosphorylated by GRKs attenuates G protein-signalling (18).

4.1.2 Heterotrimeric G proteins are classical signal transducers

Heterotrimeric G proteins transmit receptor activation to intracellular second messengers (12). Mammalian heterotrimeric G proteins consist of three distinct subunits encoded by gene families: 21 α-subunits (Gα, 39-52 kDa) encoded by 16 genes, 6 β-subunits (Gβ, 36 kDa) encoded by 5 genes and 12 γ-subunits (Gγ, 7-8 kDa) encoded by 12 genes (19). Gα, in its inactive state, is bound to GDP and forms a heterotrimer with Gβγ. Upon ligand binding, the GPCR undergoes a conformational change and couples to the G protein. As guanine nucleotide exchange factors (GEF), GPCRs induce Gα to exchange GDP for GTP, thereby activating it. The activated Gα·GTP then dissociates from Gβγ. Both, Gα·GTP and Gβγ are thus free to interact with downstream effectors. Signal termination occurs through GTP-hydrolysis facilitated by the intrinsic GTPase activity of Gα (11,20). Further, the family of regulator of G protein signalling (RGS) proteins were found to promote GTPase activity (21). Inactivation of Gα enables re-association with Gβγ and thus prepares the G protein for a new cycle of activation (11,13).

4.1.2.1 The α-subunit of heterotrimeric G proteins

Based on sequence similarities, Gα subunits have been divided into four canonical families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. The different subtypes display receptor specificity and a distinct pattern of effector activation (22).

Effectors of $G\alpha_s$

$G\alpha_s$ subunits stimulate adenylyl cyclase to convert adenosine triphosphate (ATP) to 3', 5'-cyclic adenosine monophosphate (cAMP) (23), a second messenger activating

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protein kinase A (PKA) (24). This serine/threonine kinase, which belongs to the family of AGC kinases (protein kinases A, G and C), regulates a variety of physiological functions by direct activation of proteins by phosphorylation or alteration of gene transcription (25,26).

Effectors of Gα$_{i/o}$

Classically, Gα$_{i/o}$ subunits inhibit adenylyl cyclase, thus decreasing intracellular cAMP levels and reducing PKA activation (12,27).

Effectors of Gα$_{q/11}$-subunits

Gα$_{q/11}$ activates the β-isoform of phospholipase C (PLCβ), which converts phosphatidylinositol-4,5-biphosphate (PIP$_2$) into inositol-1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). These second messengers increase intracellular Ca^{2+} by stimulating calcium release from the endoplasmic reticulum. DAG activates protein kinase C (PKC) (28,29).

In addition, Gα$_{q/11}$ directly interacts with G protein-coupled receptor kinase 2 (GRK2, originally named β-adrenergic receptor kinase (βARK)). Via its N-terminal regulator of G protein signalling (RGS) homology (RH) domain, GRK2 rapidly inactivates Gα$_{q/11}$ in a phosphorylation-independent manner (30-32).

Effectors of Gα$_{12/13}$

The most recently discovered family of Gα subunits (Gα$_{12/13}$) activates RhoGTPase nucleotide exchange factors (RhoGEF), which in turn promote nucleotide exchange on the small GTPase RhoA (33,34).

4.1.2.2 The βγ-complex of heterotrimeric G proteins

The β- and γ-subunits of the heterotrimeric G protein act as a functional unit and co-expression is required for proper folding (35). Gβ is a WD repeat-containing protein displaying a characteristic seven-bladed β-propeller-structure (36). Gγ conveys membrane attachment through isoprenylation at a C-terminal cysteine residue (35).

Downstream effectors of the Gβγ complex

Once dissociated from the Gα subunit, Gβγ is free to interact with a variety of downstream effectors (37).

Kinases

In canonical GPCR signalling, Gβγ plays a pivotal role by activating and recruiting GRK2/3 to the plasma membrane, eventually promoting receptor desensitization and downregulation (38). Furthermore, Gα$_i$-derived Gβγ can activate the mitogen-activated protein kinase (MAPK) pathway, which in turn is involved in cell cycle control (39,40).

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Ion homeostasis

Additionally, Gβγ is involved in intracellular ion homeostasis by modulating the activity of a number of ion channels. It was found to modulate voltage-dependent N- and P/Q-type Ca^{2+}-channels in neurons (41,42). Moreover, Gβγ is able to activate the G protein-gated inwardly rectifying potassium channel (GIRK) directly (43). Gβγ also stimulates PLCβ to release calcium from the endoplasmic reticulum (37).

Furthermore, Gβγ is involved in the regulation of adenylyl cyclase showing diverse effects depending on the isoforms (37,44).

4.1.2.3 Coupling specificity of GPCRs

Receptors are known to couple preferentially to specific Gα-subunits, thus eliciting a distinct cellular response. But it is also known that certain receptors are able to couple to multiple G proteins from different subclasses, simultaneously or successively (45).
In the case of the β-adrenergic receptor type 2 (β2AR), switching from canonical coupling to $Gα_s$ to $Gα_i$ is mediated by receptor phosphorylation through the second messenger-dependent kinase PKA (46).

4.1.3 Arrestins are mediators of GPCR signalling and desensitization

The family of arrestins consists of four members: the visual arrestins (arrestin-1 and arrestin-4) expressed in the retina (47,48), and the ubiquitously expressed arrestin-2 and -3 (β-arrestin 1 and 2) (49). β-arrestins were first described for dampening the β2-adrenergic response following agonist stimulation (50). The ~ 48-kDa proteins share ~70% sequence identity, but display distinct, albeit overlapping physiological roles (48,51).

Originally known for their role in "arresting" (52) GPCR signalling following phosphorylation by GRKs (14), arrestins are now increasingly recognized for also promoting G protein-independent receptor signal transduction (53,54).

4.1.3.1 Arrestins are signal transducers

A role for β-arrestins in GPCR signal transduction was first proposed for the stimulation of the mitogen-activated protein kinase (MAPK) pathway via activation of the non-receptor protein tyrosine kinase (Src) (15). Moreover, β-arrestin 2 was demonstrated to act as a scaffolding protein in the formation of stable signalling complexes containing the angiotensin II type-1 receptor (AT1R) and each of the component kinases of the extracellular signal-regulated kinase MAPK/ERK pathway (16). In addition, β-arrestins were found to be key players in endothelial growth factor receptor (EGFR) transactivation following agonist-stimulation of the β-adrenergic receptor type 1 (β1AR) (55).

Interestingly, arrestin-dependent receptor signalling is both spatially and temporally distinct from G protein-dependent signal transduction. Whereas e.g. G protein-dependent ERK1/2 activation is rapid, but transient and results in nuclear translocation of the kinase, β-arrestin-dependent ERK1/2 activation is slower, more persistent and often restricted to the cytosol (56). Thus, a time-course of a "first wave" of G protein-dependent signalling followed by a "second wave" of β-arrestin-dependent signalling could be established (56). Intriguingly, persisting β-arrestin-dependent signalling even after dissociation from the activating receptor could be demonstrated (57).

Recent data showing sustained G protein-dependent signalling by internalized GPCRs (58-61), however, challenges this model of G protein-dependent signalling switching to β-arrestin-dependent pathways. Only this year, Lefkowitz and co-workers proposed the formation of "megaplexes" composed of a single GPCR, β-arrestin and G protein (Figure 2). Simultaneous interaction of a GPCR with β-arrestin and G protein offers a possible explanation for the observation of sustained G protein-signalling after receptor internalization (62).
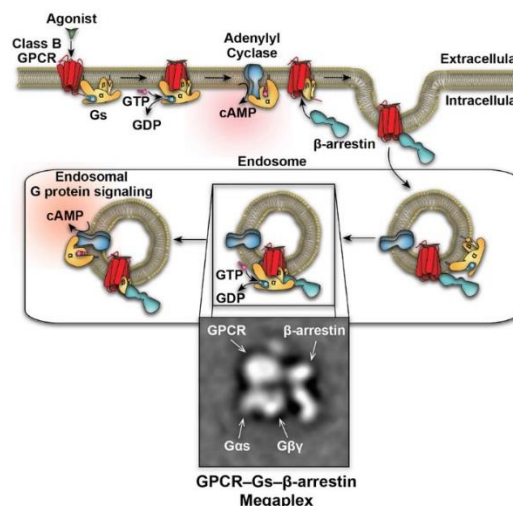
Figure 2: Megaplex consisting of a GPCR simultaneously binding a G protein and β-arrestin

Sustained G protein-signalling from within endosomes is explained by the formation of "megaplexes" containing a β2V2R receptor chimera (i.e. a β2AR ("class A" receptor) where the C-terminal tail was exchanged for the vasopressin type 2 receptor ("class B" receptor)) simultaneously engaged with a G protein and β-arrestin (62).

The formation of such "megaplexes" requires β-arrestin to interact with the receptor C-terminal tail rather than the core region, as the transmembrane core is occupied by the G protein (62). This "tail" conformation relies on a strong interaction of the receptor's C-terminal tail with β-arrestin (62,63), which has been reported for "class B" GPCRs (e.g. AT1R) (64). "Class A" GPCRs (e.g. β2AR), in contrast, lack the C-terminal

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serine/threonine clusters necessary for the formation of a stable complex with β-arrestin and thus generally bind β-arrestins only transiently resulting in rapid recycling of receptors (65). Thus, the β-arrestin-GPCR interaction pattern confers a β-arrestin conformational signature (66), which in turn is linked to β-arrestin function.

4.1.3.2 Arrestins mediate GPCR sequestration

β-arrestins do not only act as scaffolding proteins in assembling signal complexes (16), but also with regard to the components of the endocytic machinery (67). Thus, they are critically involved in receptor sequestration following agonist-stimulation (Figure 3): (i) After phosphorylation by GRKs, arrestins bind to receptors leading to sterical uncoupling of receptors from their cognate G proteins and signal termination, i.e. desensitization of the receptors (14,50,68,69). (ii) Further, arrestin-binding may promote receptor internalization via clathrin-coated pits (67,70) or alternative pathways (71), eventually resulting in receptor degradation and downregulation (72). (iii) Alternatively, packing of receptors into endocytic vesicles can also be the first step in a recycling process back to the plasma membrane (resensitization) (73-75).
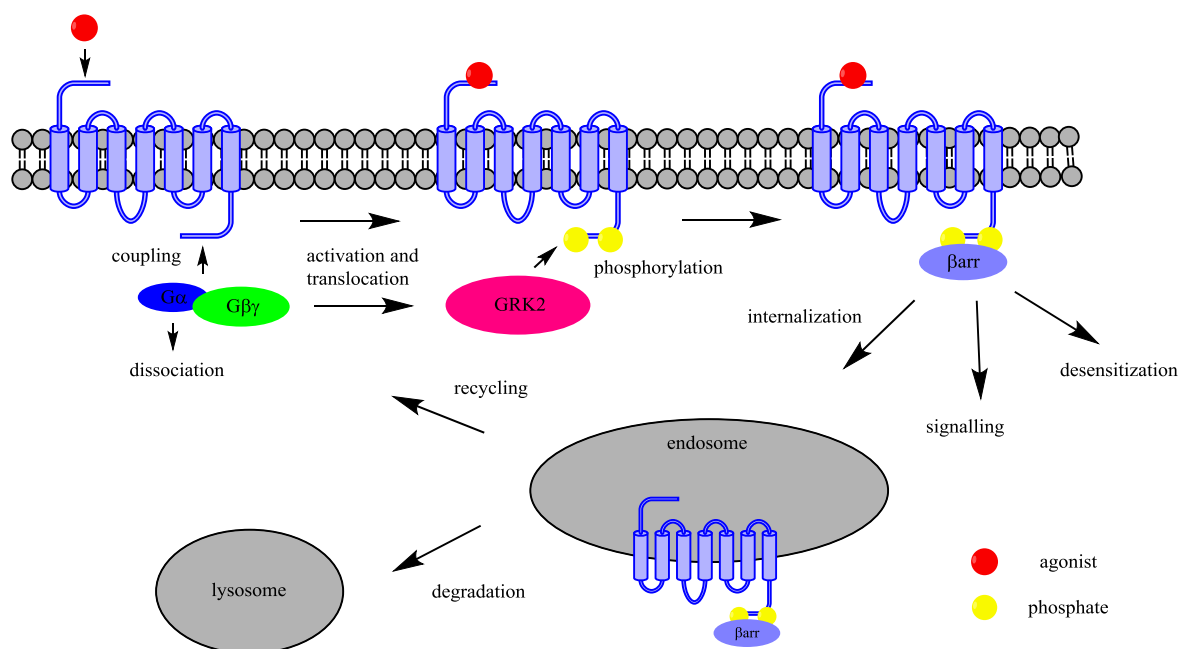
Figure 3: Arrestins adopt multiple functions

Canonical GPCR signalling involves activation of G proteins, which in turn activate distinct downstream effectors. Following phosphorylation of agonist-occupied receptors by GRKs, β-arrestin binds, which may lead to: (i) receptor desensitization through sterical uncoupling of G proteins, (ii) β-arrestin-dependent signalling events, (iii) internalization with subsequent recycling back to the plasma membrane, or lysosomal degradation. (adapted from (76) and (77)); abbreviation: βarr, β-arrestin

4.1.4 GPCR biased signalling

Whether β-arrestin predominantly partakes in signal activation or termination is linked to the conformational change induced upon receptor binding (66). Moreover, ligands may display bias by stabilizing receptor conformations (biased agonism), which favour activation of G protein-dependent or β-arrestin-dependent signalling pathways, as demonstrated for the β-blocking agent carvedilol, which blocks G protein-dependent signalling while stimulating β-arrestin-dependent activation of the MAPK pathway (78). Efforts towards therapeutic exploitation of biased agonism yielded a β-arrestin biased ligand (TRV120027) for AT1R, which promotes cardiac contractility and survival, while at the same time blocking deleterious hypertrophic signalling (79,80).

Furthermore, a role for GRKs in governing these distinct signalling patterns was suggested (81).

4.2 Physiological roles of G protein-coupled receptor kinases

G protein-coupled receptor kinases (GRK) are a family of AGC serine/threonine kinases (82), best known for orchestrating, in cooperation with arrestins, GPCR desensitization (83). They can be grouped into three subfamilies based on sequence similarities: the visual subfamily (GRK1, GRK7), the βARK subfamily (GRK2, GRK3) named originally for their role in the regulation of βAR signalling and the GRK4 subfamily (GRK4-6) (84). Members of the visual subfamily of GRKs are solely expressed in rod and cone photoreceptors in the retina. GRK4 is expressed in neurons, kidneys and testes, where protein levels are highest. The other four GRK isoforms (GRK2, GRK3, GRK5, GRK6) are ubiquitously expressed (85).

4.2.1 GRKs are multi-domain kinases

Crystal structures from all three GRK subfamilies have been solved, revealing common structural features (86-90) (Figure 4).

GRKs (~62-80 kDa) are multi-domain proteins with a conserved N-terminal region unique to the GRK family followed by a regulator of G protein signalling (RGS) homology domain (RH) (~120 amino acids). The N-terminal domain (~55 amino acids) is critically involved in promoting receptor interaction (91,92). The central kinase domain (~270 amino acids) contains the active site with the adenosine triphosphate (ATP)-binding cleft, tailed with a short AGC kinase sequence (90,93). GRK C-termini, although sharing a common function of mediating cellular localization and membrane association, are structurally highly diverse and of variable length (~105-230 amino acids) (94,95).
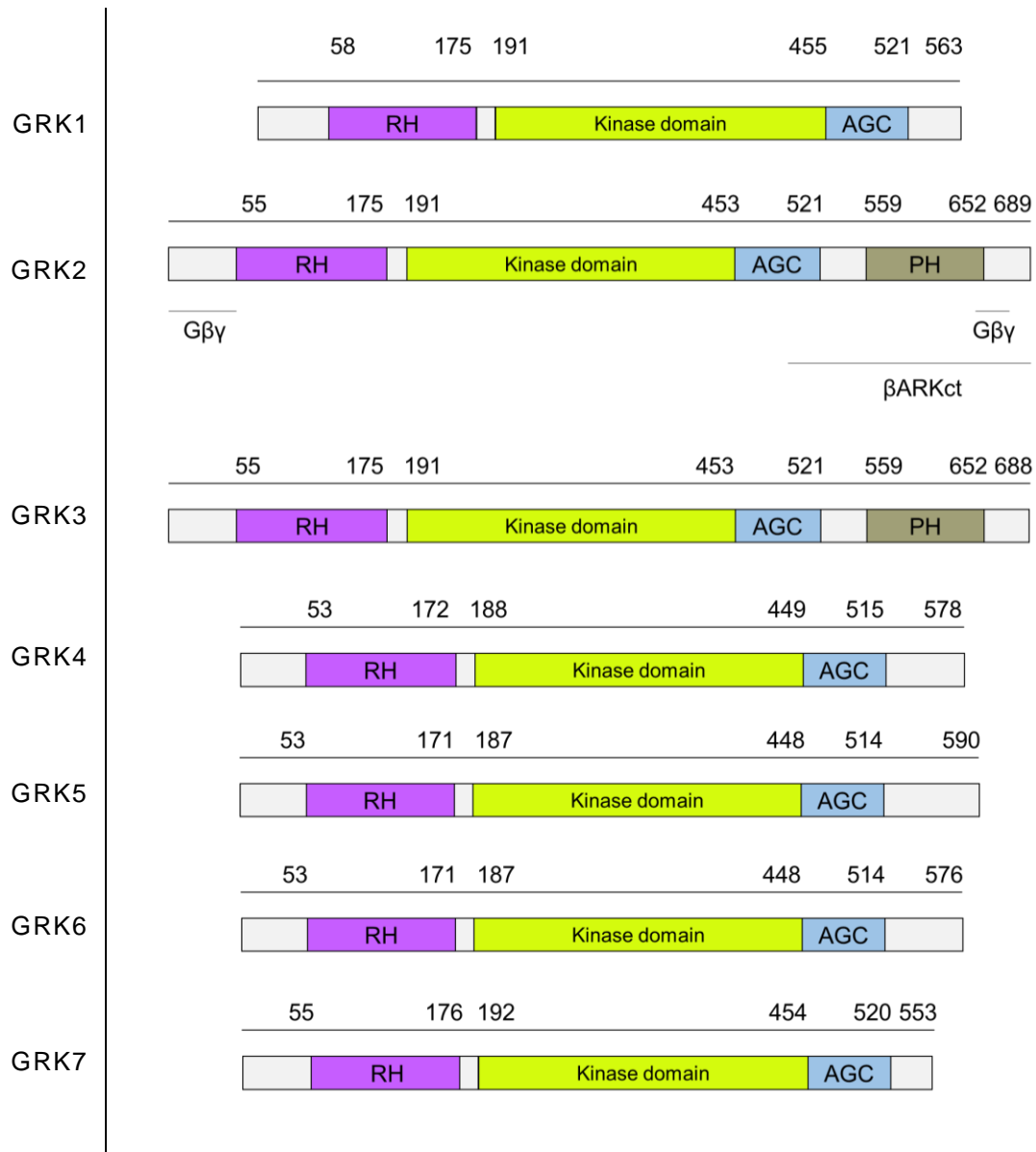
Figure 4: Structural characteristics of GRK isoforms

G protein-coupled receptors kinases are multi-domain proteins with structural variations between the different isoforms. (adapted from (96,97), includes information from the UniProt Knowledgebase); abbreviation: PH, pleckstrin homology

4.2.1.1 The visual subfamily

The visual GRKs are localized to the plasma membrane by lipid modifications. Whereas GRK1 is farnesylated, GRK7 is gernaylgeranylated (98,99).

4.2.1.2 The βARK subfamily

The cytosolic proteins GRK2/3 contain a C-terminal pleckstrin homology (PH) domain enabling binding to Gβγ and subsequent membrane-translocation and PIP_2-binding (38,100-102). This mechanism of activation is unique to the βARK subfamily members

(103). Additionally, Gβγ contributes to GRK2 activity by interacting with its N-terminal domain (104). Furthermore, a role for membrane lipids in activating GRK2 was reported (105).

4.2.1.3 The GRK4 subfamily

The N-terminal domain of the GRK4 subfamily members has been shown to interact with PIP2 thereby increasing the catalytic activity (101). GRK5 also binds to the plasma membrane via an amphipathic helix located in its C-terminal domain (106). Furthermore, GRK5 contains a nuclear localization signal (NLS) and a nuclear export signal (NES) (107). Post-translational C-terminal palmitoylation of GRK4 and GRK6 enhances lipid binding and localization to the plasma membrane (108,109).

4.2.2 GRKs are multifunctional proteins

GRKs are able to phosphorylate a number of both receptor and non-receptor substrates, and exert kinase-independent functions.

4.2.2.1 Receptor desensitization

GRKs were first described for playing a role in receptor desensitization by phosphorylating serine/threonine residues on the C-terminal tail and intracellular loops of agonist-occupied GPCRs (110,111). Phosphorylation subsequently promotes arrestin binding und G protein uncoupling by sterical hindrance, thus dampening G protein-dependent cellular responses (50,112). In addition to GRKs promoting homologous receptor desensitization, i.e. only activated agonist-occupied receptors are affected, second messenger-dependent protein kinases A (PKA) and C (PKC) are involved in heterologous desensitization, which is directed towards both inactive and active receptors (113,114).

It is noteworthy that only seven GRKs regulate over 800 GPCRs indicating that the kinases are highly promiscuous. Remarkably, GRKs may contribute to functional selectivity of receptors. The distinct phosphorylation patterns ("barcodes") of GRK2/3 and GRK5/6 affect GPCR signalling (111). This barcode hypothesis is in line with data showing that GRK2-phosphorylation of β2AR primarily leads to internalization, whereas phosphorylation by GRK6 favours the β-arrestin-mediated pathway of MAPK activation. At the same time, both GRKs contribute to receptor desensitization (115,116).

4.2.2.2 Phosphorylation of non-receptor substrates

On top of its role in regulating GPCR signalling via phosphorylation, GRK2 phosphorylates a number of cytosolic non-receptor substrates (85).

A linker role for GRK2 between GPCR activation and cytoskeletal remodelling has been suggested. GRK2 phosphorylates several members of the cytoskeleton, such as tubulin (117) or radixin (118) and ezrin (119) of the radixin/ezrin/moesin protein family.

Moreover, phosducin was reported as a substrate of GRK2 (120). Phosducin is a cytosolic protein known to interact with Gβγ and thereby may compete with GRK2 for Gβγ (121). Phosphorylation by GRK2 significantly diminishes this interaction (120).

Synucleins were also identified as GRK2-substrates, although the implications are not clear (122).

In macrophages, GRK2 was reported to mediate TNFα (tumour necrosis factor α)-induced NF-κB (nuclear factor κB)-dependent gene transcription by phosphorylation of IκBα (inhibitory κB) (123).

Moreover, GRK2 is able to phosphorylate and desensitize non-GPCR membrane receptors, as e.g. the platelet-derived growth factor receptor-β (PDGFRβ) (124).

4.2.2.3 Kinase-independent functions of GRK2

GRK2 has been demonstrated to interact with $Gα_{q/11}$ via its N-terminal RH domain. Whereas GTPase activity is modest in comparison to other RGS proteins, Gα sequestration bears a certain importance (30). Moreover, GRK2 may block $Gα_q$-mediated activation of PLCβ by competitive binding (30,125). The RH domain located in the N-terminal domain seems only to be active in the βARK subfamily and shows specific GTPase-activating protein-activity exclusively towards $Gα_q$ (30,126).

In a similar fashion, GRK2, via its C-terminal PH domain, scavenges Gβγ thereby interfering with downstream effector functions (39). An additional Gβγ interaction site is located in the N-terminal domain of GRK2, which is also capable of interrupting Gβγ signalling (104).

Moreover, GRK2 is able to modulate receptor internalization in a kinase-independent manner by recruiting phosphoinositide 3-kinase (127) or the GRK-interacting protein GIT1 (128) to the plasma membrane. GRK2 also directly interacts with clathrin via its C-terminal domain (129).

4.3 Pathophysiological roles of GRK2

A pathophysiological role for GRK2 was first proposed for heart failure more than 20 years ago (130). The number of diseases connected to impaired GRK2 function has been increasing ever since (131).

4.3.1 Heart failure

Heart failure is a clinical syndrome defined by structural and/or functional abnormalities of the heart leading to reduced cardiac performance (132). In heart failure patients, GRK2

expression levels are upregulated and activity is heightened (130). GRK2 dysregulation is thought to be deleterious in several ways.

GRK2 upregulation leads to a state of chronic desensitization and consequently impaired β-adrenergic responsiveness (130). Moreover, the β1/β2AR ratio of 80:20 in the healthy heart is changed to 60:40 in the failing heart due to selective downregulation of β1AR (133,134). Although this change is considered adaptive, as β2AR displays anti-apoptotic effects via Gα$_i$-coupling, whereas Gα$_s$ signalling increases myocyte apoptosis (135).

In addition, under conditions of oxidative stress, ERK-mediated phosphorylation at Ser670 of GRK2 promotes the binding of the mitochondrial chaperone heat-shock protein 90 (Hsp90). This leads to mitochondrial accumulation of GRK2 and consequently the promotion of pro-death signalling (136).

GRK2 upregulation in the adrenal glands is involved in the desensitization of the α-adrenergic receptor type 2 (α2AR)-mediated feedback inhibition of catecholamine release, which is thought to be deleterious to cardiac function. Hence, GRK2 contributes to the vicious cycle of enhanced activation of the sympathetic nervous system and chronic GPCR desensitization observed in heart failure (137).

Moreover, several studies point towards a beneficial role for GRK2-inhibition in heart failure.

Mice hemizygous for GRK2 gene ablation (GRK2^{+/-}) have 50% reduced GRK2 expression levels and present with a phenotype characterized by improved cardiac contractility and relaxation (138).

Moreover, GRK2-inhibition, subsequent normalization of βAR levels and resensitization of endogenous βAR signalling shows a cardioprotective effect in several animal models including a large animal model mimicking human physiology (139-141). Some of the beneficial effects observed under β-blocker treatment can be attributed to reduced sympathetic drive and consequential normalization of elevated GRK2 levels (142).

There is also support for a beneficial effect of GRK2-inhibition on the disturbed substrate metabolism in late-stage heart failure (143).

Intriguingly, myocardial GRK2 gene ablation after myocardial infarction was able to stop adverse remodelling (144).

Taken together, reduction of GRK2 activity in the heart is a well-studied approach to target the structural and physiological alterations, which accompany heart failure development.

4.3.2 Hypertension

A role for GRK2 in hypertension was suggested, because expression and activity of the kinase are enhanced in circulating lymphocytes of hypertensive individuals (145). Enhanced GRK2 expression levels could underlie the impaired β-adrenergic function

present in hypertension (145). Moreover, GRK2 inhibits protein kinase B-mediated activation of the endothelial nitric oxide synthase (eNOS) and thus, reduces NO production (146). This might explain the increased vasodilatory response observed in GRK2$^{+/-}$ mice (147).

However, vascular tone is a result of a careful balance between vasodilatory and vasoconstrictory pathways, both of which are in part GPCR-mediated. Global GRK2 knockout was found to increase both opposing pathways and eventually, resulted in spontaneous hypertension (148). GRK2$^{+/-}$ mice, in contrast, are resistant to the deleterious effects of angiotensin II on vascular structure and functionality (147). In agreement with the elevated GRK2 levels found in patients with hypertension, GRK2 overexpression led to a hypertensive state in mice (149). Therefore, GRK2 deserves further investigation as a target for hypertension, although physiological outcomes have to be carefully monitored.

4.3.3 Diabetes mellitus

A number of studies support a role for GRK2 as an inhibitor in insulin signalling. GRK2 levels were found to be upregulated in blood mononuclear cells from insulin-resistant patients and in different models of insulin resistance (150). Moreover, applying models of insulin-resistance, GRK2$^{+/-}$ mice are protected from developing impaired insulin sensitivity (150) and induced GRK2 ablation improves insulin sensitivity (151). In addition, GRK2-inhibition could ameliorate glucose homeostasis (152).

The mechanisms at a molecular level are not entirely clear yet. GRK2 interferes with β-adrenergic signalling, which plays a role in the development of insulin resistance (153). GRK2 also negatively regulates insulin-stimulated glucose transport in a kinase-independent manner via interaction with Gα$_{q/11}$ or by direct interaction with the insulin receptor substrate 1 (IRS1) (150,154,155).

4.3.4 Obesity

A role for GRK2 in age- and diet-induced adiposity was implied (150). In GRK2$^{+/-}$ mice on a high-fat diet, adiposity was lowered (150) and induced gene ablation prevented further body weight gain and the development of adiposity (151).

Given the scale of the obesity epidemic and the lack of adequate pharmacological treatment options, further research into GRK2 seems worthwhile.

4.3.5 Osteoporosis

The GPCR calcium-sensing receptor (CaR), which negatively regulates parathyroid hormone (PTH) secretion, is investigated as a potential drug target in osteoporosis (156,157). Sustained elevated levels of parathyroid hormone (PTH) increase bone loss, whereas intermittent pulses increase bone mass (158). Stimulation of the CaR by

increased extracellular calcium levels, leads to reduced PTH secretion (159) and is in part attenuated in a kinase-independent manner by GRK2 (160).

4.3.6 Cancer

A role for GRK2 in cell differentiation and survival arose from the observation of embryonic lethality in global GRK2$^{-/-}$ mice (161). This is likely due to a more general role in embryogenesis rather than a cardiac-specific function, because GRK2 knockout restricted to the heart produced viable offspring with normal heart structure and function (162).

Interestingly, GRK2 upregulation was reported for thyroid cancers, where it showed an antiproliferative effect (163). A similar effect could be observed in hepatocellular carcinoma, where GRK2 overexpression decreased cell proliferation and tumour growth. Mechanistically, a role for GRK2 in the cell cycle arrest during G2/M transition was proposed (164). In line with a role for GRK2 in cell cycle control is the finding, that transient downregulation of the kinase during G2/M transition is crucial for cell cycle progression (165).

GRK2 protein levels are also upregulated in a number of breast cancer cells lines. However, these data support a tumour-promoting role for GRK2, with elevated levels stimulating mitogenic ERK1/2 signalling and pro-survival (166).

Overall, this indicates, that further research into the underlying mechanisms of the role of GRK2 in cancer development is needed.

4.3.7 Potential of a GRK2-inhibitor

In conclusion, GRK2 has been implicated as a drug target for a variety of different conditions. Research into the pathophysiology of GRK2 has focused on heart failure, where there is still an urgent need for new medications (167).

As detailed in the next chapter, several compounds with a GRK2-inhibitory effect have been described and characterized, but to date none has reached clinical trials or market approval.

4.4 Approaches to GRK2-inhibition

The tri-domain structure of GRK2, with every domain contributing to activity, allows for a range of approaches to inhibit GRK2 effector functions (Figure 5).

Firstly, compounds interacting with the N-terminal domain of GRK2 may hinder the binding of receptor substrates. Moreover, Gα$_q$-mediated kinase-independent effector functions can be blocked. Secondly, targeting the enzyme active site provides a more general way to prevent kinase activity, but is unlikely to interfere with kinase-independent functions. Thirdly, intervening with the Gβγ-mediated GRK2 activation and membrane

translocation provides a route to inhibit both the phosphorylation of GPCR-substrates, as well as soluble substrates. To what extent kinase-independent effects are affected, however, is unclear.
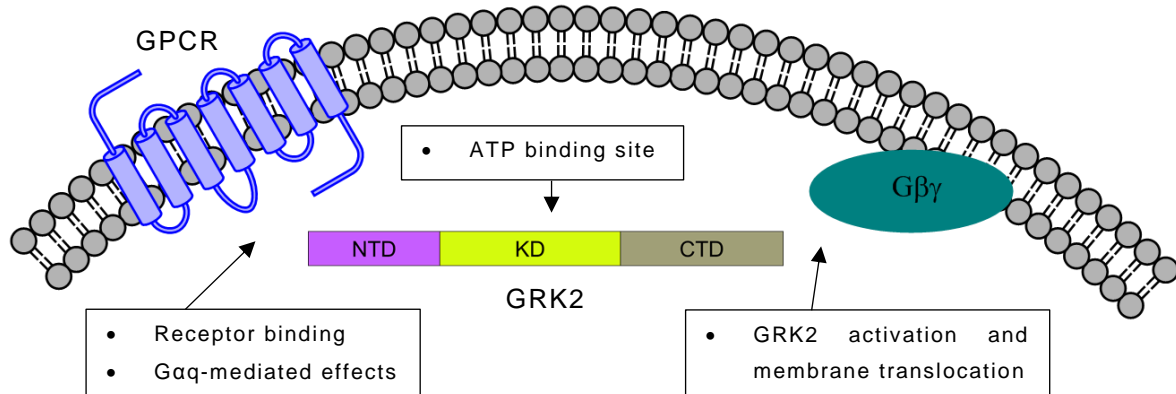
Figure 5: Domain-specific inhibition of GRK2
The multi-domain structure of GRK2 allows for multiple routes of inhibiting kinase-dependent and –independent effects (adapted from (131)).

In addition to different target sites and blocking specific effector functions, inhibitors also vary in their biochemical nature (small molecules, peptides, and proteins), potency and the selectivity they display for the seven GRK isoforms.

4.4.1 GRK2-inhibition via the N-terminal domain

RKIP is a dual-specific inhibitor of Raf-1 and GRK2
The Raf-1 kinase inhibitor protein (RKIP) is a member of the family of phosphatidylethanolamine-binding proteins (PEBP), with the capacity to inhibit both GRK2 (168) and the serine/threonine kinase Raf-1, thus inhibiting the Raf-MEK-ERK1/2-pathway by competitive inhibition of MEK phosphorylation (169). Phosphorylation of the amino acid Ser153 by the second-messenger dependent kinase PKC mediates a target switch from Raf-1 to GRK2 (168,170). RKIP is thought to interact with the N-terminus of GRK2, thereby interfering with receptor substrate binding (91,168). RKIP also shows inhibitory activity against several serine proteases (171).

RKIP is upregulated under cardiac stress and some evidence supports the notion of an adaptive role mediating cardioprotection, which is in line with the beneficial effects seen under GRK2-inhibition (172). However, RKIP, possibly by inhibiting the cardioprotective MAPK-pathway, interferes with survival pathways and plays a role in apoptosis. Another study showed that cardiac overexpression of RKIP representing levels seen under cardiac stress led to increased heart dilatation, cardiac dysfunction, upregulation of the

cardiac lipid gene metabolism and cardiomyocyte death indicating transition to heart failure (173).

Thus, RKIP is an effective GRK2-inhibitor with specificity towards receptor substrates, by targeting the receptor interaction site located in the N-terminal domain. RKIP, however, also shows cardiotoxic side effects mediated by its Raf-1 inhibitory property (173).

The amino terminal GRK2 inhibitor peptide (βARKnt)

A peptide covering the extreme N-terminus of GRK2, that is the first 14 amino acids (MADLEAVLADVYSL), inhibits β2AR-phosphorylation after stimulation with the β-adrenergic agonist isoproterenol, but it does not affect phosphorylation of the soluble substrate tubulin (174).

The extreme N-terminus of GRKs, which forms an amphipathic helix, displays significant sequence conservation. Moreover, it selectively enhances the catalytic activity towards receptor substrates, possibly by simultaneously binding phospholipids through its hydrophobic side and facilitating an intramolecular interaction with the kinase domain through its polar side (174).

The therapeutic potential of this peptide, to our knowledge, has not been further investigated.

4.4.2 Targeting the central kinase domain

An RNA-aptamer selectively inhibits GRK2

An RNA-aptamer (C13) found via an *in vitro* selection process is a potent inhibitor of GRK2-mediated phosphorylation of bovine rhodopsin. Moreover, it shows specificity for members of the GRK family (175). C13 is thought to target the kinase domain and interfere with phosphorylation by stabilizing a unique inactive conformation through interactions within and outside of the ATP binding pocket (176). Aptamers are not generally suitable for therapeutic application, but may be used in displacement assays to identify small molecule inhibitors (177). Paroxetine, a market-approved selective serotonin-reuptake inhibitor (SSRI), was identified as a selective GRK2-inhibitor through its ability to displace C13 from GRK2 (178).

Paroxetine is a GRK2 inhibitor

Paroxetine has been revealed as a GRK2-inhibitor with selectivity over other GRK subfamilies. The crystal structure shows paroxetine fitting into the enzyme active site, thus stabilizing an inactive conformation (178). Isoproterenol-stimulation in paroxetine-treated mice leads to increased myocardial contractility and enhanced inotropic response. Moreover, paroxetine treatment in a myocardial infarction-induced heart failure mouse model results in enhanced ejection fraction, fractional shortening and improved

contractility. It limits adverse ventricular remodelling and preserves structural integrity of the myocardium. GRK2-inhibition with paroxetine also normalized β-adrenergic signalling as indicated by reduced serum catecholamine levels, restored βAR density and decreased myocardial GRK2 upregulation (179).

Interestingly, paroxetine, in contrast to the β-blocker metoprolol, prevents induction of the foetal gene expression program, thus delivering improvements on top of those observed with β-blocker treatment (179).

Nevertheless, a compound approved for the treatment of psychiatric disorders is not an ideal GRK2-inhibitor. Moreover, improved selectivity and potency would be desirable. A derivative, synthesized with the desire to tackle these concerns, exhibited less activity regarding serotonin reuptake. Unfortunately, it also enhanced inhibition of other AGC kinases (93).

Protein kinase inhibitors
There are known serine/threonine kinase inhibitors, which were tested for their GRK2-inhibitory potential.

One of them, balanol, inhibits members of the AGC kinase family (180) and displays some selectivity for GRK2 over other GRKs (87). Balanol has been reported as a potent inhibitor of GRK2-mediated phosphorylation of the soluble substrate tubulin. Balanol interferes with GRK2 activity by stabilization of an inactive enzyme conformation (87).

Takeda Pharmaceutical Company Limited developed a number of compounds with a heterocyclic structure similar to balanol (181). These compounds selectively inhibit GRK2 through stabilization of the inactive conformation, as revealed from crystal structures (182). However, for reasons unknown, none of the compounds has proceeded to clinical trials.

Furthermore, one group screened an assembly of known kinase inhibitors for their selective targeting of either GRK2 or GRK5. The most potent candidate was co-crystallized with GRK2 and Gβγ to gain further insight into the structure-activity relationship (88).

4.4.3 Targeting the C-terminal interaction with Gβγ

The carboxyl terminal GRK2 inhibitor peptide (βARKct)
βARKct is a peptide consisting of the C-terminal amino acids 495-689 of GRK2 covering the Gβγ-binding site, interaction with which is crucial in activating and translocating GRK2 to the plasma membrane towards receptor substrates (139). Therefore, βARKct

acts as a competitive inhibitor of GRK2 by scavenging Gβγ-subunits and interfering with membrane translocation.

Cardiac-specific overexpression of βARKct in a transgenic heart failure model, protected animals from heart failure development. Gβγ sequestration as a mechanism of action led to less cardiac dilation, enhanced heart function and prolonged survival (183). In another transgenic mouse model with GRK2 overepression to levels found in human heart failure (3-fold increase), cardiac-specific expression of βARKct led to a normalization of the βAR-stimulated contractility (184).

Adenoviral-mediated gene delivery of βARKct was performed in several animal models of heart failure. In a clinically relevant large animal model (porcine) of post-myocardial heart failure, gene delivery resulted in improved contractile function, decreased plasma catecholamine levels, and subsequent reversal of cardiac remodelling and foetal gene expression (141). In cardiomyocytes from end-stage heart failure patients undergoing heart transplantation, βARKct was able to improve the βAR-stimulated contractile response, further supporting the maladaptive nature of GRK2 upregulation in heart failure (185).

However, the restoration of adrenergic responsiveness observed with βARKct cannot be solely attributed to GRK2-inhibition. In the context of its role in Gβγ-sequestration, βARKct has been reported to improve cardiac contractility by increasing the L-type Ca^{2+}-channel current (186).

Hence, the molecular mechanisms underlying βARKct-mediated cardiac effects are not fully elucidated yet. At the same time, this finding shed light on a novel potential target for heart failure treatment (186).

Peptides and small molecules targeting Gβγ

Studies towards interrupting the Gβγ-GRK2 interaction have yielded several GRK2-inhibitory peptides and small molecules.

A shorter GRK2CTD-derived peptide (amino acids 643-670) corresponding to the 'Gβγ binding domain' (187), inhibits GRK2-activity and phosphorylation of rhodopsin. Interestingly, one group used a phage display library screen with Gβγ as bait to elucidate requirements for binding partners and identified a protein-protein interaction "hot spot" on Gβγ (188). A mutated form (SIGK) (189), of the peptide with the highest affinity for Gβγ (SIRK) inhibited certain Gβγ effector functions, while leaving others unaffected (190). Based on the identified "hot spot", a virtual docking of a small molecule library yielded one compound (M119), which selectively interfered with Gβγ-protein interactions (191). In an animal model of heart failure, both M119 and a closely related analogue, gallein, prevented heart failure progression and improved cardiac function.

These data indicate that small molecule disruption of the Gβγ-GRK2 interaction is feasible and effective in ameliorating heart failure symptoms (192).

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A phosducin-derived peptide

Phosducin (PDC), a cytosolic protein mainly expressed in retinal photoreceptor cells and the pineal gland, has been shown to inhibit Gβγ-mediated effector functions. Moreover, a PDC-derived peptide (amino acids 215-232) containing a high-affinity Gβγ-binding site showed a moderate inhibitory effect on GRK2-mediated phosphorylation of rhodopsin (193).

4.4.4 Peptide GRK2-inhibitors

Receptor-derived peptides

A peptide comprising the first intracellular loop (amino acids 56-74) of the hamster β2AR inhibits receptor phosphorylation by GRK2, but does not show inhibitory activity towards non-receptor substrates (194).

Modification of this peptide by adding charged amino acids and truncation yielded a 40-fold more potent peptide-inhibitor (PepInh, AKFERLQTVTNYFITSE), which displayed activity towards rhodopsin (195). However, it does not interfere with GRK2-mediated phosphorylation of a peptide substrate indicating a mechanism of action differing from competition with ATP or the substrate (195). Moreover, PepInh displays specificity, as it does not interfere with activity of PKA or PKC, but only modest selectivity within the GRK family (195). In a pressure overload mouse model, the peptide retarded signs of heart failure (143).

Peptides derived from a substrate-binding site of GRK2

Peptides (KRX-683$_{107}$, KRX-683$_{124}$ and $^{383-390}$HJ loop) derived from a kinase-substrate interaction site of GRK2 have been reported to inhibit GRK2 activity *in vitro* and show favourable effects on glucose homeostasis in an animal model (152,196,197).

4.4.5 Other GRK2-inhibitors

Polyanionic compounds have been the first reported inhibitors of GRK2-mediated receptor phosphorylation, with heparin and dextran sulphate showing the highest potency (198). In addition, heparin also blocks agonist-induced phosphorylation of βAR in permeabilized cells (199). However, polyanionic compounds are inherently highly charged and as such membrane-impermeable. Furthermore, the molecular weight of both heparin and dextran sulphate impedes their clinical use.

Recently, α-hederin has been shown to block GRK2-mediated phosphorylation of the β2AR. α-hederin is a component of the dry extract of *Hedera helix*, which is used in the treatment of diseases of the respiratory tract for its secretolytic and bronchospasmolytic effects. Inhibition of GRK2 is in line with the observed increase in adrenergic responsiveness of the airways (200).

In conclusion, a number of GRK2 inhibitors have already been identified and characterized. They differ in their mechanism of action, the specific effector functions they block and their biochemical composition. Many of the described compounds are peptides or proteins (RKIP, βARKnt, βARKct, PepInh), which indicates a potential for amino acid-based structures in targeting GRK2.

4.5 The therapeutic potential of peptide drugs

The number of therapeutic peptides entering clinical trials has been exponentially increasing over the last 30 years. Whereas the majority binds extracellular targets, around 15% are directed towards targets within the cell (201).

In recent years, peptides have been increasingly recognized for their pharmacological potential. Not complying with Lipinski's rule of five (202), such polyamides were thought largely unfit for the development into pharmaceuticals. However, their distinct physicochemical properties combine some of the advantages of both larger proteins and small molecules, thus potentially filling a niche in the space of drug candidates (203).

4.5.1 Characteristics of peptides

Peptides, short proteins of less than 50 amino acids, comprise a promising class of current and future therapeutics (201,204). Peptides might prove especially valuable for modulating intracellular protein-protein interactions (PPI), which have been notoriously difficult to target. Small molecules, although suitable for fitting into binding pockets restricted in size as e.g. enzyme active sites, are usually too small to interfere successfully with the larger interfaces of PPIs. Monoclonal antibodies (mAB) and peptides both provide larger surface areas and can thus effectively complement PPIs, with the latter having better access to intracellular targets (201).

In addition, peptides demonstrate high target specificity and affinity comparable to larger proteins, but generally require lower production costs than mABs due to standard synthetic procedures. As peptides are degraded to amino acids, they exhibit a favourable toxicity profile (204). Immunogenicity is less of a risk in comparison to larger proteins such as mAB. In addition, owing to their smaller size, peptides are potentially membrane permeable, therefore offering the possibility of reaching intracellular targets, and possess better tissue penetration (203).

However, there are considerable challenges to be overcome when exploiting peptides for therapeutic application. Peptides show an overall unfavourable pharmacokinetic profile and generally poor oral bioavailability. Solubility is variable and dependent on hydrophilicity. Hydrophilicity and molecular mass may limit the membrane permeability (204). Furthermore, susceptibility to proteolytic cleavage, hydrolysis and oxidation pose challenges in terms of stability and rapid renal and hepatic clearance shortens their

plasma half-life to minutes (201,203) Conformational flexibility often limits affinity and selectivity (201).

Some of these shortcomings may be overcome by chemical modification, as outlined below.

4.5.2 Modifications

Currently, subcutaneous injection is the most common route for administration of peptide therapeutics, although alternative delivery systems are increasingly recognized (204). Nonetheless, oral application is desirable, but generally requires chemical modifications or formulation efforts (204,205).

Methods to increase the proteolytic stability include cyclisation, introduction of unnatural amino acids, N-methylation and establishment of intramolecular hydrogen bonds (206,207). The plasma half-life can be prolonged by promoting binding to serum albumin or attachment of polymeric molecules such as e.g. polyethylene glycol (PEG) (203). The membrane permeability may be enhanced by addition of lipid groups (e.g. myristoylation) (152,208). Moreover, amino acid side chains can be replaced by specific functional groups towards the development of a small molecule analogue (209).

4.5.3 Displacement assay

Alternatively, a displacement assay could be performed. This may lead to the identification of small molecules, which compete with a peptide for a common interaction partner. Such an approach was taken in the discovery of the kinase-inhibitor paroxetine, which displaced the RNA-aptamer C13 from GRK2 (178).

4.5.4 Gene therapy

Taking into account the difficulties with regard to oral delivery of peptide drugs, alternative routes of administration have to be considered. One possible technology is gene transfer therapy, i.e. introduction of recombinant DNA through viral or non-viral delivery (210,211).

Recently, a heart failure study aiming at delivering the sarco(endo)plasmic reticulum Ca^{2+}ATPase (SERCA2a) using an adeno-associated virus for delivery has advanced to Phase IIb clinical trials, where unfortunately the promising results from earlier studies could not be confirmed (212).

Nevertheless, these studies are in support of the potential of gene transfer therapy for heart failure treatment. Moreover, there is ample data supporting a gene therapy approach for GRK2-inhibition from studies using βARKct (185,210,213).

Taken together, peptides represent a viable class of therapeutics with specific advantages compared to small molecules or biomacromolecules. A promising approach

for the identification of candidate peptide drugs is e.g. a phage display library screen, as outlined in the next chapter.

4.6 Peptide phage display library screen for candidate drugs

4.6.1 Principle of a phage display library screen

First described in 1985 by Smith and co-workers, phage display technology has since become a widely applied method in drug discovery (214-216).

Peptides or proteins are displayed fused to bacteriophage coat proteins, thus directly linking phenotype to genotype. Non-lytic filamentous phage, such as e.g. M13, which infect F plasmid-containing gram-negative bacteria (usually *E.coli* strains) are used most commonly (217). M13 contains two coat proteins suitable for peptide fusion expression: (i) the minor coat protein pIII, of which five copes are expressed and (ii) the major coat protein pVIII, of which several thousand copies cover the length of the bacteriophage (218). Thus, libraries of displayed amino acid sequences can be screened for high affinity binding to target proteins.

In a process termed bio panning (Figure 6), the target compound (bait), immobilized directly or via an affinity tag, is exposed to the phage library. After removal of unbound phages by washing, the bound phages are eluted. Thereby, because of the stability of filamentous phage towards pH, denaturants and ionic strength, a variety of unspecific methods may be applied (e.g. acid, high salt concentration). Alternatively, a known interaction partner may be used for a more specific competitive elution (214,218). Following amplification of the eluted phages in *E. coli*, another round of bio panning can be performed. Finally, sequencing of the extracted DNA provides the peptide sequence (219).
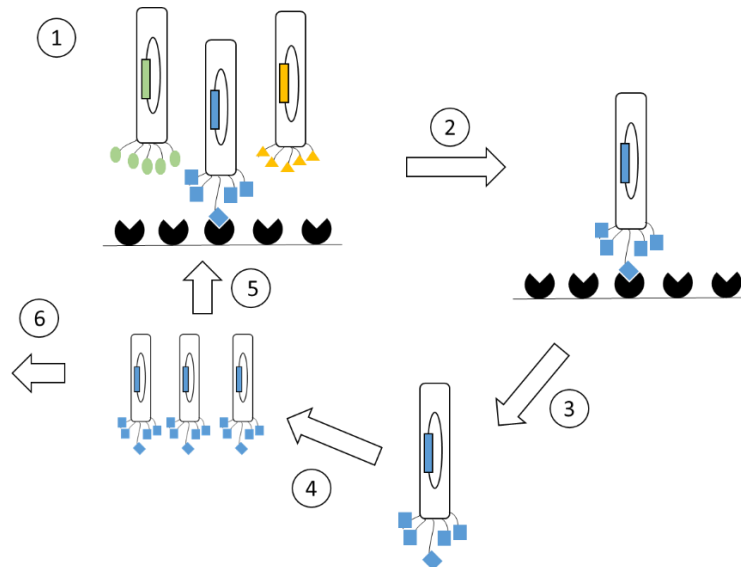
Figure 6: Schematic drawing of a phage display library screen

1. A peptide-displaying phage library is exposed to a bait-coated plate. 2. The unbound phages are removed by washing. 3. The bound phages are eluted. 4. The eluted phages are amplified. 5. The amplified phages are exposed to a bait-coated plate starting a new selection round. 6. After three rounds of panning, enriched phages may be isolated and sequenced. (adapted from (219))

4.6.2 Peptide libraries

Libraries may vary with regard to the peptides they display. Linear peptides of variable length have been used (218,220), allowing screening of ligands for targets with continuous interaction sites or residues, which are widely apart (218).

Linear peptides tend to exhibit conformational flexibility, which can reduce their affinity. In contrast, libraries displaying cyclic peptides, which are conformationally more constrained, may be more successful in the finding of high affinity ligands (221).

4.6.3 Applications of phage display technology

Intriguingly, phage display library screens yield peptides, which largely target biologically relevant sites. Hence, they often have the capacity to modulate the activity of the target protein, be it orthosterically or allosterically.

Phage display as a method may be employed in numerous scientific fields, including analysis of protein-protein interactions (218), identification of novel protein interactions partners or inhibitors (222,223), search for enzyme substrates and/or inhibitors (218,219) and target validation (215).

Commercially available peptide libraries have also successfully been employed in the search for enzyme inhibitors (219,224).

Interestingly, phage display library screening has already been used in the search for a GRK2-inhibitor.

Peptides selected from phage display libraries using Gβγ subunits as baits interfered with specific Gβγ effector functions (225). The search for small molecule inhibitors of Gβγ effector functions employing one such peptide yielded the compound M119, which blocks Gβγ-mediated GRK2 activation (191). Another prominent example of a phage display-derived GRK2-inhibitor is the RNA-aptamer C13 (175). In a displacement assay with this aptamer, paroxetine was identified as a GRK2-inhibitor (178).

These two examples underline the potential of phage display technique for the identification of potent modulators of protein function.

4.7 Aim

GRK2 is a ubiquitously expressed protein, which has been implicated in a variety of conditions. However, to date no GRK2-inhibitor has reached clinical approval.

GRK2 has been successfully inhibited by targeting the N-terminal domain thereby interfering with substrate recognition, by blocking the enzyme active site located in the central kinase domain or by preventing enzyme activation and membrane translocation mediated by Gβγ-interaction with the C-terminal domain. The latter approach has been extensively studied with the help of the βARKct peptide and proven beneficial in several heart failure animal models. Nevertheless, there is still no compound available, which interferes with the Gβγ-GRK2 interaction by directly targeting the C-terminal domain of the kinase.

Thus, the aim of this project is to find and characterize peptides, which selectively interact with the C-terminal domain of GRK2.

5 Materials and Methods

Chemicals, which were not specified otherwise, were obtained from Merck (Zug, Switzerland).

5.1 Molecular cloning

Polymerase chain reaction

Amplification of cDNA fragments for cloning was performed using 1 U/reaction *Pfu* DNA polymerase (Promega AG, Dübendorf, Switzerland) in *Pfu* DNA polymerase buffer (Promega AG, Dübendorf, Switzerland). Single colony screening of bacterial clones was performed using 1 U/reaction *Taq* DNA polymerase (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) in the supplied buffer. Primers (Microsynth AG, Balgach, Switzerland) were used at 0.4 µM, dNTPs (New England BioLabs, Bioconcept, Allschwil, Switzerland) at 200 µM and 0.1-10 ng of DNA as template. The DNA was amplified with a standard program (Table 1) using a Thermocycler 3000 (Biometra, Göttingen, Germany):

Step	Temperature	Time
1	94 °C	5 min
2	94 °C	30 s
3	50-65 °C	40 s
4	72 °C	120 s/1000 bp (*Pfu* DNA polymerase) 60 s/1000 bp (*Taq* DNA polymerase)
5	72 °C	10 min

25-30 cycles

Table 1: Standard PCR program
After initial melting of the dsDNA (step 1), 25-30 cycles of melting of dsDNA (step 2), followed by annealing (step 3) and elongation (step 4) of the primers were run. A final elongation step (step 5) was added.

The PCR products were separated by agarose gel electrophoresis.

DNA concentration measurement

The concentration of dsDNA was determined by appropriate dilution in 10 mM Tris, pH 6.8 and measurement of the absorbance at 260 nm (Biophotometer, Eppendorf, Germany). An extinction coefficient of 0.020 (µg/ml)$^{-1}$ cm^{-1} was used for the calculation of the concentration (226).

Annealing of single-stranded oligonucleotides

Single-stranded oligonucleotides were annealed using 25 µM top and bottom strand oligonucleotide, respectively, in oligonucleotide annealing buffer supplemented with 1-3% (v/v) DMSO (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) by incubation at 95 °C for 4 min, at 40 °C for 10 min and at RT for 30 min (Thermocycler 3000). Annealed oligonucleotides were detected by agarose gel electrophoresis.

Oligonucleotide annealing buffer

The oligonucleotide annealing buffer contained 1 mM EDTA and 100 mM NaCl in 10 mM Tris, pH 8.0.

Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis using 1-2% (w/v) agarose gels (Agarose MP, Roche, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) with 5 µl/100ml 1% (w/v) ethidium bromide for visualisation. Gels were prepared with TAE or TBE buffer and run with 100 V. For identification of the fragment size, a DNA ladder (GeneRuler 1kb DNA Ladder (Thermo Fisher Scientific AG, Reinach, Switzerland) or Low molecular weight DNA ladder (New England BioLabs, Bioconcept, Allschwil, Switzerland)) was run in parallel with the samples. DNA was visualised with UV-light (312 nm) using Intas Gel Capture software.

TAE buffer (1 x)

The TAE buffer was prepared with 40 mM Tris, 20 mM acetic acid, and 2 mM EDTA.

TBE buffer (1 x)

The TBE buffer was prepared by dissolving 89 mM Tris, 89 mM boric acid, and 2 mM EDTA in water.

Enzyme restriction digestion

Restriction digestion of the DNA was performed with suitable enzymes (New England BioLabs, Bioconcept, Allschwil, Switzerland) in the supplied buffers under conditions according to the manufacturer's instructions.

DNA gel extraction

DNA was excised under UV-light (365 nm) from agarose gels, extracted using the QIAquick Gel Extraction Kit (QIAGEN, Hombrechtikon Switzerland) according to the manufacturer's protocol and eluted with 30-50 µl water or 10 mM TE buffer, pH 8 (Invitrogen, Thermo Fisher Scientific AG, Reinach, Switzerland).

Ligation

DNA fragments were ligated with T4 DNA ligase (New England BioLabs, Bioconcept, Allschwil, Switzerland) in T4 DNA Ligase Reaction Buffer (New England BioLabs, Bioconcept, Allschwil, Switzerland) at 16 °C for 2-4 h or at 20 °C overnight. The concentrations of vector and fragment solutions were estimated by separation of different volumes (2, 5 and 10 µl) of 1/10 dilutions in an agarose gel. Ligation reactions were performed using 200 ng of the vector backbone and four times as many molecules of the fragment.

Heat-shock transformation

For plasmid DNA-preparation, competent *E. coli* XL1 blue (Stratagene, Agilent Technologies AG, Basel, Switzerland) were thawed on ice and, in a pre-chilled 15 ml reaction tube, 100 µl were mixed with 10 µl ligation mixture or 1 µl DNA-Midi/Maxi-Preparation, respectively, and further incubated on ice for 20 min. The bacteria-DNA mixture was heat-shocked at 42 °C for 45 s and subsequently incubated on ice for 2 min. After addition of 900 µl pre-warmed (42 °C) SOC, the mixture was incubated at 37 °C and 200 rpm for 30 min. Selective LB agar plates were pre-warmed at 37 °C, streaked with 10-100 µl of the bacteria-DNA mixture and incubated overnight at 37 °C. The next day, single colonies were used for inoculating 2 ml of selective LB medium and grown at 37 °C and 200 rpm for 3-4 h. The bacteria were pelleted by centrifuging 1 ml of the culture at 16'000 x g for 1 min. Then, the pelleted bacteria were lysed with 100 µl water and 5 µl were used as template for a single colony screening PCR.

SOC

To prepare 50 ml of SOC, 48 ml of SOB, 0.5 ml of 1 M $MgCl_2$, 0.5 ml of 1 M $MgSO_4$, and 1 ml of 20% (w/v) D(+)-glucose monohydrate were mixed.

SOB

For 200 ml SOB, 4 g of bacto-tryptone (Becton Dickinson AG, Allschwil, Switzerland), 1 g of bacto-yeast extract (Becton Dickinson AG, Allschwil, Switzerland), and 0.1 g NaCl were dissolved in water and sterilized.

LB medium

LB medium was prepared by dissolving 1% (w/v) bacto-tryptone, 0.5% bacto-yeast extract, and 1% (w/v) NaCl in water.

LB agar plates

LB agar plates contained 1.5% (w/v) bacto-agar (Becton Dickinson AG, Allschwil, Switzerland) in LB medium.

Preparation of competent bacteria

E.coli were spread on LB agar plates and incubated overnight at 37 °C. Next, a single colony was cultivated overnight in 5 ml of LB medium at 37 °C and 200 rpm. An aliquot (1 ml) of the overnight-culture was diluted in 120 ml of LB medium and grown to mid-log phase ($OD_{600\ nm}$=0.4-0.6). The bacteria were pelleted by centrifugation at 3000 rpm at 4°C for 10 min. Thereafter, the bacterial pellet was resuspended in 12 ml of TSB on ice. Aliquots were flash frozen with liquid nitrogen and stored at -80 °C.

TSB

To prepare 100 ml of TSB, 10 g PEG 3000 were dissolved in 40 ml of LB medium and the pH was adjusted to 6.1. Thereafter, 5 ml of DMSO, 2 ml of 1 M $MgSO_4$, and 2 ml of 1 M $MgCl_2$ were added. LB medium was added to a final volume of 100 ml.

Midi/Maxi-Preparations of plasmid DNA

Plasmid DNA was extracted from bacterial cultures using the Invitrogen PureLink HiPure Plasmid Purification Kit (Thermo Fisher Scientific AG, Reinach, Switzerland) according to the manufacturer's protocol. For Midi-Preparations 25 ml and for Maxi-Preparations 200 ml of bacterial overnight-culture were used. The extracted DNA was dissolved in 100-400 µl TE and stored at -20 °C.

Sequencing

Sequencing reactions were performed by Microsynth AG, Balgach, Switzerland according to the company's instructions.

Primers

The primers were purchased from Microsynth AG, Balgach, Switzerland.

The following primers were used for cloning of hexahistidine-tagged GRK2-domains into the pET-3d vector (Figure 7) (the tag-sequence is underlined):

GRK2seq16	C-terminal hexahistidine tagged GRK2NTD forward
	5'-TAT AAT CC ATG GCC GCG GAC CTG GAG GCG GTG CTG GCC GAC GTG AGC TAC CTG ATG GCG ATG GAG-3'
GRK2seq17	C-terminal hexahistidine tagged GRK2NTD reverse
	5'-TAT AAT GGA TCC TTA <u>ATG GTG ATG GTG ATG GTG</u> GTC ATT CAT GGT CAG GTG GAT GTT GAG CTC CAC ATT C-3'
GRK2seq19	N-terminal hexahistidine-tagged GRK2KD forward
	5'-TAT AAT CC ATG GCC <u>CAT CAC CAT CAC CAT CAC</u> TTC AGC GTG CAT CGC ATC ATT GGG CGC GGG-3'
GRK2seq20	N-terminal hexahistidine-tagged GRK2KD reverse
	5'-AAT GGA TCC TTA GAA AAA GGG GCT CTC TTT CAC CTC CTG-3'
GRK2seq34	C-terminal hexahistidine-tagged GRK2KD forward
	5'-TAT AAT CC ATG GCC TTC AGC GTG CAT CGC ATG ATT GGG CGC-3'
GRK2seq35	C-terminal hexahistidine-tagged GRK2KD reverse
	5'-AAT GGA TCC TTA <u>ATG GTG ATG GTG ATG GTG</u> GAA AAA GGG GCT CTC TTT CAC CTC CTG-3'
GRK2seq24	N-terminal hexahistidine-tagged GRK2CTD forward
	5'-AAT TAT AAT TC ATG ACC <u>CAT CAC CAT CAC CAT CAC</u> CGC TCC CTG GAC TGG CAG ATG GTC TTC TTG-3'
GRK2seq25	N-terminal hexahistidine-tagged GRK2CTD reverse
	5'-AAT GGA TCC TCA GAG GCC GTT GGC ACT GCC GCG CTG GAC-3'
GRK2seq32	C-terminal hexahistidine-tagged GRK2CTD forward
	5'-TAT AAT TAT AAT TC ATG ACC CGC TCC CTG GAC TGG CAG ATG GTC-3'
GRK2seq33	C-terminal hexahistidine-tagged GRK2CTD reverse
	5'-AAT GGA TCC TCA <u>ATG GTG ATG GTG ATG GTG</u> GAG GCC GTT GGC ACT GCC GCG CTG GAC-3'

Table 2: Primers for cloning of the His-tagged GRK2 domains into pET-3d

pET-3a sequence landmarks

T7 promoter	615-631
T7 transcription start	614
T7·Tag coding sequence	519-551
T7 terminator	404-450
pBR322 origin	2814
bla coding sequence	3575-4432

The maps for pET-3b, pET-3c and pET-3d are the same as pET-3a (shown) with the following exceptions: pET-3b is a 4639bp plasmid; subtract 1bp from each site beyond *Bam*H I at 510. pET-3c is a 4638bp plasmid; subtract 2bp from each site beyond *Bam*H I at 510. pET-3d is a 4637bp plasmid; the *Bam*H I site is in the same reading frame as in pET-3c. An *Nco* I site is substituted for the *Nde* I site with a net 1bp deletion at position 550 of pET-3c. As a result, *Nco* I cuts pET-3d at 546. For the rest of the sites, subtract 3bp from each site beyond position 551 in pET-3a. *Nde* I does not cut pET-3d.
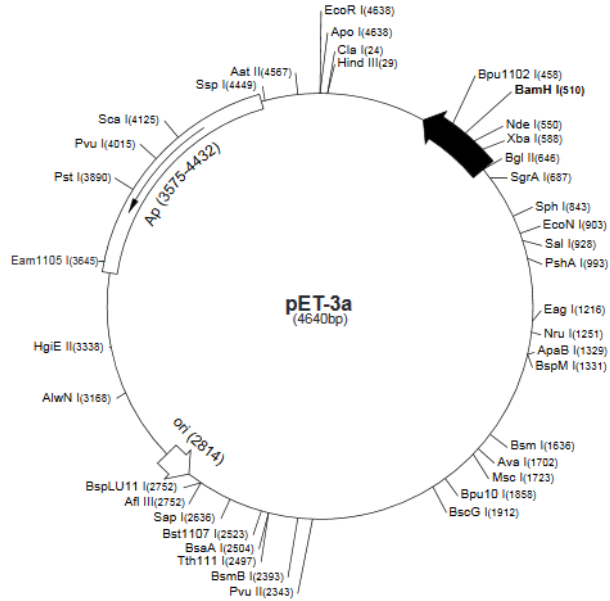
Figure 7: Vector map of pET-3d

The vector map of pET-3d (Novagen) shows a multiple cloning site and the T7 promoter.

Single-stranded oligonucleotides

Single-stranded oligonucleotides were obtained from Microsynth AG, Balgach, Switzerland.

The following single-stranded oligonucleotides were used for the generation of the WD-peptide containing plasmids (pcDNA3, Figure 8):

WD1	upper strand
	5'-GTC CAA TAC GGA TCC ATG GCC AGC ACC CTG ATC GTG GGC CAC TGG GAC GGC AAC ATG AGC CTG GTG GAC AGG AGG ACC TGA CTC GAG GTA TTG GAC-3'
WD1	lower strand
	5'-GTC CAA TAC CTC GAG TCA GGT CCT CCT GTC CAC CAG GCT CAT GTT GCC GTC CCA GTG GCC CAC GAT CAG GGT GCT GGC CAT GGA TCC GTA TTG GAC-3'
WD2	upper strand
	5'-GTC CAA TAC GGA TCC ATG TTC CTG GCC GAG GAC GCC AGC ACC CTG ATC GTG GGC CAC TGG GAC GGC AAC ATG AGC CTG TGA CTC GAG GTA TTG GAC-3'
WD2	lower strand
	5'-GTC CAA TAC CTC GAG TCA CAG GCT CAT GTT GCC GTC CCA GTG GCC CAC GAT CAG GGT GCT GGC GTC CTC GGT CAG GAA CAT GGA TCC GTA TTG GAC-3'
WD3	upper strand
	5'-GTC CAA TAC GGA TCC ATG GAG AGG AGC AGC TTC AGC AGC TTC GAC TTC CTG GCC GAG GAC GCC AGC ACC CTG ATC GTG TTC TGA CTC GAG GTA TTG GAC-3'
WD3	lower strand
	5'-GTC CAA TAC CTC GAG TCA GAA CAC GAT CAG GGT GCT GGC GTC CTC GGC CAG GAA GTC GAA GCT GCT GAA GCT GCT CCT CTC CAT GGA TCC GTA TTG GAC-3'

Table 3: Single-stranded oligonucleotides for the cloning of the WD-Peptides into pcDNA3

Comments for pcDNA3:
    5446 nucleotides

CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin ORF: bases 2151-2945
SV40 poly A: bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin ORF: bases 4450-5310
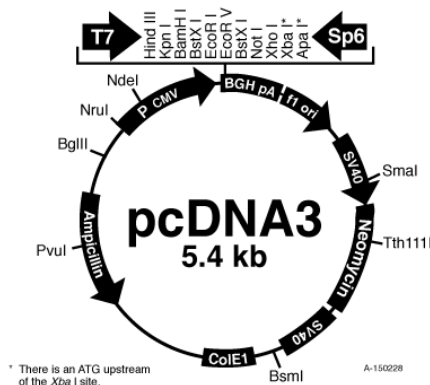
Figure 8: Vector map of pcDNA3

The vector map of pcDNA3 (Invitrogen) shows a multiple cloning site, promoters and bacterial resistance genes.

The following single-stranded oligonucleotides were used for cloning of the WD3 peptide sequence into the α-myosin heavy chain (α-MHC) vector:

WD3	upper strand
	5'-GTC CAA TAC GTC GAC ATG GAG AGG AGC AGC TTC AGC AGC TTC GAC TTC CTG GCC GAG GAC GCC AGC ACC CTG ATC GTG TTC TGA AAG CTT GTA TTG GAC-3'
WD3	lower strand
	5'-GTC CAA TAC AAG CTT TCA GAA CAC GAT CAG GGT GCT GGC GTC CTC GGC CAG GAA GTC GAA GCT GCT GAA GCT GCT CCT CTC CAT GTC GAC GTA TTG GAC-3'

Table 4: Single-stranded oligonucleotides for cloning of the WD3-Peptide into α-MHC

5.2 Protein procedures

Expression of recombinant proteins using the pET expression system

The cDNA of proteins to be overexpressed was cloned into the bacterial expression vector pET-3d (Novagen, Merck KGaA, Darmstadt, Germany), which contains the bacteriophage-derived T7 promoter under the control of the *lac* operon (227). *E.coli* BL21(DE3)pLysS were used as a host strain. The pLysS plasmid constitutively expresses

low levels of T7 lysozyme. Therefore, it represses basal protein expression by inhibition of basal levels of T7 RNA polymerase. Moreover, basal protein expression is repressed by the *lac* repressor, which upon addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) is inactivated. This leads to the transcription of the T7 RNA polymerase gene under the control of the *lac* promoter. Thereafter, T7 RNA polymerase starts the transcription of the gene sequences under the control of the T7 promoter in the pET-3d plasmids. Thus, proteins of interest are selectively overexpressed following the addition of IPTG (Figure 9).
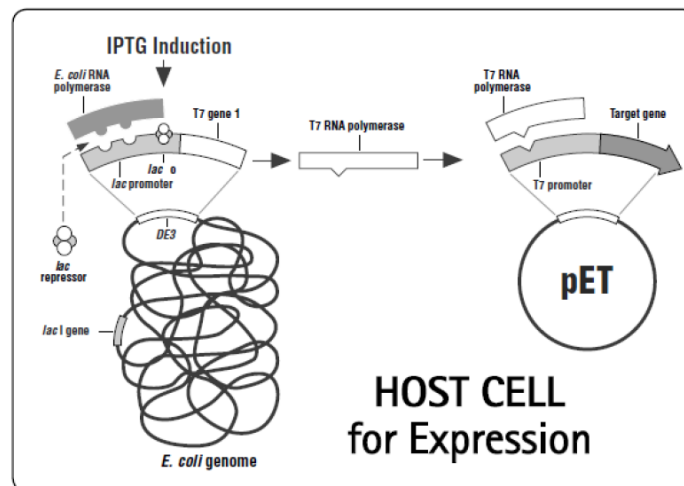
Figure 9: Protein overexpression with the pET expression system
This schema illustrates how addition of IPTG releases the *lac* repressor, thereby allowing the transcription of the T7 RNA polymerase gene. The T7 RNA polymerase in turn transcribes the target gene inserted into the pET expression vector. (pET System Manual, 11th edition, Novagen)

Single use competent BL21(DE3)pLysS (Promega AG, Dübendorf, Switzerland) were transformed by heat-shock transformation and spread on selective LB agar plates. Single colonies were picked and cultivated overnight at 37 °C and 200 rpm in 10 ml of selective LB medium. The overnight cultures were diluted 1/20 in 200 ml of selective LB medium and grown to mid-log phase under the same conditions. As an uninduced control, a sample of 10 ml of the bacterial culture was centrifuged at 4000 x g at RT for 10 min (Rotanta 460R, Hettich Laborapparate, Bäch, Switzerland). The supernatant was disposed of and the bacterial cell-pellet was flash frozen in liquid nitrogen and subsequently stored at -80 °C. Thereafter, the protein expression was induced with 1 mM IPTG for 3-4 h at 37 °C and 200 rpm. Hourly samples were taken for time-course analysis of the protein expression and treated as above. Finally, the bacterial culture was centrifuged at 4000 x g at RT for 10 min, the supernatant disposed of and the bacterial pellet stored at -20 °C until purification.

For SDS-PAGE analysis or immunoblotting, the samples taken before and after induction of the protein expression were thawed on ice, resuspended in 200 µl freshly prepared 8 M urea and incubated at RT for 30 min. The viscosity of the suspension was reduced by sonication on ice (HD2200, 2-3 x 3 sec with 30% cycle and 70% power; UW2200; MS73; BANDELIN electronic GmbH & Co.KG, Berlin, Germany). After addition of 50 µl of 5 x SDS sample buffer and 25 µl of β-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Buchs SG. Switzerland), the samples were incubated at RT for 1 h and subsequently stored at -20 °C.

<u>SDS sample buffer (5 x)</u>
To prepare 5 x SDS sample buffer, 5% (w/v) SDS (Bio-Rad Laboratories AG, Cressier, Switzerland) and 10% (v/v) glycerol were dissolved in 250 mM Tris, pH 6.8. A tiny amount of bromophenol blue was added.

Optical density measurement
The growth of bacterial cultures was monitored by measuring the optical density (OD) at 600 nm (Biophotometer, Eppendorf, Germany).

Purification of His-tagged proteins by metal affinity chromatography
Ni-NTA affinity chromatography allows the purification of recombinant proteins with a polyhistidine tag, which can be introduced by standard molecular cloning techniques.
The column was prepared by equilibrating 1-2 ml of 50% Ni-NTA slurry (QIAGEN, Hombrechtikon, Switzerland) with 10-20 ml of lysis buffer. Then, the bacterial cell pellet of a 50 ml or 200 ml culture was thawed on ice and resuspended in 10 or 20 ml of lysis buffer, respectively. The viscosity of the bacterial suspension was reduced by sonication on ice (HD2200, 5-10 x 3 sec with 30% cycle and 70% power; UW2200; MS73; BANDELIN electronic GmbH & Co.KG, Berlin, Germany). Next, the suspension was cleared by centrifugation at 4000 x g at 4 °C for 15 min (Sorvall RC 6 Plus, Thermo Electron Corporation, Rotor SLA 3000). The supernatant was incubated on the equilibrated Ni-NTA resin at 4 °C with agitation for 1 h to overnight, followed by removal of unspecifically bound proteins by washing with 20 x resin volumes of wash buffer. Thereafter, the bound proteins were eluted with 10 ml of elution buffer and collected in 2 ml aliquots. Aliquots of the flow-through, the washing step and the eluates were mixed with appropriate amounts of 5 x SDS sample buffer and analysed by SDS-PAGE or immunoblotting.

The Ni-NTA resin was regenerated by incubation with 0.5 M NaOH for 30 min and subsequently stored in 30% (v/v) ethanol at 4 °C. Thus, packed columns were used up to five times for purification of the same protein.

Lysis buffer

The lysis buffer contained 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, and 100 mM β-mercaptoethanol. The pH was adjusted to 8.0.

Wash buffer

The wash buffer was prepared as the lysis buffer, but the pH was adjusted to 6.3.

Elution buffer

The elution buffer was prepared as the lysis buffer, only the pH was adjusted to 4.3.

Protein separation by SDS-PAGE

Protein samples mixed with the appropriate volume of 5 x SDS sample buffer were separated by SDS-PAGE. Gels consisted of an upper stacking gel and a lower separation gel. Polymerisation of the stacking gel was initiated with 1 µl/ml TEMED (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 0.1% (w/v) APS (Bio-Rad Laboratories AG, Cressier, Switzerland). For starting the polymerisation of the separation gel, 0.5 µl/ml TEMED and 0.325% (w/v) APS were used. The proteins were resolved at 80-120 V in SDS running buffer for approximately 2-2 ½ h. A protein weight marker (PageRuler Plus Prestained Protein Ladder, Thermo Fisher Scientific AG, Reinach, Switzerland) was run in parallel with the samples. The stacking gels were removed with a scalpel and the separation gels were either directly stained with Coomassie brilliant blue or blotted by semi-dry blotting technique and subsequently stained and destained.

Stacking gel

Stacking gels (3.75%) were prepared with 0.125 ml/ml of 30% (w/v) acrylamide/bis-acrylamide solution (29:1, 3.3% crosslinker, Bio-Rad Laboratories AG, Cressier, Switzerland), and 0.1% (w/v) SDS in 125 mM Tris, pH 6.8.

Separation gel

Separation gels were prepared with appropriate amounts of 30% (w/v) acrylamide/bis-acrylamide solution and 0.1% (w/v) SDS in 375 mM Tris, pH 8.8.

SDS running buffer

For the preparation of the SDS running buffer, 192 mM glycine, 25 mM Tris, and 0.2% (w/v) SDS were dissolved in water.

Staining solution

The staining solution was prepared with 10% (v/v) acetic acid, 20% (v/v) methanol and 0.05% (w/v) Coomassie brilliant blue R 250.

<u>Destaining solution</u>
The destaining solution contained 10% (v/v) acetic acid and 20% (v/v) methanol.

Immunoblot detection of proteins
After separation by SDS-PAGE and removal of the stacking gel, the separation gel was incubated with transfer buffer for 45 min with agitation. A PVDF membrane (Immobilon-P, 0.45 μm, Merck & Cie, Schaffhausen, Switzerland) was activated in methanol for 7 min and subsequently equilibrated in transfer buffer for 30 min. In addition, two filter papers (Extra thick blot paper, Bio-Rad Laboratories, Cressier, Switzerland) were equilibrated in transfer buffer for 30 min. The separated proteins were transferred to the membrane by semi-dry blotting technique with 20 V and 0.04 A for 40 min (Trans-Blot SD semi-dry Transfercell, Bio-Rad Laboratories AG, Cressier, Switzerland). The membranes were washed five times with water to remove residual methanol and incubated overnight in PBS at 4° C. Then, the membranes were blocked with 20 ml of blocking buffer for 1 h at RT with agitation, followed by incubation with an appropriate primary antibody diluted in blocking buffer for 1 h at RT with agitation. After washing 4 x 5 min with PBST 0.05%, the membranes were incubated with enzyme-conjugated secondary antibody diluted in blocking buffer for 30 min at RT with agitation. Next, the membranes were washed 6 x 5 min with PBST 0.05% and 4 x 5 min with PBS to remove residual detergent. The visualization was performed with ECL Western Blotting detection reagent (Amersham, GE Healthcare, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) according to the manufacturer's instructions. X-ray films (Super RX, Fuji Medical x-ray film, Schenk, Röntgenbedarf AG, Hettlingen, Switzerland) were exposed for 20 s to 2 min, developed (Tetenal, Roentoroll HC, Tetenal AG & Co, Germany) and fixed (Tetenal Superfix MRP, Tetenal AG & Co, Germany)

<u>Transfer buffer</u>
The transfer buffer contained 192 mM glycine, 25 mM Tris, 0.2% (w/v) SDS and 20% (v/v) methanol.

<u>PBS</u>
PBS was prepared by dissolving 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 in water.

<u>Blocking buffer</u>
The blocking buffer was prepared by dissolving 5% (w/v) BSA (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) in PBST 0.05% (PBS with 0.5% (v/v) Tween 20).

Antibodies

The following primary antibodies were used for immunoblot detection of proteins or ELISAs:

anti-GRK2 antibodies were raised in rabbit against purified recombinant GRK2 expressed in Sf9 cells (173); anti-GRK2CTD antibody is a mouse monoclonal antibody raised against amino acids 468-689 of human GRK2 (sc-13143, Santa Cruz Biotechnology, LabForce AG, Muttenz, Switzerland); HRP-conjugated anti-M13 antibody is a monoclonal antibody purified from mouse ascites fluid, which reacts specifically with the bacteriophage M13 major coat protein product of gene VIII (27-9421-01, Amersham, GE Healthcare, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland); anti-SRSF1 antibody is a mouse monoclonal antibody epitope mapping near the N-terminus of the SF2/ASF protein (sc-33652, Santa Cruz Biotechnology, LabForce AG, Muttenz, Switzerland); anti-WDR76 antibodies were produced in rabbit against an antigen sequence containing the WD3 sequence (HPA040626, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland).

The following secondary antibodies were used for immunoblot detection of proteins or ELISAs:

Peroxidase-conjugated AffiniPure F(ab')$_2$ Fragment Goat-Anti-Rabbit (111-036-046)/goat anti-mouse (115-036-071) IgG, Fc Fragment Specific (Jackson ImmunoResearch Laboratories, USA) as applicable.

Acetone precipitation

Proteins were precipitated by mixing 0.5 ml of protein solution with 1 ml ice-cold (-20 °C) acetone and centrifugation at 13'200 x g for 20 s. The supernatant was disposed of and the protein pellet dissolved in 1 x SDS sample buffer. The samples were sonicated on ice (HD2200, 3-5 x 3 sec with 30% cycle and 10% power; UW2200; MS73; BANDELIN electronic GmbH & Co.KG, Berlin, Germany) and separated by SDS-PAGE.

SDS sample buffer (1 x)

To prepare the 1 x SDS sample buffer, 2% (w/v) SDS, 10% (v/v) glycerol and 300 mM β-mercaptoethanol were dissolved in 62.5 mM Tris, pH 6.8. A tiny amount of bromophenol blue was added.

Desalting columns (PD-10)

Buffer exchange of protein solutions with PD-10 desalting columns (GE Healthcare, Glattbrugg, Switzerland) was performed according to the manufacturer's instructions. The proteins were eluted with the desired buffer in aliquots of 1 ml.

Protein Concentration measurement

The protein concentration was quantified by Bradford's assay (228) using Roti-Quant reagent (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The assay was performed in 96-well plates (F8 Maxisorp Nunc-Immuno Module, Thermo Fisher Scientific AG, Reinach, Switzerland) according to the manufacturer's protocol. The absorbance was read at 595 nm with a microplate reader (VersaMax ELISA Microplate Reader, Molecular Devices, Sunnyvale CA, USA) and evaluated using SoftMax Pro 5.4.5 software.

ELISA procedure

Peptides or proteins were coated onto 96-well plates in carbonate buffer (pH: 1-2 units above the pI of the peptide or protein) and incubated overnight at 4 °C. Subsequent incubation steps were carried out at RT with agitation. The plates were blocked with ELISA blocking buffer for 1-2 h. The washing steps were carried out with TBST 0.05%. Interaction partners or antibodies were added in blocking buffer and incubated for 1 h. The ABTS detection reaction was prepared freshly by adding 36 µl of 30% H_2O_2 to 21 ml ABTS-Stock solution and 100 µl were added to each well. The substrate reaction was allowed to proceed for 30 min before the absorbance was read at 410 nm (VersaMax ELISA Microplate Reader, Molecular Devices, Sunnyvale CA, USA). The data were evaluated with SoftMax Pro 5.4.5 software.

Peptides were obtained from EMC microcollections GmbH, Tübingen, Germany and were N-terminally acetylated in order to increase stability.

Carbonate buffer

The carbonate buffer contained 100 mM $NaHCO_3$.

ELISA blocking buffer

The ELISA blocking buffer contained 2% (w/v) BSA in TBST 0.05% or alternatively 0.1-0.2% milk (frema Reform Instant-Magermilchpulver, Granovita GmbH, Heimertingen, Germany) in TBST 0.05%.

TBST 0.05%

To prepare TBST 0.05%, 150 mM NaCl and 50 mM Tris were dissolved and the pH was adjusted to 7.5. Thereafter, 0.05% (v/v) Tween 20 were added.

ABTS-Stock solution

The ABTS-Stock solution was prepared by dissolving 22 mg ABTS in 100 ml of 50 mM sodium citrate solution, pH 4.0.

5.3 Peptide phage display library screening

For the phage display peptide library screening, the Ph.D.-7 library (New England BioLabs, Bioconcept, Allschwil, Switzerland) was used, which displays linear heptapeptides.

Peptides interacting with the target protein were selected in three consecutive rounds of panning. Thereby, the eluted phages from earlier rounds were amplified and used as input phage for the next round. The eluted phages from the third round were amplified individually. Next, the interaction with the target protein was assessed with an ELISA and the peptide sequences of the phages showing the strongest binding were determined.

Surface panning

For coating of the bait-protein, wells (F8 Maxisorp Nunc Immuno-Module, Thermo Fisher Scientific AG, Reinach, Switzerland) received 10-100 µg/ml of bait-protein in carbonate buffer and were incubated at 4 °C with gentle agitation in a humidified container overnight. Streptavidin (New England BioLabs, Bioconcept, Allschwil, Switzerland) was used in parallel as a positive control. The next day, the coating solution was poured off and wells were blocked for 2 h at 4°C. Wells were washed six times with TBST 0.1% (v/v) Tween 20 for the first round of panning and 0.5% (v/v) Tween 20 for the second and third round of panning using a wash bottle. A 100-fold representation of the library (10^{11} plaque forming units (pfu)) was diluted with 100 µl of TBST 0.01% or 0.05%, respectively, added to the coated wells and gently rocked at RT for 60 min. Unbound phages were removed by washing ten times with TBST and bound phages were eluted with 100 µl of phage display elution buffer by rocking gently for 10-20 min. The eluate was neutralized with 15 µl of 1 M Tris, pH 9.1 and stored at 4 °C until amplification. For the streptavidin control experiment, the bound phages were eluted by gently rocking with 0.1 mM biotin (New England BioLabs, Bioconcept, Allschwil, Switzerland) in TBS for 30 min.

Carbonate buffer

The carbonate buffer contained 100 mM $NaHCO_3$ and the pH was adjusted to 8.6.

Phage display blocking buffer

The phage display blocking buffer was prepared by dissolving 0.5% (w/v) BSA in carbonate buffer. The buffer for the streptavidin control experiment was supplemented with 0.1 µg/ml streptavidin.

Phage Display elution buffer

For the phage display elution buffer, 0.1% (w/v) BSA were dissolved in 0.2 M glycine, pH 2.2.

Phage precipitation

E. coli ER2738 (New England BioLabs, Bioconcept, Allschwil, Switzerland) were streaked on selective (20 µg/ml tetracycline (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) in 1:1 ethanol/water) LB agar plates and incubated at 37 °C overnight. Next, a single colony was used to inoculate 20 ml of selective LB medium. The culture was grown to early-log phase ($OD_{600\ nm}$=0.01-0.05) at 200 rpm at 37 °C. Eluted phages from the first and second round were amplified by infection of the early-log phase culture and incubation at 37 °C and 200 rpm for 4.5 h. Third round eluates were not amplified, but directly used for ELISA or sequencing. The cultures were centrifuged with 12'000 x g at 4 °C for 10 min (Sorvall RC 6 Plus, Thermo Electron Corporation, Rotor SS34). After re-centrifuging the supernatant, the phages were precipitated at 4 °C overnight by adding 2.7 ml of precipitation buffer to 16 ml of the supernatant. The precipitated phages were pelleted by centrifugation at 12'000 x g at 4 °C for 15 min. The supernatant was decanted and the phage pellet re-centrifuged for 3 min. After removal of the residual supernatant, the phage pellet was resuspended in 1 ml of TBS and incubated at RT for 60 min. The phage suspension was transferred to a fresh tube, followed by centrifugation at 14'000 rpm for 5 min. Thereafter, the supernatant was transferred to a fresh tube and the phages were re-precipitated by addition of 167 µl of precipitation buffer and incubation on ice for 60 min. The phage suspension was centrifuged at 14'000 rpm and 4 °C for 10 min. Next, the supernatant was discarded and the pellet re-centrifuged for 90 s. The residual supernatant was removed, the phages were resuspended in 200 µl TBS, incubated at RT for 60 min and centrifuged at 14'000 rpm for 1 min. The supernatant containing the amplified phages to be used for subsequent panning rounds, was transferred to a fresh tube, incubated at 65 °C for 15 min to kill residual bacteria and stored at 4 °C.

Precipitation buffer

The precipitation buffer contained 20% (w/v) PEG 8000 (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) in a 2.5 M NaCl solution.

Determination of the phage eluate titer

For the determination of the phage titer, serial dilutions of the eluted phages (10^8-10^{11} for the amplified first and second round eluates, 10^4-10^7 for the unamplified third round eluates) in LB medium were prepared and 10 µl were used to infect 200 µl of *E.coli* ER2738 mid-log culture. After vortexing for 10 s and incubation at RT for 1-5 min, the infected bacteria were transferred to 3 ml of previously melted Top Agar, immediately vortexed and spread on selective LB agar plates for blue/white screening. The plates were allowed to cool down for 5-10 min and incubated at 37 °C overnight. A phage titer, determined by counting blue plaques only, of ~10^{13-14} pfu/ml was expected. For the second and third round panning experiments, a volume corresponding to 10^9-10^{11} pfu was used as input phage.

For the preparation of Top Agar, 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.5% (w/v) NaCl, and 0.7% (w/v) bacto-agar were dissolved in water.

The LB agar plates were prepared by adding IPTG (50 µg/ml in DMF) and x-gal (40 µg/ml in DMF) to selective LB medium supplemented with 1.5% (w/v) bacto-agar.

Amplification of single plaques

Blue plaques (around 10-20) from a freshly titered plate (<1-3 days, <100 plaques) were amplified in 1 ml of a 1/100 dilution of an *E.coli* ER2738 overnight culture at 37 °C and 200 rpm for 4.5-5 h. The cultures were pelleted at 14'000 rpm for 30 s. Whereas the bacterial pellet was stored at -20 °C for subsequent alkaline lysis and sequencing, the supernatant was re-centrifuged in a fresh tube. The upper 80% of the phage-containing supernatant were transferred to a fresh tube and incubated at 65 °C for 15 min to kill residual bacteria. Finally, the amplified phage stock was stored at 4 °C.

Phage ELISA

Multi-well plates were coated with 10-100 µg/ml of target protein dissolved in 100 µl of carbonate buffer or streptavidin control at 4 °C overnight with gentle agitation in a humidified container. Control wells received 100 µl incubation buffer without target protein. The wells were blocked with 0.5% (w/v) BSA in carbonate buffer at 4 °C for 2 h. Then, wells were washed six times with TBST 0.5%. The amplified phage stock from the third panning round was diluted 1:1 with blocking buffer, and 100 µl were added to a well coated with target protein and a control well, respectively, and incubated at RT for 2 h with agitation. Unbound phages were removed by washing six times with TBST 0.5%. Bound phages were detected by addition of an M13-specific antibody-conjugate diluted in blocking solution (1/5000) at RT for 1 h with agitation. After removal of unbound antibody-conjugate by washing six times with TBST 0.5%, the ABTS substrate was added. The absorbance was read at 415 nm after 10, 30 and 45 min (Bio-Rad Model 680 Microplate Reader, Microplate Manager 5.2 Software).

Alkaline lysis for preparation of DNA

The DNA was isolated from bacterial pellets by alkaline lysis. To this end, the pellets from bacteria infected with a single phage each, were resuspended in 100 µl of Buffer R3 (Pure link HiPure Plasmid Filter Purification Kit, Invitrogen) and incubated at RT for 5 min. The bacterial cells were lysed by incubation with 200 µl of Buffer L7 (Pure link HiPure Plasmid Filter Purification Kit, Invitrogen) on ice for 5 min. The suspension was mixed with 150 µl of Buffer N3 (Pure link HiPure Plasmid Filter Purification Kit, Invitrogen), briefly vortexed and incubated on ice for 15 min. The mixture was centrifuged

at 16'000 x g for 5 min. The supernatant was transferred to a fresh tube and DNA was precipitated by the addition of 1 ml of 100% ice-cold ethanol, followed by vortexing and incubation at -20 °C for 5 min. The precipitated DNA was pelleted by centrifugation at 16'000 x g for 5 min. Thereafter, the pellet was resuspended in 250 µl of 0.3 M sodium acetate, pH 4.8, mixed with 700 µl of 100% ice-cold ethanol, vortexed and centrifuged at 16'000 x g for 5 min. The DNA pellet was dried and resuspended in 20 µl water and dissolved by incubation for 30 min at RT. The plasmid DNA (1.2 µg) was sequenced with primer -96 gIII (5'-CCC TCA TAG TTA GCG TAA CG-3'). Experiments yielding the consensus site (His-Pro-Gln) for the streptavidin control were considered successful.

Protein database search of identified peptides for sequence similarities
A basic local alignment search tool (BLAST) search of the NCBI protein database was performed to search for sequence similarity with known human proteins (taxid: 9606) (229). The non-redundant protein sequences database was searched with the blastp (protein-protein BLAST) algorithm. Search parameters were adjusted to search for a short input sequence by default.

5.4 *In vitro* phosphorylation assay

Expression and purification of His-tagged proteins from Sf9 insect cells
Proteins of interest were expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Thermo Fisher Scientific AG, Reinach, Switzerland) according to the manufacturer's instructions. For protein purification, the insect cells were washed once with 10 ml of ice-cold PBS, followed by resuspension of the pellet with 10 ml of lysis buffer. The cell suspension was sonicated (3 x 3 sec with 30% cycle and 33% power) and centrifuged at 4700 x g at 4 °C for 30 min. The supernatant was incubated with 0.5 ml equilibrated Ni-NTA resin for 1 h on ice. Unbound proteins were removed by washing with 20 ml of wash buffer, after which the bound proteins were eluted with 5 ml of elution buffer.
Protein expression and purification from Sf9 insect cells were performed in the Molecular Pharmacology Unit, ETH Zurich, Switzerland by Stefan Wolf.

Lysis buffer
The lysis buffer contained 300 mM NaCl and 50 mM HEPES supplemented with 1% (v/v) IGEPAL CA-630 (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland), 1 mM PMSF (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) and 100 µl Protease Inhibitor Cocktail (P8340, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland). The pH was adjusted to 7.5.

To prepare the wash buffer, 300 mM NaCl and 50 mM HEPES were dissolved in water and supplemented with 30 mM imidazole (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland). The pH was adjusted to 7.5.

For the elution buffer, 300 mM NaCl, 50 mM HEPES, and 300 mM imidazole were dissolved in water and the pH was adjusted to 7.5.

Phosphorylation reaction

To assess the GRK2-inhibitory activity of the peptides, a kinase assay was performed. The phosphorylation was performed in a reaction mixture of 50 µl containing 130 nM of His-tagged GRK2 purified from Sf9 cells infected with a recombinant baculovirus, 300 nM of His-tagged protein substrate purified from bacterial culture and the compound of interest at different concentrations in DMSO at a final concentration of 2% in assay buffer. After incubation on ice for 30 minutes, the reaction was started by addition of 5 µl of ATP ((Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland)/γ^{32}P-ATP (10 µCi/ml, PerkinElmer Inc., Germany)) to give a final volume of 50 µl. The reaction took place at 30 °C. After 30 min, the reaction was stopped by the addition of 20 µl Bolt 4x LDS sample buffer (Invitrogen, Thermo Fisher Scientific AG, Reinach, Switzerland) and 8 µl of Bolt 10 x sample reducing agent (Invitrogen, Thermo Fisher Scientific AG, Reinach, Switzerland). After an incubation at 70 °C for 10 minutes, phosphorylated proteins were separated by SDS-PAGE at 165 V using 12% Bolt Bis-Tris Plus gels (Invitrogen, Thermo Fisher Scientific AG, Reinach, Switzerland) and MOPS SDS running buffer.

The assay buffer contained 20 mM Tris, 5 mM $MgCl_2$, and 2 mM EDTA dissolved in water. The pH was adjusted to 7.5.

Detection of phosphorylated proteins by autoradiography

X-ray films (Amersham Hyperfilm ECL, GE Healthcare, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) were exposed to the acrylamide gels for a couple of hours to overnight at -80 °C and developed as described earlier. Subsequently, exposed films were analysed using Image J software and GraphPad Prism for statistical analysis.

5.5 Cell culture

Cell thawing

A frozen aliquot of HEK293A cells (3 x 10^6 cells, Invitrogen, Thermo Fisher Scientific AG, Reinach, Switzerland) was removed from liquid nitrogen storage, thawed in a 37 °C water bath and slowly added to 10 ml complete medium. The cell suspension was centrifuged at 300 rpm at RT for 5 min. Next, the cell pellet was resuspended in 10 ml of complete medium. One third and two thirds, respectively, of the cell suspension were cultivated in 10 ml of complete medium on a culture dish (TPP tissue culture dish 60 cm^2, TPP Techno Plastic Products AG, Trasadingen, Switzerland) at 37 °C under 5% CO_2 in a humidified incubator.

Complete medium

The complete medium contained DMEM (D6429, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) supplemented with 10% (v/v) FBS (Hyclone, Lot RZG35920, Thermo Fisher Scientific AG, Reinach, Switzerland) and 100 U/ml penicillin/100 µg/ml streptomycin (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland).

Cell cultivation

Adherent HEK293A cells were cultured in complete medium at 37 °C and 5% CO_2 in a humidified incubator. Cell culture medium was changed at least every three days.

Cell splitting

Adherent cells were washed with PBS (Dulbecco's phosphate buffered saline, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) and trypsinized with 0.05% Trypsin-EDTA (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) for 2-3 min. Trypsin was inactivated by the addition of 10% FBS-containing medium (three volumes).

Determination of the cell number

For determination of the cell number, the cell suspension was mixed with an equal volume of 0.4% (w/v) Trypan Blue (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) in PBS. The cells were counted using a Neubauer hemocytometer (LO-Laboroptik GmbH, Bad Homburg, Germany) according to the manufacturer's instructions. After applying 10 µl of the 1:1 diluted cell suspension, the number of living cells in four squares was counted. The cell concentration (cells/ml) was calculated with the following formula:

$$\text{concentration} = \frac{\text{number of cells} \times 10'000}{\text{number of squares}} \times \text{dilution factor} \times 100$$

Calcium phosphate transfection

Adherent cells, which had been cultured to 80-90% confluency, were transiently transfected using the calcium phosphate transfection method. The cells were split 1:3 and incubated for 3-4 h at 37 °C in a humidified incubator under 5% CO_2.

The following reaction mixture was prepared:

1	plasmid DNA	20 µg
2	water	to a volume of 450 µl
3	2.5 M $CaCl_2 \cdot 2 H_2O$	50 µl

Table 5: Reaction mixture for the calcium phosphate transfection

The plasmid DNA was diluted in water to a volume of 450 µl and 50 µl of 2.5 M $CaCl_2$ were added.

This mixture (Table 5) was added dropwise to 500 µl of 2 x BBS while vortexing. After an incubation of 20 min at RT, the transfection mixture was added dropwise to the previously split cells. After 12-16 hours, cells were washed with PBS and supplied with new complete medium.

<u>BBS (2 x)</u>

To prepare 2 x BBS, 280 mM NaCl, 1.5 mM Na_2HPO_4, and 50 mM BES (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) were dissolved in water and the pH was adjusted to 6.95.

Measurement of the intracellular free calcium concentration with FURA-2 (230)

Measurements were performed 36-48 h after the transfection. The adherent cells were washed with PBS, trypsinized and centrifuged at 300 rpm for 5 min at RT. The cells were loaded with FURA-2 by resuspension in 2.5 ml of complete medium supplemented with 2.5 µl of 1 mM FURA-2AM (dissolved in DMSO), followed by incubation at 37 °C under 5% CO_2 for 30 min. After centrifugation of the cells, FURA-2AM was deesterified by an incubation with 10 ml of Fura Buffer for 30 min at RT in the dark. For the measurement, the cells (1-2 x 10^6) were centrifuged and resuspended in 2 ml of Fura Buffer. The samples were alternatively excited at intervals of 0.08 s at 340 and 380 nm and the emission was recorded at 509 nm. The measurement was performed using a Perkin Elmer LS 55 Fluorescence Spectrometer and FLWinLab software.

Fura Buffer

The Fura buffer was prepared by dissolving 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.6 mM $CaCl_2$, 1 g/l D(+)-glucose monohydrate and 20 mM Na^+-HEPES in water and adjusting the pH to 7.3.

5.6 Establishment of transgenic mouse lines

All animal experiments were performed in accordance with National Institutes of Health guidelines, and approved by the local committee on animal care and use (University of Zurich).

Preparation of linearized plasmid DNA for generation of transgenic mouse lines

Plasmid DNA for generation of transgenic mouse lines was prepared using the EndoFree Plasmid Maxi Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's protocol. To this end, 100 ml of bacterial culture were used and DNA was dissolved in 200 µl of endofree TE-Buffer. Plasmid DNA sequences were removed by restriction digestion with NotI. After purification by agarose gel electrophoresis, linearized DNA was diluted to 2 ng/µl.

Transgene introduction

Transgenic mice were generated in the Molecular Pharmacology Unit, ETH Zurich, Switzerland by Dr. Joshua AbdAlla as previously described (173).

Preparation of genomic DNA from mouse biopsies for the genotyping PCR

Ear-punch biopsies were digested overnight at 57 °C and 1000 rpm in 200-220 µl of Lysis buffer supplemented with 10 µl of proteinase K solution (20 mg/ml, Roche Proteinase K, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland). The reaction was stopped by incubation for 20 min at 99 °C in a thermomixer (Eppendorf, Schönenbach, Switzerland). Samples were centrifuged at 23000 x g at 4 °C for 5 min. The supernatant containing the genomic mouse DNA was used as a template for the genotyping PCR.

<u>Lysis buffer</u>

The lysis buffer contained 2.5 ml of 10% sodium lauroyl sarcosinate (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland), 1 ml of 5 M NaCl, and 2.5 g Chelex-100 (Bio-Rad Laboratories AG, Cressier, Switzerland) in a total volume of 50 ml.

Genotyping PCR

The PCR reaction was performed using *Taq* Polymerase in the supplied buffer supplemented with 50 µg/ml BSA (New England BioLabs, Bioconcept, Allschwil, Switzerland), 250 µM $MgCl_2$ (Promega AG, Dübendorf, Switzerland), and 100 µM dNTPs.

Primers specific for the α-MHC-vector were used at 0.625 µM, namely MHC2 (5'-AGG ACT TCA CAT AGA AGC CTA GCC CAC ACC-3') as a forward primer and HGH2 (5'- ATT AGG ACA AGG CTG GTG GGC ACT GGA GTG-3') as a reverse primer. The PCR reaction was carried out in a reaction volume of 40 µl with 1.5 µl of genomic mouse DNA prepared as described above. Amplifications were performed with a standard program using a Thermocycler 3000 (Biometra, Göttingen, Germany):

Step	Temperature	Time
1	95 °C	2 min
2	95 °C	45 s
3	60 °C	60 s
4	72 °C	60 s
5	72 °C	10 min
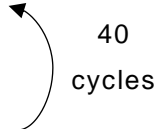
40 cycles

Table 6: Standard PCR program for the genotyping of transgenic mice
An initial melting step (step 1) at 95 °C for 2 min was followed by 40 cycles of melting of dsDNA at 95 °C for 45 s (step 2), annealing of the primers at 60 °C for 60 s (step 3) and elongation of the primers at 72 °C for 60 s (step 4). A final elongation step (step 5) at 72 °C for 10 min was added and samples were subsequently cooled down to 4 °C.

The amplified fragments were mixed with 5x Green GoTaq Flexi Buffer (Promega AG, Dübendorf, Switzerland) and separated by agarose gel electrophoresis in 2% (w/v) agarose gels prepared with 10 µl/100 ml 1% (w/v) ethidium bromide for visualization.

Abdominal aortic constriction
Abdominal aortic constriction was performed in the Molecular Pharmacology Unit, ETH Zurich, Switzerland, essentially as described (143,231).

5.7 Statistical analysis

Data were analysed using GraphPad Prism 6 software. Groups were analysed using two-tailed student's t-test and p-values < 0.05 were considered significant. Results are given as mean ± SEM.

6 Results

6.1 Cloning, protein expression and purification of GRK2 domains

GRK2 is a drug target for a variety of pathologies (4.3). Inhibition of the multi-domain kinase can be achieved by targeting any of the three domains (Figure 10).
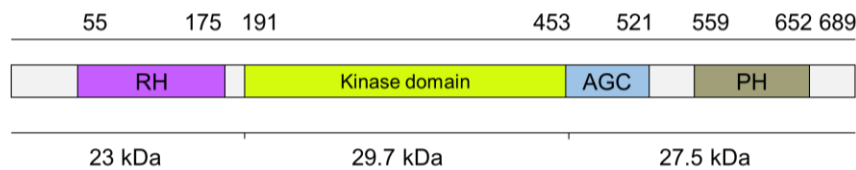
Figure 10: GRK2 is an enzyme with multiple domains

GRK2 consists of three distinct domains: The kinase domain containing the catalytically active site, flanked by the N-terminal domain mediating the interaction with the receptor and the C-terminal domain, which upon activation by the Gβγ-complex (Gβγ) facilitates translocation to the cell membrane (90-95).

To screen a phage display peptide library for domain-specific interaction partners, the different GRK2 domains were overexpressed individually in the pET bacterial expression system, which allows expression of a given protein under the control of the T7 promoter. A hexahistidine tag was attached to each domain to enable protein purification by Ni-NTA chromatography.

To this end, plasmids for bacterial expression containing the corresponding cDNA sequences for the three GRK2 domains were generated.

6.1.1 Generation of GRK2 domain-containing plasmids

For the N-terminal domain of GRK2 (GRK2NTD), the cDNA corresponding to the first 190 amino acids of GRK2 was amplified by PCR using suitable primers (GRK2seq16/17) for introduction of a His-tag C-terminally. The PCR also introduced the NcoI/BamHI recognition sites for restriction endonucleases. After PCR, the amplified fragments were cut by restriction digestion with NcoI and BamHI and inserted into the NcoI/BamHI cut pET-3d plasmid.
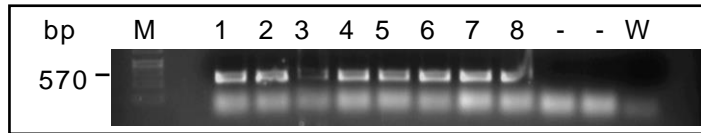
Figure 11: Cloning of C-terminally His-tagged GRK2NTD into pET-3d

Single colony screening PCR of bacterial clones: Separation by agarose gel electrophoresis showed the amplification of an approximately 570 bp fragment for clones 1-8. Control clones (-) and water control (W) were negative.

Single colony screening (Figure 11) showed that all eight colonies were positive for the GRK2NTD. Clone 6 was chosen for DNA sequencing, which confirmed the identity of the DNA and its correct insertion into pET-3d (13.1).

For cloning of the central kinase domain (GRK2KD), the cDNA coding for amino acids 191-453 of GRK2 was amplified introducing His-tags N-terminally or C-terminally, respectively, with suitable primers (GRK2seq19/20, GRK2seq34/35) (Figure 12). After introduction of the NcoI/BamHI recognition sites for restriction endonucleases by PCR, the amplified fragments were cut and inserted into the NcoI/BamHI cut pET-3d plasmid.

A

B
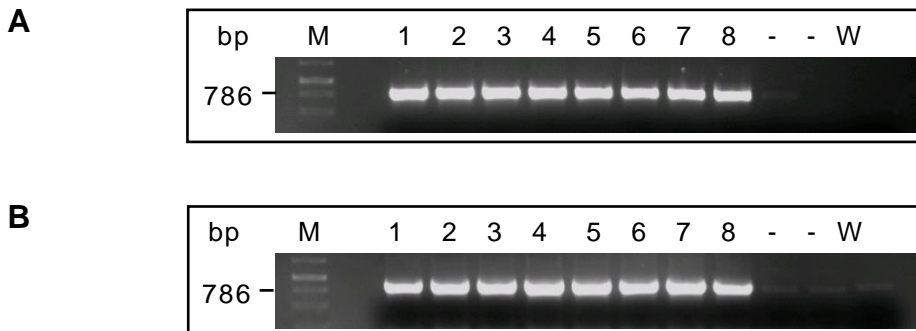
Figure 12: Cloning of N-terminally (A) and C-terminally (B) His-tagged GRK2KD into pET-3d

Single colony screening PCR of bacterial clones: Separation by agarose gel electrophoresis showed the amplification of an approximately 786 bp fragment for clones 1-8 (A and B). Control clones (-) and water controls showed only a slight or no signal.

Again, almost all bacterial colonies were positive for the DNA of interest (Figure 12). Clones 2 (6xNHisGRK2KD) and 1 (GRK2KD6xCHis) were chosen for DNA sequencing, which confirmed the identity of the DNA and its correct insertion into pET-3d (13.2, 13.3).

Cloning of the C-terminal domain was carried out by amplification of the cDNA encoding for amino acids 454-689 of GRK2 with primers (GRK2seq24/25, GRK2seq32/33) introducing His-tags N- or C-terminally, respectively (Figure 13). Recognition sites for the BspHI and BamHI restriction endonucleases were also inserted by PCR. After cutting the amplified fragments with BspHI and BamHI, they were inserted into the NcoI/BamHI cut pET-3d plasmid. This was possible, because BspHI and NcoI produce the same overhang (5'-CATG).
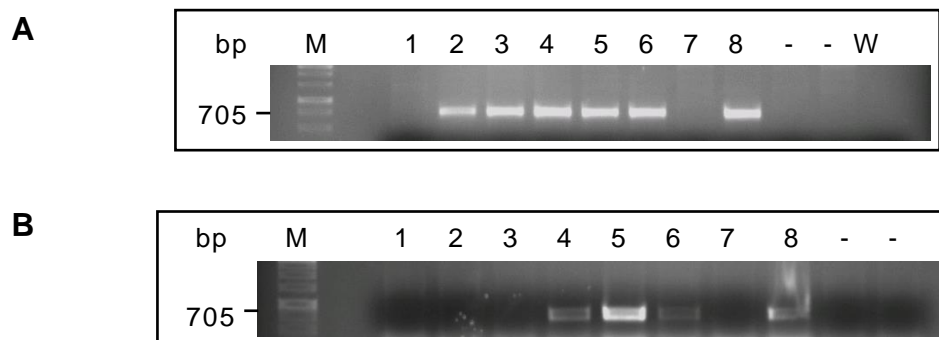
Figure 13: Cloning of N-terminally (A) and C-terminally (B) His-tagged GRK2CTD into pET-3d

Single colony screening PCR of bacterial clones: Separation by agarose gel electrophoresis showed the amplification of an approximately 705 bp fragment for clones 4-6 and 8 (A) and 2-6 and 8 (B). Control clones (-) and water control (W) were both negative.

Single colony screening showed that six out of eight colonies were positive for the N-terminally His-tagged and four out of eight for the C-terminally His-tagged GRK2CTD (Figure 13). For DNA sequencing, clones 2 (6xNHisGRK2CTD) and 4 (GRK2CTD6xCHis) were chosen, which confirmed the identity of the DNA and its correct insertion into pET-3d (13.4, 13.5).

6.1.2 Protein expression and purification of GRK2 domains

The generated pET-3d plasmids were used for the expression of His-tagged GRK2 domains in *E.coli* BL21(DE3)pLysS.

Protein expression and purification of GRK2NTD

The plasmid coding for C-terminally His-tagged GRK2NTD (GRK2NTD6xCHis) was transformed into *E.coli* BL21(DE3)pLysS and protein expression was induced with 1 mM IPTG. Uninduced controls plus samples isolated 1, 2 and 3 h after IPTG-induction were separated by SDS-PAGE. A band of 24 kDa was induced in the GRK2NTD samples, but was absent in the controls. The relative molecular mass of ~24 kDa corresponds well with the expected mass of GRK2NTD6xCHis. Immunoblot detection with a polyclonal

GRK2-specific antibody (1/2000) confirmed the identity of the IPTG-induced 24-kDa protein as GRK2NTD in samples 1, 2 and 3. Maximum induction of GRK2NTD was already achieved after 1 h of induction.
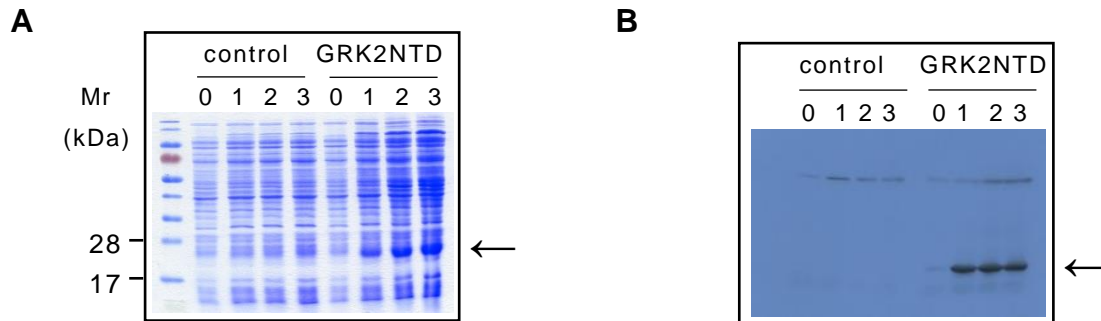
Figure 14: Protein expression of GRK2NTD6xCHis

Aliquots before (0) and 1, 2 and 3 hours (1-3) after IPTG-induced protein expression were resolved by SDS-PAGE followed by Coomassie brilliant blue staining (A) or detected by immunoblotting with a polyclonal GRK2-specific antibody (B).

Next, the GRK2NTD6xCHis was purified by Ni-NTA chromatography. To this end, pelleted bacteria from 50 ml of culture were resuspended with 10 ml of lysis buffer. After reduction of the viscosity by sonication, the suspension was centrifuged and the cleared supernatant incubated with 2 ml equilibrated Ni-NTA resin overnight. Unbound proteins were removed by washing with 30 ml of wash buffer, followed by elution of the bound proteins with 1.5 ml of elution buffer, pH 5.9 and subsequently 1.5 ml of elution buffer, pH 4.3. The purified protein was collected in four aliquots of 0.75 ml each. The overall yield of the purified protein was only 3 mg from 200 ml of bacterial culture.

The Coomassie brilliant blue staining of the flow-through, washes and eluates separated by SDS-PAGE showed no band with the expected size of ~24 kDa (data not shown), probably due to the low amount of the target protein (GRK2NTD6xCHis). Thus, eluates E1-E3 were precipitated with acetone to concentrate the purified protein. Separation of the acetone-precipitated eluates E1-E3 by SDS-PAGE revealed a weak band in all eluates (Figure 15, A). Immunoblot detection with a polyclonal GRK2-specific antibody showed equal amounts of purified protein in eluates E1-E3 (Figure 15, B).
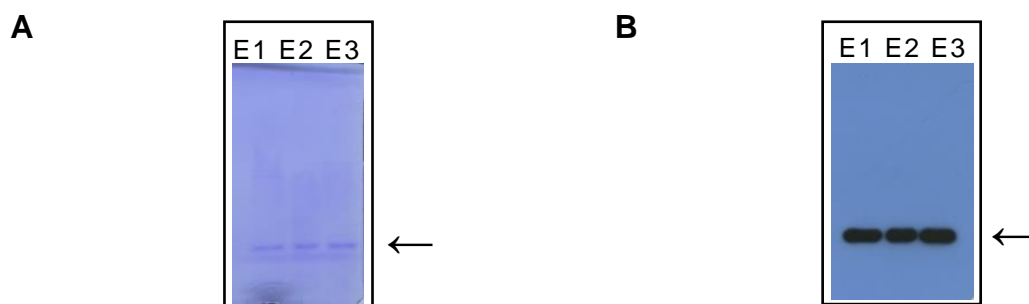
Figure 15: GRK2NTD6xCHis was purified by Ni-NTA chromatography

Eluates E1-E3 from the protein purification of GRK2NTD6xCHis were resolved by SDS-PAGE (A). Proteins were stained with Coomassie brilliant blue (A) and GRK2NTD was detected by immunoblotting with a polyclonal GRK2-specific antibody (B).

Protein expression and purification of GRK2KD

The plasmid coding for the N-terminally or C-terminally His-tagged GRK2KD, respectively, (6xNHisGRK2KD, GRK2KD6xCHis) was transformed into *E.coli* BL21(DE3)pLysS and protein expression was induced with 1 mM IPTG. Separation of the proteins from uninduced samples and samples after hours 1-3 by SDS-PAGE revealed no expression of the C-terminally His-tagged protein (data not shown). For the N-terminally His-tagged protein, by contrast, analysis by SDS-PAGE showed the strong induction of a protein of ~28 kDa in samples 1, 2 and 3 increasing over time (Figure 16, A). The relative molecular mass of 6xNHisGRK2KD is ~30 kDa. Thus, GRK2KD runs slightly lower in the SDS-PAGE gel than expected. This effect might be a result of the basic character of the protein (pI=8.6) or of the N-terminal position of the His-tag, which can result in the purification of an incompletely translated or a degraded protein.

For the purification of 6xNHisGRK2KD, bacteria from a 200 ml bacterial expression culture were resuspended with 20 ml of lysis buffer, sonicated and centrifuged. The cleared supernatant was incubated with 2 ml of Ni-NTA resin overnight with agitation. After washing with 40 ml of wash buffer, the purified protein was eluted at pH 4.3 and collected in five 2 ml aliquots. From 200 ml of bacterial culture, up to 8 mg protein could be purified.

Aliquots taken during the purification steps were resolved by SDS-PAGE (Figure 16, B). Coomassie blue staining showed reasonable purification of a ~ 28-kDa protein.
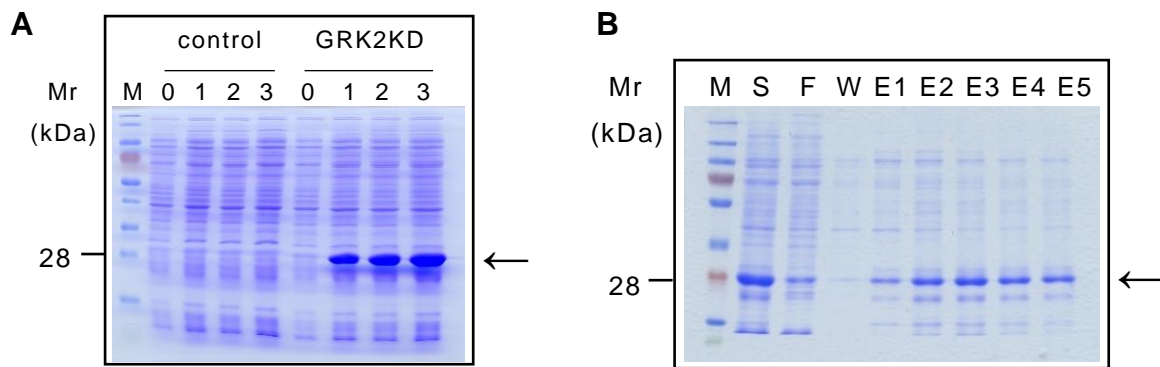
Figure 16: GRK2KD was purified by Ni-NTA chromatography

Aliquots taken before (0) and 1, 2 and 3 hours after (1-3) IPTG-induced protein expression were resolved by SDS-PAGE (A).

Aliquots from the supernatant (S), flow-through (F), wash (W) and eluates (E1-E5) from the purification of 6xNHisGRK2KD were resolved by SDS-PAGE. Proteins were stained with Coomassie brilliant blue. M, Marker

Protein expression and purification of GRK2CTD

E.coli BL21(DE3)pLysS were transformed with the plasmid coding for N-terminally or C-terminally His-tagged GRK2CTD, respectively, (6xNHisGRK2CTD, GRK2CTD6xCHis). Samples before and 1, 2 and 3 h after induction of protein expression with 1 mM IPTG were resolved by SDS-PAGE. Coomassie brilliant blue staining showed the strong induction of a protein of ~30 kDa, which increased during the 3 h induction period (Figure 17). As the relative molecular mass of His-tagged GRK2CTD is ~28 kDa, GRK2CTD runs slightly higher in the SDS-PAGE gel than expected.
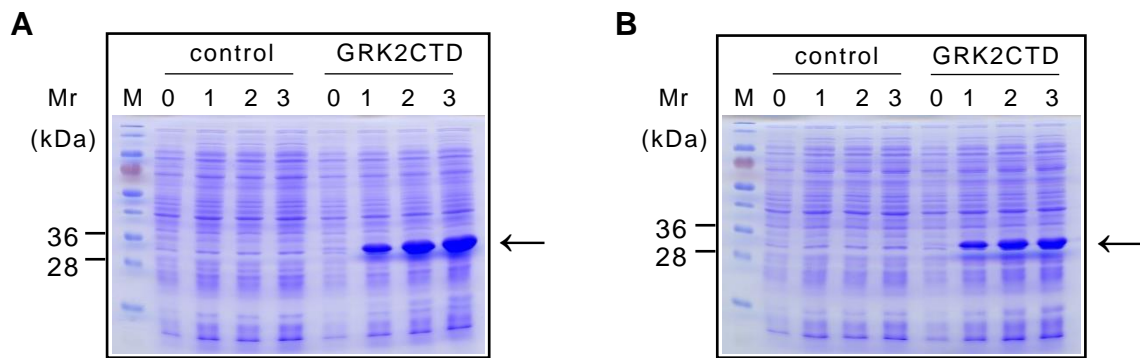
Figure 17: Protein expression of GRK2CTD
Aliquots before (0) and after 1, 2 and 3 hours (1-3) of IPTG-induced protein expression of N-terminally (A) and C-terminally (B) His-tagged GRK2CTD were resolved by SDS-PAGE. Proteins were stained with Coomassie brilliant blue. M, Marker

For purification, 200 ml of bacterial expression culture were pelleted, followed by resuspension with 20 ml of lysis buffer. After sonication and centrifugation, the cleared supernatant was incubated with 2 ml of Ni-NTA resin overnight with agitation. After removal of unbound protein by washing with 40 ml of wash buffer, the purified protein was eluted at pH 4.3 and collected in five 2 ml aliquots. Up to 15 mg of purified protein could be obtained from 200 ml of bacterial culture.

Aliquots of the purification procedure were resolved by SDS-PAGE (Figure 18). Coomassie brilliant blue staining showed the enrichment of a ~ 30-kDa protein. Again, the band is slightly higher than expected for the His-tagged GRK2CTD with a relative molecular mass of ~28 kDa.
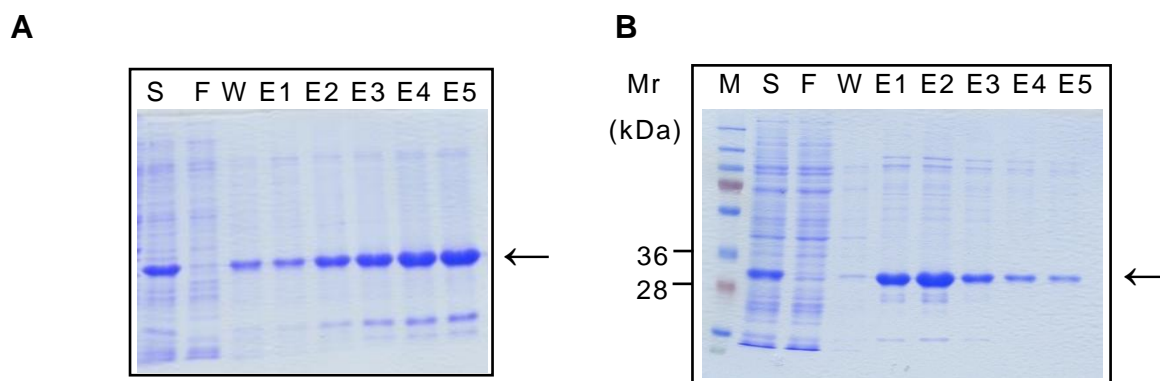
Figure 18: GRK2CTD was purified by Ni-NTA chromatography

Aliquots from the supernatant (S), flow-through (F), wash (W) and eluates (E1-E5) of the purification of 6xNHisGRK2CTD (A) and GRK2CTD6xCHis (B) were resolved by SDS-PAGE. Proteins were stained with Coomassie brilliant blue. M, Marker

6.2 Peptide phage display library screening with GRK2CTD as bait

6.2.1 Panning procedure

After purification of the recombinant GRKCTD6xCHis in high amounts, it was used as a bait in three rounds of panning with a library displaying 1 x 10^9 different heptapeptides as described in chapter 5.3. A control experiment used streptavidin.

For the first round of panning, one well of a 96-well plate was coated overnight at 4 °C with 125 µl of purified GRK2CTD (35 µg/ml) in carbonate buffer (pH 8.6) and, as a control, one well was coated with 125 µl streptavidin (100 µg/ml) in carbonate buffer. The phage titer of the amplified eluates was determined by streaking dilutions (10^8-10^{11}) on selective LB agar plates. Counting of the phage plaques yielded a phage titer of 8.6·10^{12} pfu/ml for GRK2CTD and 1.25·10^{12} pfu/ml for the streptavidin control experiment, respectively. Consequently, input phage volumes corresponding to 10^{11} pfu for the second round of panning were calculated to be 12 µl (GRK2CTD) and 80 µl (streptavidin control experiment), respectively. The amplified eluate was applied onto the plate coated with GRK2CTD in a total volume of 150 µl.

The second round of panning used wells coated with 125 µl of protein solution (100 µg/ml each) in carbonate buffer. The phage titer of the amplified eluates was determined to be approximately 10^{13} pfu/ml for both GRK2CTD and the streptavidin control experiment.

For the third round of panning, 10 µl of amplified eluates corresponding to approximately 10^{11} pfu were diluted with 140 µl TBS and used as input phage.

For the third round of panning, wells were again coated with 125 µl of protein solution (100 µg/ml each) in carbonate buffer. In contrast to the first two rounds, eluates were not amplified, but rather diluted (10^4-10^7) and titered directly. The phage titer was determined to be approximately 10^7 pfu/ml for both GRK2CTD and streptavidin.

Amplified phage stocks were prepared for 11 clones from the last titering steps for both GRK2CTD and the streptavidin control experiment.

6.2.2 Phage ELISA

An ELISA was performed to evaluate binding of the eluted phages to purified GRK2CTD6xCHis. To this end, wells were coated overnight at 4 °C with 125 µl of GRK2CTD6xCHis solution (125 µg/ml) in carbonate buffer (pH 8.6) or empty buffer as a control. For the streptavidin control experiment, streptavidin was used instead of purified GRK2CTD6xCHis. After blocking with carbonate buffer supplemented with 0.5 % BSA for 2 h at 4 °C, wells were washed six times with TBST 0.5%. Thereafter, 100 µl of amplified phage stocks 1:1 diluted with blocking buffer were added to the protein-coated wells or control wells. After incubation for 2 h at RT with shaking, wells were washed as above and 200 µl of M13-specific monoclonal antibody (1/5000 in blocking buffer) were added. After incubation for 1 h at RT with shaking, wells were washed again as above and 200 µl of freshly prepared ABTS-substrate solution were added to each well. After allowing the substrate reaction to proceed for 30 min, the absorbance was read at 415 nm. Phages showing the strongest signal in this assay (Figure 19) were sequenced.
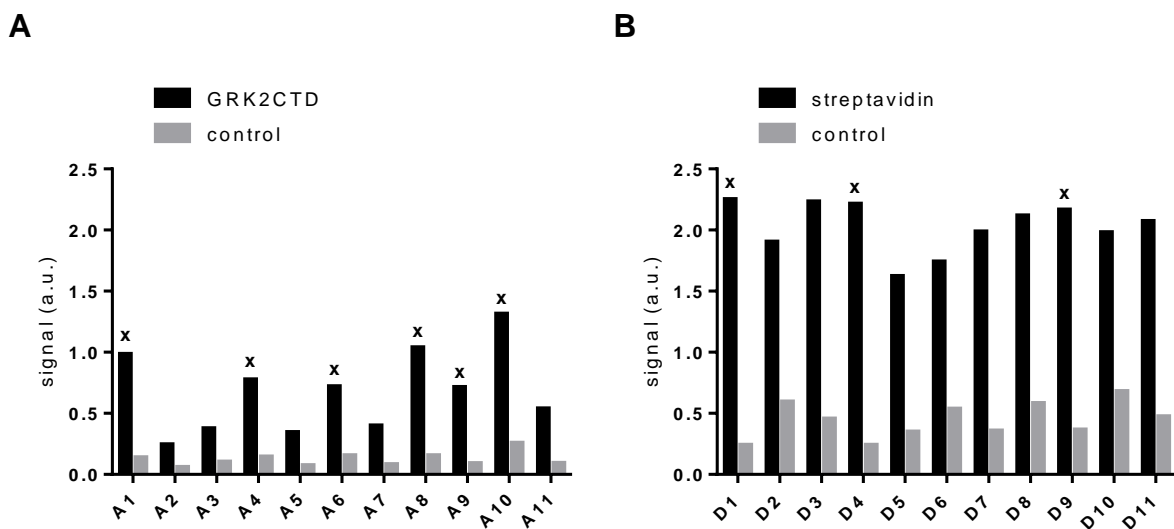
Figure 19: Evaluation of binding by a phage ELISA
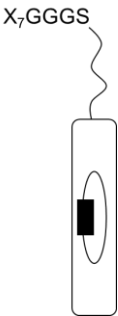
(A) Binding of eluted phages to GRK2CTD. (B) Binding of eluted phages from the streptavidin control experiment to streptavidin.

6.2.3 Peptide results

The DNA for sequencing was prepared by alkaline lysis for clones A1, A4, A6, A8, A9 and A10 and resuspended with 20 µl of water. For the sequencing, 1.2 µg DNA and 4 pmol of the primer -96gIII were used. The results are displayed in Table 7.

Clones D1, D4 and D9 of the streptavidin control experiment were also sequenced. They all yielded the expected consensus sequence site (WLFNHPQ) (232).

A

X$_7$GGGS

B

Clone	Sequence
A1	ASTLIVF
A4	IRYVPQT
A6	HGGVRLY
A8	HYTDFRW
A9	IVSLQTP
A10	HYIDFRW

Table 7: Peptide sequences

(A) Schematic drawing of a phage displaying a random peptide sequence with a GGGS-linker. (B) Peptide sequences of eluted phages.

6.3 Development of a GRK2-interacting peptide based on the panning results

6.3.1 BLAST sequence analysis

Phage display technology may be utilized as a source to identify novel interaction partners for a specific bait-protein (233,234). Therefore, the NCBI protein database was searched for sequence similarity of the phage display-derived heptapeptides with known human proteins. Agreement in at least six continuous amino acids was considered as a hit (Table 8).

Protein	Sequence	Protein sequence
WDR76	ASTLIVF	…SFSSFDFLAED**ASTLIV**GHWDGNMSL…
Cadherin 20	IRYVPQT	…MLPEIESLF**RYVPQT**CAVNS…
–	HGGVRLY	–
–	HYTDFRW	–
NFATc2	IVSLQTP	…PESSGR**IVSLQT**ASNPIECS…
–	HYIDFRW	–

Table 8: Peptide sequence homology with known proteins
A BLAST search of the NCBI protein database identified sequence homology (i.e. at least six continuous amino acids in common) with known proteins for three of the six phage display-derived peptides.

The peptide sequence ASTLIVF showed homology with the WD repeat-containing protein 76 (WDR76), IRYVPQT with cadherin 20 and IVSLQTP with the nuclear factor of activated T-cells (NFAT).

WD repeat-containing proteins are abundant in the mammalian proteome (235). Intriguingly, Gβ is a prominent member of this protein family and displays the characteristic seven-bladed propeller structure (Figure 20).
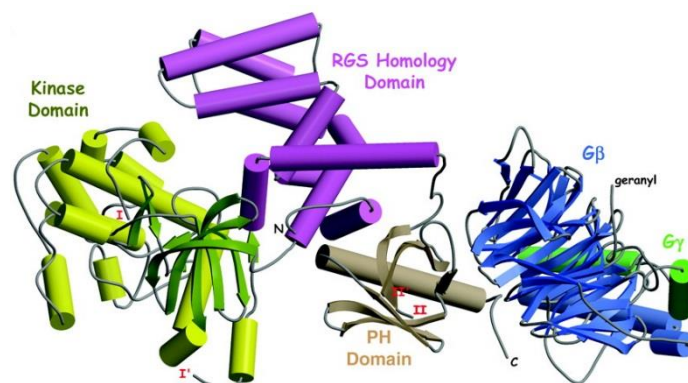
Figure 20: Model of the crystal structure of GRK2 in complex with Gβγ
The crystal structure of bovine GRK2 with Gβ1γ2 shows the characteristic WD domain structure of Gβγ interacting with the C-terminal domain of the kinase (236).

With regard to the known interaction of the WD repeat-containing protein Gβ with GRK2CTD, this peptide (ASTLIVF) was considered the most promising one for further investigation.

6.3.2 Sequence elongation based on WDR76

Thus, the original phage display-derived peptide (ASTLIVF) was elongated based on the sequence of WDR76 (Table 9).

original peptide	**ASTLIV**F
WDR76	…RNERSSFSSFDFLAED**ASTLIV**GHWDGNMSLVDRRTPGTSY…
WD1 peptide	**ASTLIV**GHWDGNMSLVDRRT
WD2 peptide	FLAED**ASTLIV**GHWDGNMSL
WD3 peptide	ERSSFSSFDFLAED**ASTLIV**F

Table 9: Sequence elongation of WD-peptides

The original peptide from the peptide phage display library screen was elongated C-terminally (WD1), N-terminally (WD3) or in both directions (WD2) to a length of ~20 amino acids.

The longer peptides (WD1 and WD3) were subsequently characterized for their potential to interact with GRK2. WD2 showed strong aggregation and therefore could not be tested.

6.3.3 An ELISA shows selective binding of GRK2CTD to WD3

To investigate the interaction of the WD1 and WD3 peptides with GRK2CTD, an ELISA binding experiment was performed by coating WD1 or WD3, respectively, at increasing concentrations. To enhance coating efficiency, BSA was added to a total protein concentration of 4 µg/ml (237). GRK2CTD was added at a constant concentration of 2 µg/ml. Bound GRK2CTD was detected using a primary GRK2CTD-specific polyclonal antibody (1/300) and a secondary HRP-conjugated antibody (1/4000). The substrate reaction was allowed to proceed for 30 min, after which the absorbance was read at 410 nm.

GRK2CTD shows concentration-dependent binding to WD3 (Figure 21). For WD1, in contrast, a concentration-dependent increase in GRK2CTD-binding cannot be observed. Although at 10 µg/ml, WD1 shows a significant interaction with GRK2CTD, WD1 shows no concentration-dependent interaction as can be seen for WD3.
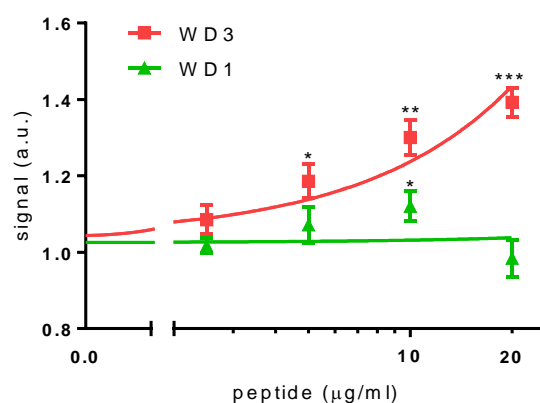
Figure 21: GRK2CTD shows selective binding to WD3 in an ELISA

Wells coated with increasing amounts (2.5, 5, 10 and 20 µg) of the elongated peptides WD1 and WD3, respectively, were exposed to GRK2CTD at a constant concentration of 2 µg/ml.

Data of four experiments are displayed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test. Linear curve fitting was performed using GraphPad Prism 6.

6.4 The GRK2-interacting peptide shows GRK2-inhibitory features

6.4.1 WD3 interferes with the binding of GRK2CTD to Gβ3

Blockade of the Gβγ-binding to GRK2 via its C-terminal domain confers kinase inhibition (191,192), as Gβγ subunits are essential for kinase activation and targeting to the plasma membrane (38). Similarly, a compound interacting with the GRK2CTD could inhibit GRK2 function by interfering with this protein-protein interaction. Thus, the effect of the peptides WD1 and WD3, respectively, on the Gβ-GRK2CTD interaction was examined in an inhibition ELISA. The Gβ-subunit directly interacts with GRK2 (238,239), whereas the Gγ-subunit mediates membrane anchoring of the Gβγ-GRK2-complex via its prenylation site (240). Thus, the assay was performed using only Gβ-subunits. More specifically, Gβ3 and a shorter splice variant, Gβ3-s, were used.

6.4.1.1 GRK2CTD binds to Gβ3 and Gβ3-s

To verify the interaction of Gβ3 and Gβ3-s with GRK2CTD, a direct binding ELISA was performed using recombinant His-tagged proteins purified from bacterial cultures. Gβ3 and Gβ3-s, respectively, were coated at different concentrations ($6 \cdot 10^{-7}$ M, $3 \cdot 10^{-7}$ M,

1.5·10⁻⁷ M, 7.5·10⁻⁸ M, 3.75·10⁻⁸ M, 1.88·10⁻⁸ M, 9·10⁻⁹ M) and GRK2CTD was added at a constant concentration of 6 µg/ml. As a control, wells were coated with BSA. A polyclonal GRK2-specific antibody (1/300) was used as a primary and a HRP-conjugated antibody (1/10'000) as a secondary antibody. Absorbance was read at 410 nm 30 min after addition of the substrate ABTS.

Whereas GRK2CTD did not interact with BSA, GRK2CTD interacted with both Gβ3 and Gβ3-s in a concentration-dependent manner (Figure 22).
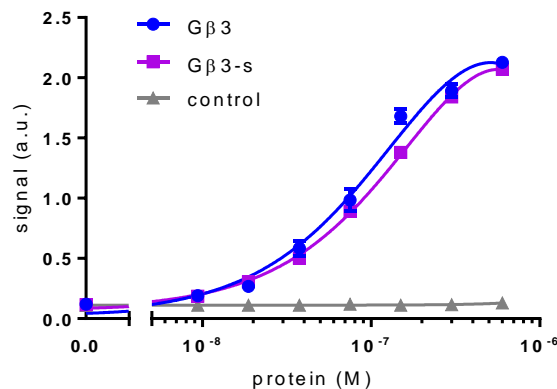
Figure 22: GRK2CTD binds Gβ3 and Gβ3-s

Binding of GRK2CTD to Gβ3 and Gβ3-s, respectively, was determined by ELISA. BSA was used as a control.

Data of three independent experiments are displayed as mean ± SEM.

6.4.1.2 WD3 inhibits the binding of GRK2CTD to Gβ3

Based on the binding assay described above, an inhibition ELISA was performed by coating with a constant concentration (6·10⁻⁷ M) of Gβ3 and Gβ3-s, respectively. After removal of unbound protein by washing steps, GRK2CTD was added at a constant concentration (6 µg/ml) and pre-mixed with increasing amounts of the elongated peptides WD1 and WD3, respectively. For detection, a primary polyclonal GRK2CTD-specific antibody (1/300) and a secondary HRP-conjugated antibody (1/4000) were used. Absorbance was read at 410 nm 30 minutes after addition of the substrate.

The results show that WD3 significantly interfered with the binding of GRK2CTD to Gβ3. WD3 also showed a tendency to inhibit the interaction of GRK2CTD with Gβ3-s. In contrast, WD1 did not affect the interaction of GRK2CTD with Gβ3 or Gβ3-s at concentrations up to 100 µg/ml (Figure 23: A, B).

A

B
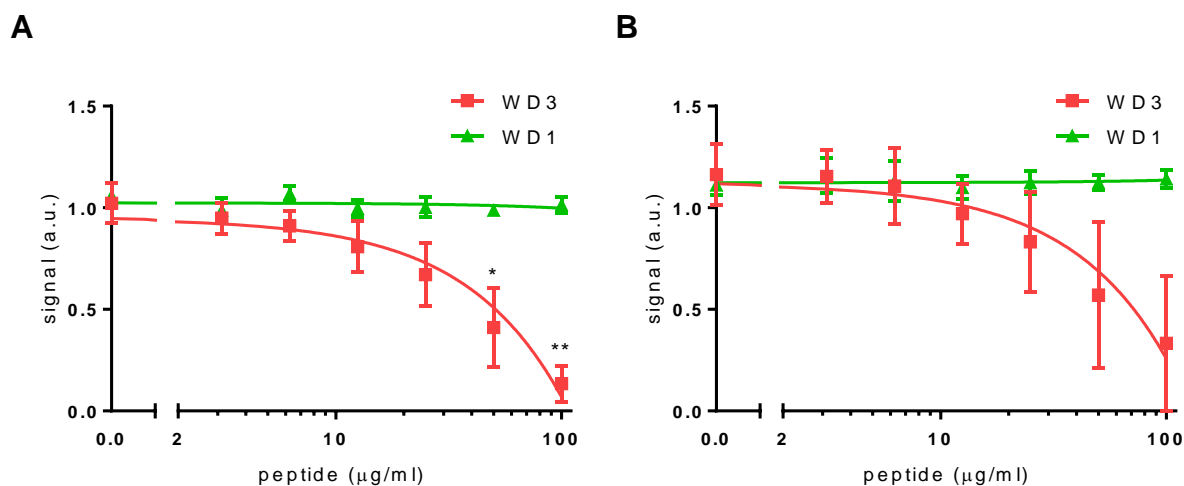
Figure 23: WD3 interferes with the binding of GRK2CTD to Gβ3 and Gβ3-s

Binding of GRK2CTD to Gβ3 (A) and Gβ3-s (B), in the presence of different concentrations of WD1 and WD3 was assessed in an ELISA.

Data of three independent experiments are displayed as mean ± SEM. Statistical analysis was performed using an unpaired student's t-test.

6.4.2 Assessment of kinase activity

To evaluate the effect of the peptides, WD1 and WD3, on the kinase activity of GRK2 a phosphorylation assay using a soluble substrate of GRK2 was performed.

6.4.2.1 WD3 inhibits the phosphorylation of the splicing factor SRSF1 by GRK2

The serine/arginine-rich splicing factor 1 (SRSF1) regulates splicing of cardiac Ca^{2+}/calmodulin-dependent kinase IIδ (CAMKIIδ), thereby participating in the functional transition from juvenile-to-adult heart characterized by increased workload (241). The cytoplasmic splicing variant CAMKIIδC phosphorylates phospholamban (PLB), thereby accelerating Ca^{2+}-reuptake into the sarcoplasmic reticulum and consequently increasing the rate of relaxation following contraction (242). Overexpression of CAMKIIδC results in a heart failure phenotype with disturbed Ca^{2+}-handling (243).

SRSF1 contains several phosphorylation sites, including a cluster located in the C-terminal arginine/serine-rich (RS) domain (UniProt Knowledgebase). SRSF1 has recently been identified as a novel substrate of GRK2 (Quitterer, U., unpublished data).

Phosphorylation was performed using 130 nM of recombinant GRK2 purified from Sf9 insect cells infected with a baculovirus encoding for GRK2 and 300 nM of recombinant SRSF1 purified from *E.coli* as a substrate. Compounds under investigation were added at increasing concentrations dissolved in DMSO yielding a final concentration of 2% total DMSO. The GRK2-stimulated incorporation of phosphate into the SRSF1 substrate was

detected by the addition of [γ-32P]-labelled ATP (10 µCi/ml, total ATP 5 µM). After separation by SDS-PAGE, gels were exposed to X-ray films for autoradiography. The control in the absence of peptide was set to 100% and values were calculated accordingly.

Whereas WD3 successfully inhibited the phosphorylation of SRSF1 by GRK2 (59 ± 9%, p=0.0087, n=3, Figure 24: A, C), WD1 did not lead to a reduced signal (102 ± 4%, p=0.6126, n=3, Figure 24: B, C).
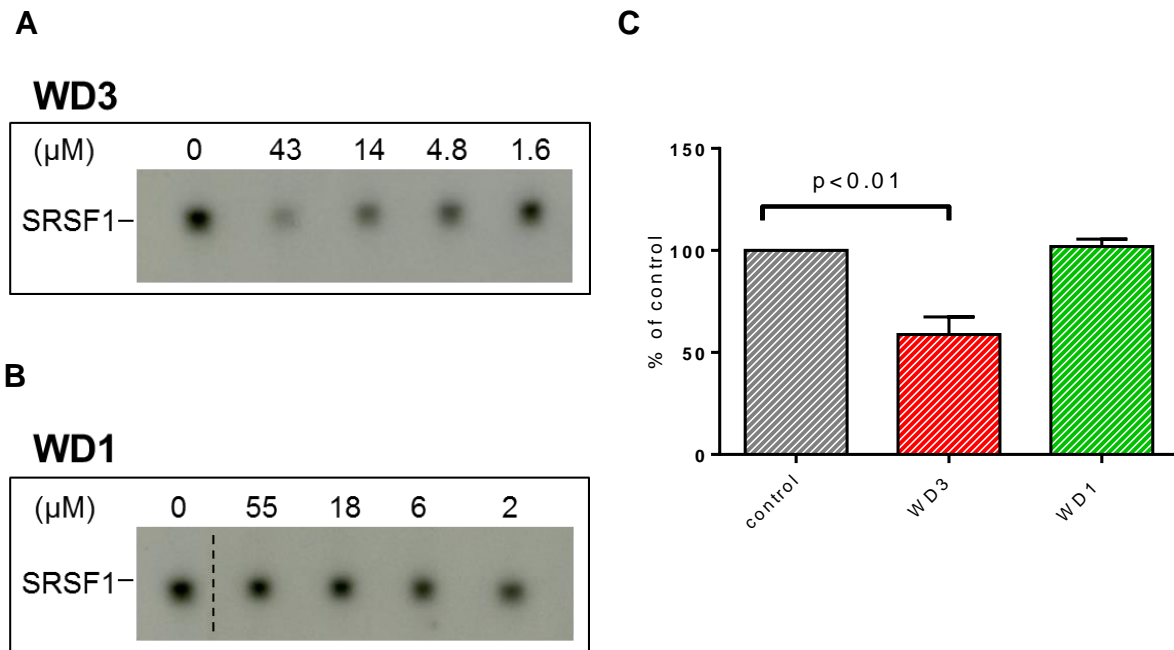
Figure 24: WD3 inhibits the phosphorylation of SRSF1 by GRK2

WD1 and WD3 were tested at different concentrations in phosphorylation assays using GRK2 and SRSF1 as a substrate.

Representative autoradiograms showing phosphorylation of SRSF1 in the presence of different concentrations of WD3 (A) and WD1 (B). (C) Statistical analysis of three independent experiments (WD3: 43 µM, WD1: 55 µM), (unpaired student's t-test).

6.4.2.2 An ELISA shows binding of SRSF1 to GRK2

Since SRSF1 is a novel substrate for GRK2 phosphorylation, the GRK2-SRSF1 protein interaction was investigated by ELISA technique. To this end, GRK2 purified from Sf9 cells infected with a GRK2-expressing baculovirus was coated at different concentrations and subsequently SRSF1 purified from bacterial culture was added at a constant concentration of 5 µg/ml. BSA was used as a control. Detection was performed using a primary mouse monoclonal antibody against SRSF1 (1/300) and a secondary

HRP-conjugated goat anti-mouse antibody (1/4000). The substrate reaction was allowed to proceed for 30 min, before the absorbance was read at 410 nm.

The results of the ELISA showed that SRSF1 shows a concentration-dependent interaction with GRK, whereas SRSF1 does not interact with BSA, which was used as a control protein.
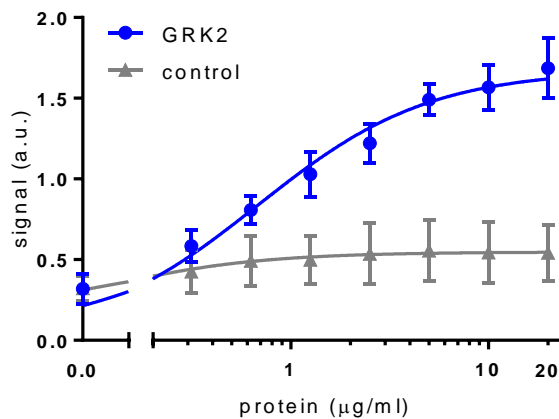
Figure 25: An ELISA shows binding of SRSF1 to GRK2

Binding of SRSF1 (5 µg/ml) to GRK2 or BSA, which was used as a control. Data of three independent experiments are displayed as mean ± SEM.

6.4.3 Inhibition of GRK2-mediated receptor desensitization

The bradykinin receptor B2 (B2R) is a ubiquitously expressed GPCR with roles in the cardiovascular system, pain modulation and airway function (244). In the renin-angiotensin-aldosterone system (RAAS), B2R plays an important counterpart to AT1R by acting as a vasodepressor through stimulation of NO release by eNOS (245).

Upon binding of the endogenous peptide-agonist bradykinin, B2R couples most prominently to $G\alpha_q$, thus eliciting a rise in the intracellular free Ca^{2+} concentration. Moreover, B2R has been also shown to couple to $G\alpha_i$ and $G\alpha_{12/13}$ (244).

Homologous desensitization of B2R may be initiated by various GRKs including GRK2 and GRK5 (246).

Fura-2 is a fluorescent dye used for measuring Ca^{2+} levels photospectrometrically (230). The acetoxymethylester derivative (Fura-2AM) is able to permeate the cell membrane. Intracellular esterases hydrolyse it to the membrane-impermeable Ca^{2+}-sensitive carboxylic form, Fura-2. Whereas the free dye shows an absorbance maximum at ~340 nm, binding of Ca^{2+} shifts it to ~380 nm (emission: 509 nm). Thus, alternating excitation at 340 nm and 380 nm allows the determination of the ratio between calcium-

free and calcium-bound Fura-2, thereby correcting for differences in dye concentration or loading efficiency (247).

6.4.3.1 Generation of plasmids expressing WD-Peptides

The WD-Peptides (WD1-3) were cloned by annealing of single-stranded oligonucleotides into pcDNA3 for expression in mammalian HEK293A cells. The identity and its correct insertion into pcDNA3 was confirmed by DNA sequencing (13.6, 13.7, and 13.8).

6.4.3.2 WD3 increases the bradykinin-stimulated Ca²⁺-signal in HEK293A cells

HEK293A cells were transiently transfected with 20 µg of plasmid DNA coding for WD1, WD2 or WD3, respectively. Cells transfected with pcDNA3 were used as a negative control and cells transfected with pcDNA3RKIP were used as a positive control (248). The rise in the free intracellular calcium concentration, $[Ca^{2+}]_i$, after stimulation of the endogenously expressed B2R with 100 nM bradykinin (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) was measured with Fura-2 and was expressed as fluorescence ratio at 340 nm and 380 nm. The maximum response after stimulation with bradykinin (as indicated by ΔI) was statistically analysed in comparison to the control (pcDNA3, $\Delta I = 0.43 \pm 0.06$).

Whereas expression of WD1 and WD2 showed no effect on the bradykinin-stimulated intracellular free Ca^{2+} concentration ($\Delta I = 0.49 \pm 0.11$, $p = 0.2112$, n=4, Figure 26: A, D; 0.41 ± 0.12, $p = 0.6155$, n=4, Figure 26: B, D) relative to the control, the expression of WD3 led to a significant increase in the bradykinin-stimulated intracellular $[Ca^{2+}]_i$ ($\Delta I = 0.59 \pm 0.10$, $p = 0.0159$, n=4, Figure 26: C, D). As a positive control, the dual-specific GRK2 and Raf-1 kinase inhibitor, RKIP, was used. The expression of RKIP also led to a significant enhancement of the bradykinin-stimulated Ca^{2+}-signal ($\Delta I = 0.51 \pm 0.05$, $p = 0.0081$, n=4, Figure 26: D).
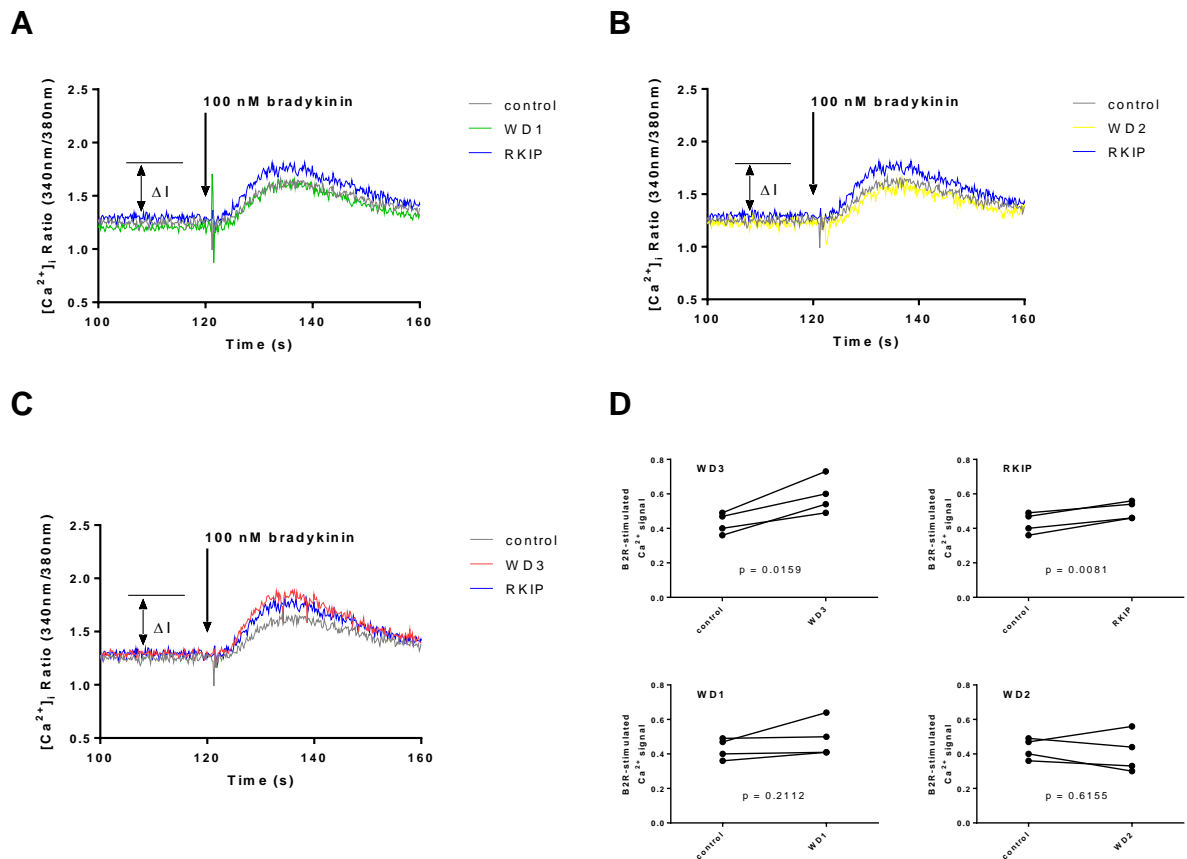
Figure 26: WD3 sensitizes the bradykinin-stimulated Ca^{2+} signal of endogenously expressed B2R

HEK293A cells were transiently transfected with plasmid DNA encoding WD1, WD2 or WD3 and stimulated with 100 nM of bradykinin. Cells transfected with pcDNA3 were used as a negative and cells transfected with pcDNA3RKIP as a positive control. (A-C) Representative calcium measurements of WD-expressing cells. (D) Statistical analysis of the maximum [Ca^{2+}]$_i$ peak (ΔI) of four independent experiments (paired student's t-test).

6.5 In vivo characterization of the GRK2-inhibitory peptide

6.5.1 Generation of a plasmid for cardiac-specific expression of WD3

For the generation of a transgenic mouse model with cardiac-specific expression of the WD3 peptide, the corresponding DNA was cloned into a vector containing the α-myosin heavy chain promoter, thereby restricting protein expression to cardiac tissue (249).

To this end, annealing of single-stranded oligonucleotides was performed and inserted into the SalI/HindIII cut α-MHC plasmid by a ligation reaction. Bacterial clones were screened by PCR using vector-specific primers (HGH2 and MHC2).
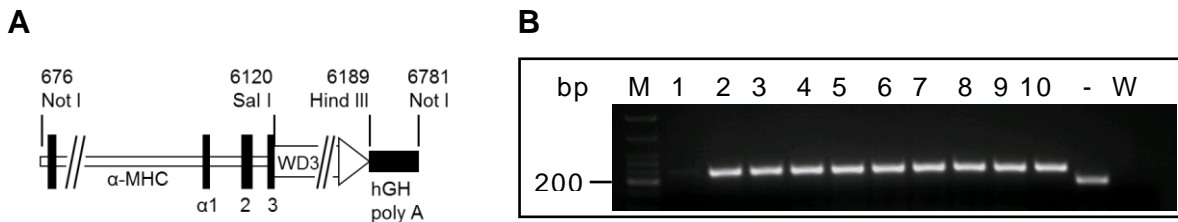
Figure 27: Generation of a plasmid coding for WD3 under the control of the α-myosin heavy chain (α-MHC) promoter

Schematic drawing of the vector construct (A). Single colony screening PCR shows the amplification of a ~250 bp fragment for samples 2-10, whereas the negative control (-) shows the amplification of an only ~130 bp long fragment (B). The observed difference reflects the amplification of the vector sequence without insert, which corresponds well with the fragment size of approximately 100 bp. The water control (W) is negative.
M, Marker

The DNA was sequenced (13.9) and endotoxin-free DNA was prepared from clone 2 (Figure 27) and subsequently linearized by restriction digestion with NotI.

6.5.2 Establishment of a transgenic mouse model

Introduction of the transgene into the mouse strain C57BL/6J was performed in the Molecular Pharmacology Unit of ETH Zurich, Switzerland by Dr. Joshua Abd Alla according to a method described elsewhere (173).

6.5.3 Genotyping of transgenic animals by PCR

Animals were genotyped by PCR using genomic DNA isolated from ear punch biopsies as template and vector-specific primers (HGH2 and MHC2). Mice were born at mendelian frequency (Figure 28).
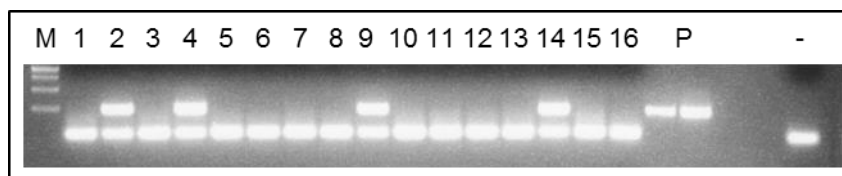
Figure 28: Identification of the α-MHCWD3 transgene in the genomic DNA by PCR

Isolated genomic DNA was amplified with vector-specific primers (HGH2 and MHC2) and separated on an agarose gel. M, Marker; P, positive control; -, negative control

6.5.4 The GRK2-inhibitory peptide shows a cardioprotective effect *in vivo*

To study the effect of WD3 on the development of cardiac hypertrophy, a model of chronic pressure overload induced by abdominal aortic constriction (AAC) was applied (250). In this model, pressure overload is induced by ligation of the aorta above the renal arteries (Figure 29, lower right panel), which leads to increased blood pressure proximal to the constriction and consequently chronic pressure overload-induced hypertrophy (Figure 29, lower left panel) (250). GRK2-inhibition has been shown to reduce the development of signs of cardiac dysfunction and heart failure after AAC (143).

Cardiac-specific expression of WD3 retarded the development of cardiac hypertrophy after AAC in comparison to a non-transgenic wild-type mouse (Figure 29, upper panel). This finding presents the first evidence of a cardioprotective effect of WD3 *in vivo*.
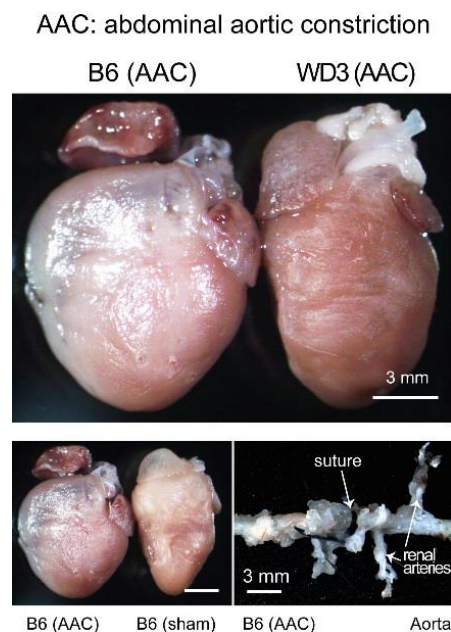
Figure 29: WD3 shows a cardioprotective effect in a pressure overload mouse model

WD3 retards AAC-induced cardiac hypertrophy compared to a wild-type control mouse (B6), upper panel. AAC induces chronic pressure overload and cardiac hypertrophy compared to sham-operated animals (sham), lower left panel. Suprarenal suture of the aorta, lower right panel. The aorta was dissected two weeks after ligation of the aorta above the suprarenal arteries.

7 Discussion

The ubiquitously expressed serine/threonine kinase GRK2 is a key player in many physiological processes (4.2.2). Particularly, GRK2 has been extensively studied for its role in phosphorylating GPCRs and thereby attenuating signalling events in cooperation with arrestins (14,110). Recently, GRK2 phosphorylation of cytosolic non-receptor substrates as well as kinase-independent functions have gained increasing attention (251). Furthermore, GRK2 has been implicated in such varied conditions as heart failure, diabetes, inflammation and cancer (131). Although efforts towards the finding of a GRK2-inhibitor have been ongoing for more than 25 years (198) and, in the case of heart failure, progressed as far as studying the effects in a large animal model (141), no compound has reached clinical trials yet. Thus, there is still a need for new inhibitors.

The modular structure of GRK2 allows for inhibition by targeting any of the three domains. However, the diverse GRK2 effector roles are affected differently depending on the approach chosen. Firstly, for the N-terminal domain containing the receptor recognition site, RKIP has been shown to interfere with the phosphorylation of receptor substrates (168,170). Secondly, kinase domain inhibitors such as paroxetine are expected to prevent phosphorylation of all GRK2 substrates. Thirdly, the GRK2-inhibitory potential of interfering with the Gβγ-mediated GRK2 activation and membrane translocation has been extensively studied with the help of the GRK2-derived peptide βARKct (183,184,239). Nevertheless, the mechanism of action is probably more accurately described as an inhibition of Gβγ effector functions, one of which is the said activity towards GRK2. It has to be remembered, however, that some of the beneficial effects observed with βARKct are thought to be GRK2-independent (186,238). It follows, that βARKct and small molecules employing the same inhibitory mechanism (192), are inadequate for studying the outcome of targeting the C-terminal domain of GRK2 with regard to substrate-specificity and kinase-independent effects. Still, inhibiting the Gβγ-GRK2CTD interaction is a well-established approach to GRK2-inhibition. Moreover, it confers specificity towards members of the βARK-subfamily, as membrane localization for the other isoforms employs different mechanisms (98,106,108,109).

In the presented study, direct targeting of GRK2CTD was investigated as a hitherto unexplored approach to GRK2-inhibition. More specifically, peptides interacting with GRK2CTD were sought for subsequent characterization of their inhibitory properties.

Small molecules have historically been preferred as pharmacologically active substances due to their advantageous pharmacokinetic features, good oral availability and the ease of formulation. However, a substantial proportion of the GRK2-inhibitors studied so far are peptides or proteins (RKIP, βARKct, the various peptide inhibitors described in

chapter 4.4.4) indicating that this could be a promising approach. In addition, interfering with Gβγ-mediated kinase activation requires targeting of a protein-protein interaction site, which is known as a challenging task (252). Peptides are considered to have the potential for effectively blocking protein-protein interactions (PPIs) (201,207,253). PPI interfaces are characterized by a wider contact area compared to protein-small molecule interfaces. These generally contain so-called "hot spots", which are characterized by facilitating high-affinity interactions (254). Intriguingly, phage display library screening has been reported to preferentially identify these hot-spots, making it a useful technique in the search for inhibitors of PPIs (254).

7.1 Identification of a GRK2-interacting peptide

7.1.1 Protein expression and purification of GRK2-domains

For the expression of recombinant GRK2 domains, a bacterial protein expression system was chosen (227). Generally, bacterial expression of recombinant proteins generates high yields of protein for subsequent purification. Disadvantages include protein aggregation and the formation of inclusion bodies, which require harsher conditions for purification. Moreover, the formation of the native conformation can be hindered (255). For the purpose of this study, namely the expression of GRK2 domains for a peptide phage display library screen and protein-protein interactions studies by ELISA technique, neither activity nor the native conformation was required.

In this study, protein expression levels of GRK2KD and GRK2CTD were high, as revealed by SDS-PAGE. Although expression of the C-terminally His-tagged GRK2CTD was lower than that of the N-terminally His-tagged protein. Other groups have reported that the position of the His-tag may affect protein yield (256) and stability (257). The protein expression level of GRK2NTD was rather low, which could reflect instability of the protein. The expressed GRK2-domains could be purified by metal affinity chromatography as evaluated by SDS-PAGE.

7.1.2 A phage display library screen identifies several peptides

The purified C-terminally His-tagged GRK2CTD was used as bait in a peptide phage display library screen, which yielded six 7-mer peptide sequences (Table 7). Two of the peptide sequences (HYIDFRW and HYTDFRW) were identical except for one position. However, as these sequences reappeared in subsequent library screens using different bait-proteins, they unlikely display a consensus sequence, which specifically interacts with a certain protein.

7.1.3 A protein database search was performed for the peptides

A BLAST search for the identified peptide sequences was performed. A BLAST analysis of phage-display derived peptides may yield novel protein interaction partners or inhibitors of protein-protein interactions (222,223).

Peptide IRYVPQT shows sequence homology with cadherin 20

For the sequence IRYVPQT, sequence homology with cadherin 20 was revealed in the BLAST analysis (Table 8).

Cadherin 20 has been identified as a member of the cadherin family of cell-cell adhesion molecules. Cadherins contain extracellular Ca^{2+}-binding domains, transmembrane domains and cytoplasmic domains interacting with intracellular proteins linking them to the cytoskeleton (258). As no further experiments were performed with this peptide, the implications of the finding is not clear.

Peptide IVSLQTP shows sequence homology with NFAT

The BLAST analysis for the peptide sequence IVSLQTP revealed sequence homology with NFAT (Table 8).

The nuclear factor of activated T-cells, cytoplasmic 3 (NFATc3) has recently gained attention as an interaction partner of GRK5.

Gαq, a mediator of cardiac hypertrophic signalling (259,260), stimulates a rise in the intracellular calcium level, thereby promoting calmodulin-mediated nuclear translocation of GRK5 (261). Nuclear accumulation of GRK5 in turn promotes transcription of hypertrophic genes by (i) phosphorylation of histone deacetylase 5 (HDAC5) inducing de-repression of the myocyte enhancer factor 2 (MEF2) (262) and (ii) enhancing DNA binding of NFAT in a kinase-independent manner (261,263).

For GRK2, however, neither nuclear localization (107) nor interaction with NFAT (263) have been reported so far. Thus, the significance of finding a peptide derived from NFAT through a phage display library screen using the GRK2CTD as bait, is unclear.

Moreover, the NFAT-isoform (NFATc2) identified in the BLAST analysis with homology to the peptide sequence IVSLQTP, differs from the one reported to interact with GRK5 (NFATc3).

Peptide ASTLIVF shows sequence homology with WDR76

For the peptide sequence ASTLIVF, the BLAST analysis showed sequence homology with WDR76 (Table 8).

WD repeat-containing proteins are characterized by a series of repetitive approximately 40 amino acids long sequences with a signature C-terminal WD (tryptophan, aspartic acid) dipeptide. WD domains were first identified in the β-subunit of bovine transducin, the main G protein in retinal cells (36,264). Solving of the crystal structure of the

prototypical WD repeat-containing protein Gβγ revealed a seven-bladed β-propeller structure (Figure 30, lower middle panel) (36,265). The propeller blades consisting of four anti-parallel β-strands display a toroidal shape building a funnel (Figure 30, lower left panel). One WD repeat spans strand D of one blade and strands A, B and C of the following blade (Figure 30, lower right panel). Hence, a WD repeat does not correspond to a single blade of the propeller structure. The result of this unique architecture is a highly symmetrical protein stabilized by numerous hydrogen bonds and displaying multiple surfaces for protein-protein interaction (235).
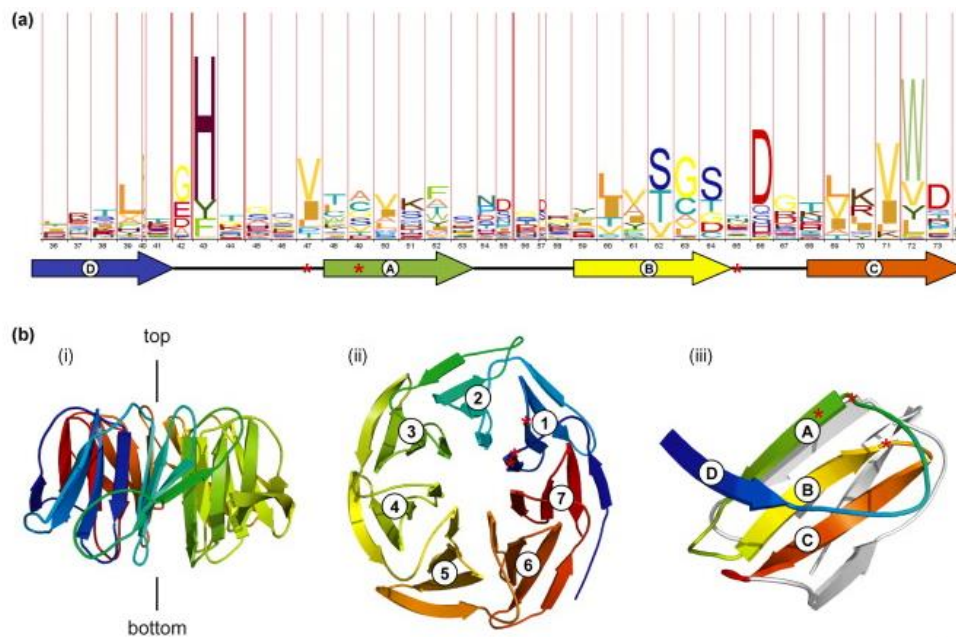
Figure 30: Sequence and structure of WD repeat-containing proteins
WD repeat-containing proteins consist of ~40 amino acid spanning sequences with a characteristic C-terminal WD-dipeptide (upper panel). The highly rigid tertiary protein structure builds a funnel-like shape (lower left panel). A seven-bladed β-propeller structure is found most commonly for WD repeat-containing proteins lower middle panel). One WD repeat consists of strand D of one blade and strands A-C of the next blade (lower right panel) (235).

Interestingly, whereas secondary and tertiary protein structures are rigidly defined among WD repeat-containing proteins, the primary amino acid sequence is highly variable making the identification of a protein as a WD repeat-containing protein somewhat challenging. Very few amino acid residues are conserved such that e.g. the name-giving WD-dipeptide is not a strict requirement. Still, some key elements are frequently found (Figure 30, upper panel): (i) a GH (glycine, histidine) dipeptide at the C-terminal end of strand D, (ii) three amino acids with small side chains (serine, glycine) at the C-terminal

end of strand B, (iii) and an aspartic acid residue six amino acids before the signature WD dipeptide (235,264,266).

WD repeat-containing proteins are known to interact with PH domains, as e.g. Gβγ interacts with GRK2 via its PH domain (267). Moreover, Gβγ was also found to interact with other WD repeat-containing proteins, such as RACK1 or WDR26 (268,269). The characteristic β-propeller architecture forms multiple possible protein interactions surfaces on the bottom, at the top and at the sides (268). Thus, a role for WD repeat-containing proteins in scaffolding protein assemblies was proposed (270).

The human WD repeat-containing protein 76 (WDR76) displays the characteristic seven-bladed tertiary protein structure. It was shown to interact *in vivo* with CUL4-DDB1 ubiquitin ligase, for which other WD repeat-containing proteins serve as molecular adaptors mediating substrate targeting (271). The WDR76 orthologue from *Saccharomyces cerevisiae*, Cmr1, in cooperation with other proteins was found to be involved in maintenance of genome integrity. More specifically, it mitigates stress-induced DNA damage during replication (272).

7.1.4 Sequence elongation of the peptide sequence ASTLIVF

The approach of elongating the peptide sequence ASTLIVF according to the homologous sequence of WDR76 was based on the structural relatedness of WDR76 and Gβγ. Moreover, targeting of PPIs with small molecules is challenging, because of the necessity to cover a larger protein interface. Thus, it could be that medium-sized compounds (1000-2000 Da) are better suited for inhibition (207).

In addition, the GRK2-inhibitory peptides reported so far are roughly between 14 and 28 amino acids in length (174,187,188,195). For the PepInh, truncation even resulted in the loss of activity (194). This could indicate a requirement for a longer sequence for GRK2-inhibition.

7.1.5 A GRK2-interacting peptide is identified by ELISA

Binding ELISAs and competition ELISAs are versatile methods to study PPIs (273).

In the presented study, ELISA technique was chosen to examine the interaction of GRK2CTD with the elongated peptides WD1 or WD3, respectively. GRK2CTD was shown to interact with WD3 (Figure 21: A), whereas the interaction with WD1 was not significant (Figure 21: B). Interestingly, both peptides, WD1 and WD3, contain the sequence identified in the phage display library screen (ASTLIV). However, WD3 in comparison to WD1 contains more (five vs. four) charged amino acids, which could facilitate electrostatic interactions with GRK2CTD. Similarly, the binding of βARKct and Gβγ has been proposed to rely on electrostatic interactions (188). To rule out unspecific binding of the GRK2CTD to WD3, a WD3-scrambled sequence could be tested for its interaction with GRK2CTD. The differences in charge between the two peptides, WD1 and WD3,

could also affect the coating efficiency. Coating of equal amounts of peptides could be confirmed by detection with a suitable antibody.

Taken together, GRK2CTD binds to WD3, which is an elongated version of a peptide identified in a phage display library screen.

7.2 The GRK2-interacting peptide shows GRK2-inhibitory features *in vitro*

To characterize the GRK2-inhbitory features of the elongated peptides, three different *in vitro* assays were performed. First, the effect of the peptides on the Gβ-GRK2CTD protein-protein interaction, which is essential for kinase activation and translocation to the plasma membrane, was studied. Second, an *in vitro* phosphorylation assay was chosen to assess, whether the peptides have any influence on the kinase activity. Third, the impact of the peptides on the desensitization of the endogenously expressed bradykinin receptor type 2 was investigated in transiently transfected HEK cells.

7.2.1 The GRK2-inhibitory peptide interferes with the Gβ-GRK2CTD interaction

In this study, binding of the GRK2CTD to both Gβ3 and Gβ3-s could be revealed by ELISA technique (Figure 22). This finding is in line with other studies reporting an interaction of GRK2 with Gβγ in general (38,187), and Gβ3 and Gβ3-s specifically (274).

Interestingly, a Gβ3-polymorphism (C825T) linked to the increased occurrence of the shorter splice variant (Gβ3s) (275,276), was found to be associated with hypertension. For this shorter splice variant, Gβ3-mediated ubiquitination of GRK2 is impaired leading to protein accumulation. Enhanced GRK2 levels, in turn, explain the association with a hypertensive state (274).

One group identified the GRK2-inhibitory compound M119 in a competition ELISA with Gβγ and SIGK, a peptide showing a high affinity for Gβγ (191). This indicates the applicability of a competition ELISA for the identification of compounds interfering with specific protein-protein interactions.

Here, it was shown that WD3 significantly interferes with the Gβ3-GRK2CTD interaction in a competition ELISA (Figure 23: A). For the shorter splice variant, Gβ3-s, a trend towards disruption of the Gβ-GRK2CTD interaction could be observed, which was not significant (Figure 23: B). WD1, in contrast did neither influence the binding of GRK2CTD to Gβ3 nor Gβ3-s (Figure 23: A, B).

The different effects observed for Gβ3 and Gβ3-s could be attributed to variations in coating efficiency. Equal coating could be confirmed by immunochemical detection. Moreover, the seven-bladed propeller structure found most often in WD repeat-containing proteins is predicted to be most stable (277). Gβ3-s, however, lacks around 40 amino acids (275), which could affect the stability of the protein and consequently the outcome of the assay. The high standard deviation as observed for the competition assay with GRK2CTD and Gβ3-s (Figure 22) could be an indication for such unstable experimental conditions.

Nevertheless, the results presented here indicate that WD3 interferes with the Gβγ-GRK2CTD interaction essential for kinase activation.

7.2.2 The GRK2-inhibitory peptide inhibits GRK2 activity in an *in vitro* phosphorylation assay

Several studies have used *in vitro* phosphorylation assays to investigate the kinase activity of GRK2 in the presence and/or absence of specific compounds (175,178,278). GRK2 phosphorylates a variety of different substrates, most prominently GPCRs (110). In addition, there is an increasing number of studies reporting cytosolic non-receptor substrates of GRK2 (85).

In the presented study, SRSF1, a novel substrate of GRK2 (Quitterer, U., unpublished data) has been used to investigate the impact of the two peptides (WD1 and WD3) on the activity of GRK2. WD3 significantly reduced the phosphorylation of SRSF1 by GRK2, which indicates an inhibition of the kinase function. In contrast, WD1 did not influence the activity of GRK2 towards SRSF1. Remarkably, the potency of WD3 is comparable to paroxetine (Quitterer, U., unpublished data), which has recently been identified as a selective GRK2-inhibitor (178).

However, the inhibitory effect of WD3 in this system was not expected. The GRK2-derived peptide (amino acids 643-670) containing the 'Gβγ binding domain' has been shown to inhibit Gβγ-mediated GRK2-activity towards a receptor substrate (187). In this study, WD3 has been shown to interfere with the Gβ-GRK2CTD interaction (Figure 23). To test such a mode of action, a reconstituted system containing a membrane-embedded receptor and Gβγ would be required (187). The *in vitro* phosphorylation setup used here lacks both Gβγ and a receptor substrate. Nevertheless, the effect observed could be explained by competition of WD3 with the chosen substrate SRSF1 for binding to GRK2. Thus, it would be interesting to perform the phosphorylation assay using different substrates, particularly a receptor substrate. However, receptors are hydrophobic and inherently expressed at low levels, making the experimental procedure more challenging.

Moreover, selectivity of the peptide towards other kinases should be addressed in further experiments. Targeting of GRK2CTD as an allosteric site would be expected to alleviate the problem of unselective kinase-inhibition, which stems from a high sequence conservation of the ATP-binding site (279). Nevertheless, testing of other kinases, e.g. of the AGC kinase family or its subfamily of GRKs, would be desirable, as it could also support the proposed mode of action.

7.2.3 The GRK2-inhibitory peptide inhibits desensitization of the GPCR B2R

The B2R, upon agonist-stimulation with bradykinin, couples to $G\alpha_q$ (280), which leads to increased intracellular calcium levels (281). GRK2 has been reported to phosphorylate B2R in human fibroblasts (282) and to impair agonist-stimulated B2R-signalling as indicated by reduced intracellular calcium levels (283). Moreover, a study using the GRK2-inhibitor RKIP showed enhanced calcium signalling in HEK cells upon stimulation of B2R with its cognate ligand bradykinin (248). B2R is endogenously expressed in HEK cells (281), hence ensuring a stable experimental system to study GRK2-inhibitory effects.

In the presented study, the bradykinin-stimulated rise in the intracellular free calcium concentration in HEK cells was used to investigate the GRK2-inhibitory effect of the transiently expressed peptides WD1, WD2 and WD3. Of the three peptides, only WD3 led to a significantly enhanced maximum calcium signal of agonist-stimulated B2R (Figure 26). These results indicate that WD3 inhibits GRK2-mediated attenuation of bradykinin-stimulated calcium signalling (desensitization).

One study showed that downregulation of endogenous RKIP expression levels using a miRNA diminished the GRK2-inhibitory capacity of RKIP (248). In the presented study, equal amounts of DNA were transfected. However, peptide expression levels were not assessed. Thus, the GRK2-inhibitory effect observed with WD3 in comparison to WD1 and WD2 could also be a result of different peptide expression levels. Immunoblot detection of the peptide expression levels could exclude such an effect.

GRK2 has also been reported to regulate β2AR (284) and α2AR (137), which are both known to couple to $G\alpha_s$ and $G\alpha_i$ (45,46). Moreover, GRK2 upregulation has been shown to contribute to the impaired signalling of these receptors in heart failure (130,137). Therefore, elucidation of the effects of the peptides (WD1, WD2 and WD3) on the desensitization of these $G\alpha_{s/i}$-coupled receptors would be interesting.

7.3 The GRK2-interacting peptide WD3 shows GRK2-inhibitory features *in vivo*

To investigate the *in vivo* effects of the peptide WD3, a transgenic mouse model with cardiac-specific expression of the peptide sequence was established (Figure 28). These mice were then subjected to AAC as an animal model of chronic pressure overload-induced heart failure (143). WD3 retarded the development of cardiac deterioration as compared with a non-transgenic wild-type control mouse.

This result is in agreement with the effects observed in a study using PepInh, a peptide GRK2-inhibitor derived from the first intracellular loop of the hamster β2AR. Mice with cardiac-specific overexpression of PepInh, showed an improved heart weight to body weight ratio compared to wild-type mice after long-term AAC. Furthermore, their heart function was improved as indicated by measuring the left-ventricular ejection fraction (143).

The transgenic mouse line with cardiac-specific expression of WD3 can be utilized to characterize the *in vivo* effects of WD3 further. Protein expression levels could be assessed by immunoblot detection. Moreover, gene expression profiling could reveal alterations in metabolic or growth-promoting pathways, which have been shown to be affected by GRK2-inhibition in previous studies (143,173).

7.4 Evaluation of the GRK2-inhibitory peptide

7.4.1 Potency and selectivity

The potency of WD3, the GRK2-inhibitory peptide development in this study, was not determined. Nevertheless, the results obtained in the *in vitro* phosphorylation assay indicate an effect comparable to paroxetine (Figure 24 and Quitterer, U., unpublished data). Moreover, the GRK2-inhibitory effect of WD3 is sufficient to exert a cardioprotective effect in an *in vivo* pressure overload model of heart failure.

Another point, which was not addressed in the presented work, is the selectivity of WD3. As mentioned before, targeting of the GRK2CTD is expected to confer selectivity within the GRK-family, because only the members of the βARK-subfamily (GRK2 and GRK3) are activated by Gβγ (100,101). Whereas GRK2 has been widely studied, less is known about the (patho)physiological role of GRK3. In contrast to GRK2 gene ablation, GRK3-deficient mice develop normally, except for a loss of odorant-receptor desensitization. This is probably a result of the fact that GRK3 is expressed at high levels in the olfactory epithelium (285). The other ubiquitously expressed GRK-isoforms are GRK5 and GRK6 (85). GRK5, the other major GRK-isoform in the heart, is also upregulated in heart failure and thought to contribute to heart failure (286). Thus, selectivity within the GRK-family might not be a requirement, at least not for the treatment of heart failure.

7.4.2 Mechanism of action

The methodical approach outlined in the presented study assumes a mode of action, by which the peptide, WD3, interferes with the Gβγ-mediated activation of GRK2. In this study, the competition ELISA with Gβ3 and GRK2CTD supports such an inhibitory mechanism (6.4.1.2). Interestingly, the common binding site shared by Gβγ effectors identified in one study, suggests that mostly hydrophobic interactions are critical for Gβγ-effector interactions (188). WD3 encompasses a hydrophobic region, which backs the effect observed in the competition ELISA. However, electrostatic interactions have been proposed to underlie the binding of βARKct to Gβγ (188). A role for charged amino acids in GRK2-inhibition is also supported by studies with anionic GRK2-inhibitors (96,198). Elongation of the original peptide-sequence identified in the phage display library screen added negatively charged amino acids (6.3.2), which would be in line with the proposed mode of action of these inhibitors (e.g. heparin). To find out, which residues are essential for the GRK2-inhibitory effect of WD3, more studies are needed.

Additionally, the C-terminal domain of GRK2 also contributes to enzyme activation by interacting with anionic phospholipids of the membrane (100,105). Moreover, agonist-occupied GPCRs are potent allosteric activators of GRK activity by inducing a transition toward an active closed conformation. Mutations in the C-terminal tail impaired receptor phosphorylation, suggesting a role for GRK2CTD in receptor-mediated kinase activation (287). Therefore, WD3 as a GRK2CTD-interacting peptide (6.3.3) could also interfere with these mechanisms of activation.

However, the surprising finding, that WD3 inhibits GRK2-mediated phosphorylation of the soluble substrate SRSF1 (Figure 24) is not sufficiently clarified by the above suggested mechanisms of action. WD3 might interfere with the GRK2-SRSF1 interaction, which would explain the results presented in this study. This would be intriguing, because there are little data on the interaction of GRK2 with soluble substrates with regard to the specific sites of interaction. Receptors are known to interact via the GRK2NTD, an interaction blocked by RKIP (91,168). In recent years, there has been an increasing interest in GRK2 non-receptor substrates (85). Thus, substrate-specific inhibition of GRK2-mediated phosphorylation would certainly be of value. However, further studies are needed to decipher the substrate-specificity of WD3-mediated GRK2-inhibition.

7.4.3 Safety of GRK2-inhibition

Systemic and organ-specific side effects of inhibiting the ubiquitously expressed GRK2 need to be addressed.
Even more so considering that GRK2 gene ablation results in embryonic lethality (161). As cardiomyocyte-specific gene ablation produces viable offspring with normal heart structure and function, embryonic lethality in GRK2 gene ablated mice is probably due to

extra cardiac effects (162). Nevertheless, an increased prevalence of cardiac congenital defects has been found to be associated with paroxetine use during the first trimester of pregnancy (288), underscoring the role of GRK2 in cardiac development.

Concerns regarding a long-term enhancement of β-adrenergic signalling stem from animal models of βAR-overexpression (289,290). These mice presented with a phenotype marked by increased cardiac contractility, which later developed into heart failure (289). Furthermore, adrenergic stimulation is inherently toxic to the heart and promotes apoptosis (291).

This problem could be overcome by a time-restricted short-term application after myocardial infarction to halt the development of maladaptive processes. Such an approach was taken by one group studying the effects of adenovirus-mediated βARKct gene delivery in a large animal model (141). Another possibility would be to limit the use to acute heart failure, as is already the current treatment regimen for inotropic drugs (132). Furthermore, paroxetine has been marketed in 1992 and shows a safety profile comparable to other antidepressants (292). This supports data from GRK2$^{+/-}$ mice, which develop normally and display improved cardiac function (138).

7.5 Outlook

7.5.1 Further development of the prototype

As mentioned before, peptide drugs generally display an unfavourable pharmacokinetic profile (4.5.1). However, there are several options for improving the pharmacokinetic and –dynamic profile of WD3. An alanine-scan could identify amino acid residues, which are essential for the GRK2-inhibitory effect of WD3 (197) and thus, guide approaches towards truncation, introduction of (un)natural amino acids or N-methylation. However, truncation might also results in loss of activity as observed for the GRK2-inhibitory peptide, PepInh (194).

Moreover, cyclisation of the peptide structure could be attempted. Due to their more rigid secondary structure, cyclic peptides tend to display higher affinity for their target proteins. However, cyclisation might also result in loss of activity. Protein-protein interactions are often hydrophobic in nature and do not display highly rigid stereochemical requirements (201). Therefore, they can be accommodated by a number of peptide conformations and sequences. A more rigid peptide conformation might still not fit into the protein-protein interface.

Alternatively, a competition screen, as was performed for the C13 aptamer and identified paroxetine as a GRK2-inhibitor (178), could be performed with WD3 in a similar manner.

7.5.2 Research Applications

The novel approach of targeting the GRK2CTD identified the GRK2-interacting peptide WD3 (Figure 21). In contrast to the compounds interfering with the Gβγ-GRK2 interaction

(βARKct, M119, gallein), WD3, by directly binding Gβγ, might help in elucidating GRK2-specific Gβγ-effector functions. Moreover, WD3 could help in identifying the GRK2-interaction site of known interaction partners or substrates.

8 Conclusion

GRK2 has been implicated as a drug target in a variety of conditions, most importantly for the treatment of heart failure. Efforts towards a clinically approved drug, however, have been unsuccessful to date.

In the presented study, a novel approach is taken by direct targeting of the C-terminal domain of GRK2, thus potentially interfering with Gβγ-mediated kinase activation. Screening of a peptide phage display library and subsequent modification of the most promising candidate, led to the development of a peptide inhibitor prototype (WD3), which shows GRK2-inhibitory features in several *in vitro* assays. Moreover, cardiac-specific expression of the WD3 peptide sequence exerts a cardioprotective effect in an animal model of chronic pressure overload.

Thus, the WD3 peptide could serve as lead structure for the development of a more potent and drug-like compound. The WD3-peptide could also prove useful in elucidating GRK2-mediated Gβγ effector functions, as it specifically inhibits the Gβγ-GRK2 interaction.

However, further research is needed to characterize the interaction of WD3 with GRK2 and the *in vivo* effects in more detail.

9 Acknowledgments

10 Curriculum vitae

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11 Publications

Patent application

Abd Alla, J., Graemer, M., Wolf, S., and Quitterer, U. (2016), Invention of a prototype, which is cardioprotective by inhibition of GRK2-mediated phosphorylation of a novel non-receptor GRK2 substrate, the Serine/arginine-rich splicing factor 1 (SRSF1), patent application approved by ETH Transfer (ETH 2016-138)

Journal article

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13 Appendix

13.1 Sequencing of GRK2NTD6xCHis

Sequencing of pET3dGRK2NTD6xCHis (amino acids 1-190 of GRK2), clone 6

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NATTTTGTTTACTTTAAGAAGGAGATATACCATGGCCGCGGACCTGGAGGCGGTGCTGGCCGACGTGAGCTACCTGATGGC
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GCAGAAGTACCTGGAGGACCGGGGCGAGGTGACCTTTGAGAAGATCTTTTCCCAGAAGCTGGGGTACCTGCTCTTCCGAGA
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GGAGGAGGAGCGTGTGGCCCGCAGCCGGGAGATCTTCGACTCATACATCATGAAGGAGCTGCTGGCCTGCTCGCATCCCTT
CTCGAAGAGTGCCACTGAGCATGTCCAAGGCCACCTGGGGAAGAAGCAGGTGCCTCCGGATCTCTTCCAGCCATACATCGA
AGAGATTTGTCAAAACCTCCGAGGGGACGTGTTCCAGAAATTCATTGAGAGCGATAAGTTCACACGGTTTTGCCAGTGGAA
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GAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGG
GTTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCACAGGACGGGTGTGGTCGCCATGATCGCGTAGTCGATAGTGGCT
CCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGTGCTCCGAGAACGGGTGCGCATAGAAATTGC
ATCAACGCATATAGCGCTAGCAGCACGCCATAGTGACTGGCGATGCTGTCGGAATGGACGATATCCCGCAAGAGGCCCGGC
AGTACCGGCATAACCAAGCCTATGCCTACAGCATCCAGGG
```

CCATGG: NcoI restriction site

ATG: Start codon

XXX: hexahistidine tag

TAA: Stop codon

GGATCC: BamHI restriction site

```
Query   4    GCGGACCTGGAGGCGGTGCTGGCCGACGTGAGCTACCTGATGGCGATGGAGAAGAGCAAG   63
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct   270  GCGGACCTGGAGGCGGTGCTGGCCGACGTGAGCTACCTGATGGCCATGGAGAAGAGCAAG   329

Query   64   GCCACGCCGGCCGCGCGCGCCAGCAAGAAGATACTGCTGCCCGAGCCCAGCATCCGCAGT   123
             |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct   330  GCCACGCCGGCCGCGCGCGCCAGCAAGAAGATCCTGCTGCCCGAGCCCAGCATCCGCAGT   389

Query   124  GTCATGCAGAAGTACCTGGAGGACCGGGGCGAGGTGACCTTTGAGAAGATCTTTTCCCAG   183
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   390  GTCATGCAGAAGTACCTGGAGGACCGGGGCGAGGTGACCTTTGAGAAGATCTTTTCCCAG   449

Query   184  AAGCTGGGGTACCTGCTCTTCCGAGACTTCTGCCTGAACCACCTGGAGGAGGCCAGGCCC   243
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   450  AAGCTGGGGTACCTGCTCTTCCGAGACTTCTGCCTGAACCACCTGGAGGAGGCCAGGCCC   509

Query   244  TTGGTGGAATTCTATGAGGAGATCAAGAAGTACGAGAAGCTGGAGACGGAGGAGGAGCGT   303
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   510  TTGGTGGAATTCTATGAGGAGATCAAGAAGTACGAGAAGCTGGAGACGGAGGAGGAGCGT   569

Query   304  GTGGCCCGCAGCCGGGAGATCTTCGACTCATACATCATGAAGGAGCTGCTGGCCTGCTCG   363
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   570  GTGGCCCGCAGCCGGGAGATCTTCGACTCATACATCATGAAGGAGCTGCTGGCCTGCTCG   629

Query   364  CATCCCTTCTCGAAGAGTGCCACTGAGCATGTCCAAGGCCACCTGGGGAAGAAGCAGGTG   423
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   630  CATCCCTTCTCGAAGAGTGCCACTGAGCATGTCCAAGGCCACCTGGGGAAGAAGCAGGTG   689

Query   424  CCTCCGGATCTCTTCCAGCCATACATCGAAGAGATTTGTCAAAACCTCCGAGGGGACGTG   483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   690  CCTCCGGATCTCTTCCAGCCATACATCGAAGAGATTTGTCAAAACCTCCGAGGGGACGTG   749

Query   484  TTCCAGAAATTCATTGAGAGCGATAAGTTCACACGGTTTTGCCAGTGGAAGAATGTGGAG   543
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   750  TTCCAGAAATTCATTGAGAGCGATAAGTTCACACGGTTTTGCCAGTGGAAGAATGTGGAG   809

Query   544  CTCAACATCCACCTGACCATGAATGAC   570
             |||||||||||||||||||||||||||
Sbjct   810  CTCAACATCCACCTGACCATGAATGAC   836
```

13.2 Sequencing of 6xNHisGRK2KD

Sequencing of pET3d6xNHisGRK2KD (amino acids 191-453 of GRK2), clone 2

```
MMNGGTTCTCTGATATTTTGTTTACTTTAAGAAGGAGATATACCATGGCCCATCACCATCACCATCACTTCAGCGTGCATC
GCATCATTGGGCGCGGGGGCTTTGGCGAGGTCTATGGGTGCCGGAAGGCTGACACAGGCAAGATGTACGCCATGAAGTGCC
TGGACAAAAAGCGCATCAAGATGAAGCAGGGGGAGACCCTGGCCCTGAACGAGCGCATCATGCTCTCGCTCGTCAGCACTG
GGGACTGCCCATTCATTGTCTGCATGTCATACGCGTTCCACACGCCAGACAAGCTCAGCTTCATCCTGGACCTCATGAACG
GTGGGGACCTGCACTACCACCTCTCCCAGCACGGGGTCTTCTCAGAGGCTGACATGCGCTTCTATGCGGCCGAGATCATCC
TGGGCCTGGAGCACATGCACAACCGCTTCGTGGTCTACCGGGACCTGAAGCCAGCCAACATCCTTCTGGACGAGCATGGCC
ACGTGCGGATCTCGGACCTGGGCCTGGCCTGTGACTTCTCCAAGAAGAAGCCCCATGCCAGCGTGGGCACCCACGGGTACA
TGGCTCCGGAGGTCCTGCAGAAGGGCGTGGCCTACGACAGCAGTGCCGACTGGTTCTCTCTGGGGTGCATGCTCTTCAAGT
TGCTGCGGGGGCACAGCCCCTTCCGGCAGCACAAGACCAAAGACAAGCATGAGATCGACCGCATGACGCTGACGATGGCCG
TGGAGCTGCCCGACTCCTTCTCCCCTGAACTACGCTCCCTGCTGGAGGGGTTGCTGCAGAGGGATGTCAACCGGAGATTGG
GCTGCCTGGGCCGAGGGGCTCAGGAGGTGAAAGAGAGCCCCTTTTTCTAAGGATCCGGCTGCTAACAAAGCCCGAAAGGAA
GCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
CTGAAAGGAGGAACTATATCCGGATATCCACAGGACGGGT
```

CCATGG: NcoI restriction site

ATG: Start codon

XXX: hexahistidine tag

TAA: Stop codon

GGATCC: BamHI restriction site


```
Query  67    ACTTCAGCGTGCATCGCATCATTGGGCGCGGGGGCTTTGGCGAGGTCTATGGGTGCCGGA  126
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835   ACTTCAGCGTGCATCGCATCATTGGGCGCGGGGGCTTTGGCGAGGTCTATGGGTGCCGGA  894

Query  127   AGGCTGACACAGGCAAGATGTACGCCATGAAGTGCCTGGACAAAAAGCGCATCAAGATGA  186
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  895   AGGCTGACACAGGCAAGATGTACGCCATGAAGTGCCTGGACAAAAAGCGCATCAAGATGA  954

Query  187   AGCAGGGGGAGACCCTGGCCCTGAACGAGCGCATCATGCTCTCGCTCGTCAGCACTGGGG  246
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  955   AGCAGGGGGAGACCCTGGCCCTGAACGAGCGCATCATGCTCTCGCTCGTCAGCACTGGGG  1014

Query  247   ACTGCCCATTCATTGTCTGCATGTCATACGCGTTCCACACGCCAGACAAGCTCAGCTTCA  306
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1015  ACTGCCCATTCATTGTCTGCATGTCATACGCGTTCCACACGCCAGACAAGCTCAGCTTCA  1074

Query  307   TCCTGGACCTCATGAACGGTGGGGACCTGCACTACCACCTCTCCCAGCACGGGGTCTTCT  366
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1075  TCCTGGACCTCATGAACGGTGGGGACCTGCACTACCACCTCTCCCAGCACGGGGTCTTCT  1134

Query  367   CAGAGGCTGACATGCGCTTCTATGCGGCCGAGATCATCCTGGGCCTGGAGCACATGCACA  426
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1135  CAGAGGCTGACATGCGCTTCTATGCGGCCGAGATCATCCTGGGCCTGGAGCACATGCACA  1194

Query  427   ACCGCTTCGTGGTCTACCGGGACCTGAAGCCAGCCAACATCCTTCTGGACGAGCATGGCC  486
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1195  ACCGCTTCGTGGTCTACCGGGACCTGAAGCCAGCCAACATCCTTCTGGACGAGCATGGCC  1254

Query  487   ACGTGCGGATCTCGGACCTGGGCCTGGCCTGTGACTTCTCCAAGAAGAAGCCCCATGCCA  546
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1255  ACGTGCGGATCTCGGACCTGGGCCTGGCCTGTGACTTCTCCAAGAAGAAGCCCCATGCCA  1314

Query  547   GCGTGGGCACCCACGGGTACATGGCTCCGGAGGTCCTGCAGAAGGGCGTGGCCTACGACA  606
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1315  GCGTGGGCACCCACGGGTACATGGCTCCGGAGGTCCTGCAGAAGGGCGTGGCCTACGACA  1374

Query  607   GCAGTGCCGACTGGTTCTCTCTGGGGTGCATGCTCTTCAAGTTGCTGCGGGGGCACAGCC  666
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1375  GCAGTGCCGACTGGTTCTCTCTGGGGTGCATGCTCTTCAAGTTGCTGCGGGGGCACAGCC  1434

Query  667   CCTTCCGGCAGCACAAGACCAAAGACAAGCATGAGATCGACCGCATGACGCTGACGATGG  726
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1435  CCTTCCGGCAGCACAAGACCAAAGACAAGCATGAGATCGACCGCATGACGCTGACGATGG  1494
```

```
Query  727   CCGTGGAGCTGCCCGACTCCTTCTCCCCTGAACTACGCTCCCTGCTGGAGGGGTTGCTGC   786
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1495  CCGTGGAGCTGCCCGACTCCTTCTCCCCTGAACTACGCTCCCTGCTGGAGGGGTTGCTGC   1554

Query  787   AGAGGGATGTCAACCGGAGATTGGGCTGCCTGGGCCGAGGGGCTCAGGAGGTGAAAGAGA   846
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1555  AGAGGGATGTCAACCGGAGATTGGGCTGCCTGGGCCGAGGGGCTCAGGAGGTGAAAGAGA   1614

Query  847   GCCCCTTTTTC   857
             |||||||||||
Sbjct  1615  GCCCCTTTTTC   1625
```

13.3 Sequencing of GRK2KD6xCHis

Sequencing of pET3dGRK2KD6xCHis (amino acids 191-453 of GRK2), clone 1

TATTTTGTTTTACTTTAAGAAGGAGATATACCATGGCCTTCAGCGTGCATCGCATCATTGGGCGCGGGGGCTTTGGCGAGG
TCTATGGGTGCCGGAAGGCTGACACAGGCAAGATGTACGCCATGAAGTGCCTGGACAAAAAGCGCATCAAGATGAAGCAGG
GGGAGACCCTGGCCCTGAACGAGCGCATCATGCTCTCGCTCGTCAGCACTGGGGACTGCCCATTCATTGTCTGCATGTCAT
ACGCGTTCCACACGCCAGACAAGCTCAGCTTCATCCTGGACCTCATGAACGGTGGGGACCTGCACTACCACCTCTCCCAGC
ACGGGGTCTTCTCAGAGGCTGACATGCGCTTCTATGCGGCCGAGATCATCCTGGGCCTGGAGCACATGCACAACCGCTTCG
TGGTCTACCGGGACCTGAAGCCAGCCAACATCCTTCTGGACGAGCATGGCCACGTGCGGATCTCGGACCTGGGCCTGGCCT
GTGACTTCTCCAAGAAGAAGCCCCATGCCAGCGTGGGCACCCACGGGTACATGGCTCCGGAGGTCCTGCAGAAGGGCGTGG
CCTACGACAGCAGTGCCGACTGGTTCTCTCTGGGGTGCATGCTCTTCAAGTTGCTGCGGGGGCACAGCCCCTTCCGGCAGC
ACAAGACCAAAGACAAGCATGAGATCGACCGCATGACGCTGACGATGGCCGTGGAGCTGCCCGACTCCTTCTCCCCTGAAC
TACGCTCCCTGCTGGAGGGGTTGCTGCAGAGGGATGTCAACCGGAGATTGGGCTGCCTGGGCCGAGGGGCTCAGGAGGTGA
AAGAGAGCCCCTTTTTCCACCATCACCATCACCATTAAGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCT
GCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGA
ACTATATCCGGATATCCACAGGACGGGTGTGGTCGCCATG

CCATGG: NcoI restriction site

ATG: Start codon

XXX: hexahistidine tag

TAA: Stop codon

GGATCC: BamHI restriction site

```
Query  46    CTTCAGCGTGCATCGCATCATTGGGCGCGGGGGCTTTGGCGAGGTCTATGGGTGCCGGAA   105
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  836   CTTCAGCGTGCATCGCATCATTGGGCGCGGGGGCTTTGGCGAGGTCTATGGGTGCCGGAA   895

Query  106   GGCTGACACAGGCAAGATGTACGCCATGAAGTGCCTGGACAAAAAGCGCATCAAGATGAA   165
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  896   GGCTGACACAGGCAAGATGTACGCCATGAAGTGCCTGGACAAAAAGCGCATCAAGATGAA   955

Query  166   GCAGGGGGAGACCCTGGCCCTGAACGAGCGCATCATGCTCTCGCTCGTCAGCACTGGGGA   225
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  956   GCAGGGGGAGACCCTGGCCCTGAACGAGCGCATCATGCTCTCGCTCGTCAGCACTGGGGA   1015

Query  226   CTGCCCATTCATTGTCTGCATGTCATACGCGTTCCACACGCCAGACAAGCTCAGCTTCAT   285
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1016  CTGCCCATTCATTGTCTGCATGTCATACGCGTTCCACACGCCAGACAAGCTCAGCTTCAT   1075

Query  286   CCTGGACCTCATGAACGGTGGGGACCTGCACTACCACCTCTCCCAGCACGGGGTCTTCTC   345
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1076  CCTGGACCTCATGAACGGTGGGGACCTGCACTACCACCTCTCCCAGCACGGGGTCTTCTC   1135

Query  346   AGAGGCTGACATGCGCTTCTATGCGGCCGAGATCATCCTGGGCCTGGAGCACATGCACAA   405
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1136  AGAGGCTGACATGCGCTTCTATGCGGCCGAGATCATCCTGGGCCTGGAGCACATGCACAA   1195
```

121

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Query  406   CCGCTTCGTGGTCTACCGGGACCTGAAGCCAGCCAACATCCTTCTGGACGAGCATGGCCA   465
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1196  CCGCTTCGTGGTCTACCGGGACCTGAAGCCAGCCAACATCCTTCTGGACGAGCATGGCCA   1255

Query  466   CGTGCGGATCTCGGACCTGGGCCTGGCCTGTGACTTCTCCAAGAAGAAGCCCCATGCCAG   525
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1256  CGTGCGGATCTCGGACCTGGGCCTGGCCTGTGACTTCTCCAAGAAGAAGCCCCATGCCAG   1315

Query  526   CGTGGGCACCCACGGGTACATGGCTCCGGAGGTCCTGCAGAAGGGCGTGGCCTACGACAG   585
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1316  CGTGGGCACCCACGGGTACATGGCTCCGGAGGTCCTGCAGAAGGGCGTGGCCTACGACAG   1375

Query  586   CAGTGCCGACTGGTTCTCTCTGGGGTGCATGCTCTTCAAGTTGCTGCGGGGGCACAGCCC   645
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1376  CAGTGCCGACTGGTTCTCTCTGGGGTGCATGCTCTTCAAGTTGCTGCGGGGGCACAGCCC   1435

Query  646   CTTCCGGCAGCACAAGACCAAAGACAAGCATGAGATCGACCGCATGACGCTGACGATGGC   705
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1436  CTTCCGGCAGCACAAGACCAAAGACAAGCATGAGATCGACCGCATGACGCTGACGATGGC   1495

Query  706   CGTGGAGCTGCCCGACTCCTTCTCCCCTGAACTACGCTCCCTGCTGGAGGGGTTGCTGCA   765
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1496  CGTGGAGCTGCCCGACTCCTTCTCCCCTGAACTACGCTCCCTGCTGGAGGGGTTGCTGCA   1555

Query  766   GAGGGATGTCAACCGGAGATTGGGCTGCCTGGGCCGAGGGGCTCAGGAGGTGAAAGAGAG   825
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1556  GAGGGATGTCAACCGGAGATTGGGCTGCCTGGGCCGAGGGGCTCAGGAGGTGAAAGAGAG   1615

Query  826   CCCCTTTTTCC   836
             |||||||||||
Sbjct  1616  CCCCTTTTTCC   1626
```

13.4 Sequencing of 6xNHisGRK2CTD

Sequencing of pET3d6xNHisGRK2CTD (amino acids 454-689 of GRK2), clone 4

```
TGNTATTTTTGTTTACTTTAAGAAGGAGATATACCATGACCCATCACCATCACCATCACCGCTCCCTGGACTGGCAGATGG
TCTTCTTGCAGAAGTACCCTCCCCCGCTGATCCCCCCACGAGGGGAGGTGAACGCGGCCGACGCCTTCGACATTGGCTCCT
TCGATGAGGAGGACACAAAAGGAATCAAGTTACTGGACAGTGATCAGGAGCTCTACCGCAACTTCCCCCTCACCATCTCGG
AGCGGTGGCAGCAGGAGGTGGCAGAGACTGTCTTCGACACCATCAACGCTGAGACAGACCGGCTGGAGGCTCGCAAGAAAG
CCAAGAACAAGCAGCTGGGCCATGAGGAAGACTACGCCCTGGGCAAGGACTGCATCATGCATGGCTACATGTCCAAGATGG
GCAACCCCTTCCTGACCCAGTGGCAGCGGCGGTACTTCTACCTGTTCCCCAACCGCCTCGAGTGGCGGGGCGAGGGCGAGG
CCCCGCAGAGCCTGCTGACCATGGAGGAGATCCAGTCGGTGGAGGAGACGCAGATCAAGGAGCGCAAGTGCCTGCTCCTCA
AGATCCGCGGTGGGAAACAGTTCATTTTGCAGTGCGATAGCGACCCTGAGCTGGTGCAGTGGAAGAAGGAGCTGCGCGACG
CCTACCGCGAGGCCCAGCAGCTGGTGCAGCGGGTGCCCAAGATGAAGAACAAGCCCGCGCTCGCCCGTGGGTGGAGCTGAGCA
AGGTGCCGCTGGTCCAGCGCGGCAGTGCCAACGGCCTCTGAGGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG
GCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGA
GGAACTATATCCGGATATCCACAGGACGGGTGTGGTCGCCATGATCGCGTAGTCGATAGTGGCTCCAAGTAGCGAAGCGAG
CAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGTGCTCCG
```

ATG: Start codon

CATGA: BspHI restriction site, not reconstituted

XXX: hexahistidine tag

TGA: Stop codon

GGATCC: BamHI restriction site

```
Query  59    CCGCTCCCTGGACTGGCAGATGGTCTTCTTGCAGAAGTACCCTCCCCCGCTGATCCCCCC   118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1625  CCGCTCCCTGGACTGGCAGATGGTCTTCTTGCAGAAGTACCCTCCCCCGCTGATCCCCCC   1684

Query  119   ACGAGGGGAGGTGAACGCGGCCGACGCCTTCGACATTGGCTCCTTCGATGAGGAGGACAC   178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1685  ACGAGGGGAGGTGAACGCGGCCGACGCCTTCGACATTGGCTCCTTCGATGAGGAGGACAC   1744

Query  179   AAAAGGAATCAAGTTACTGGACAGTGATCAGGAGCTCTACCGCAACTTCCCCCTCACCAT   238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1745  AAAAGGAATCAAGTTACTGGACAGTGATCAGGAGCTCTACCGCAACTTCCCCCTCACCAT   1804
```

```
Query  239   CTCGGAGCGGTGGCAGCAGGAGGTGGCAGAGACTGTCTTCGACACCATCAACGCTGAGAC   298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1805  CTCGGAGCGGTGGCAGCAGGAGGTGGCAGAGACTGTCTTCGACACCATCAACGCTGAGAC   1864

Query  299   AGACCGGCTGGAGGCTCGCAAGAAAGCCAAGAACAAGCAGCTGGGCCATGAGGAAGACTA   358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1865  AGACCGGCTGGAGGCTCGCAAGAAAGCCAAGAACAAGCAGCTGGGCCATGAGGAAGACTA   1924

Query  359   CGCCCTGGGCAAGGACTGCATCATGCATGGCTACATGTCCAAGATGGGCAACCCCTTCCT   418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1925  CGCCCTGGGCAAGGACTGCATCATGCATGGCTACATGTCCAAGATGGGCAACCCCTTCCT   1984

Query  419   GACCCAGTGGCAGCGGCGGTACTTCTACCTGTTCCCCAACCGCCTCGAGTGGCGGGGCGA   478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1985  GACCCAGTGGCAGCGGCGGTACTTCTACCTGTTCCCCAACCGCCTCGAGTGGCGGGGCGA   2044

Query  479   GGGCGAGGCCCCGCAGAGCCTGCTGACCATGGAGGAGATCCAGTCGGTGGAGGAGACGCA   538
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2045  GGGCGAGGCCCCGCAGAGCCTGCTGACCATGGAGGAGATCCAGTCGGTGGAGGAGACGCA   2104

Query  539   GATCAAGGAGCGCAAGTGCCTGCTCCTCAAGATCCGCGGTGGGAAACAGTTCATTTTGCA   598
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2105  GATCAAGGAGCGCAAGTGCCTGCTCCTCAAGATCCGCGGTGGGAAACAGTTCATTTTGCA   2164

Query  599   GTGCGATAGCGACCCTGAGCTGGTGCAGTGGAAGAAGGAGCTGCGCGACGCCTACCGCGA   658
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2165  GTGCGATAGCGACCCTGAGCTGGTGCAGTGGAAGAAGGAGCTGCGCGACGCCTACCGCGA   2224

Query  659   GGCCCAGCAGCTGGTGCAGCGGGTGCCCAAGATGAAGAACAAGCCGCGCTCGCCCGTGGT   718
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2225  GGCCCAGCAGCTGGTGCAGCGGGTGCCCAAGATGAAGAACAAGCCGCGCTCGCCCGTGGT   2284

Query  719   GGAGCTGAGCAAGGTGCCGCTGGTCCAGCGCGGCAGTGCCAACGGCCTCTGA   770
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2285  GGAGCTGAGCAAGGTGCCGCTGGTCCAGCGCGGCAGTGCCAACGGCCTCTGA   2336
```

13.5 Sequencing of GRK2CTD6xCHis

Sequencing of pET3dGRK2CTD6CHis (amino acids 454-689 of GRK2) clone 4

```
TTCTCTGATATTTTTGTTTACTTTAAGAAGGAGATATAC‌CATGA‌CCCGCTCCCTGGACTGGCAGATGGTCTTCTTGCAGAA
GTACCCTCCCCCGCTGATCCCCCCACGAGGGGAGGTGAACGCGGCCGACGCCTTCGACATTGGCTCCTTCGATGAGGAGGA
CACAAAAGGAATCAAGTTACTGGACAGTGATCAGGAGCTCTACCGCAACTTCCCCCTCACCATCTCGGAGCGGTGGCAGCA
GGAGGTGGCAGAGACTGTCTTCGACACCATCAACGCTGAGACAGACCGGCTGGAGGCTCGCAAGAAAGCCAAGAACAAGCA
GCTGGGCCATGAGGAAGACTACGCCCTGGGCAAGGACTGCATCATGCATGGCTACATGTCCAAGATGGGCAACCCCTTCCT
GACCCAGTGGCAGCGGCGGTACTTCTACCTGTTCCCCAACCGCCTCGAGTGGCGGGGCGAGGGCGAGGCCCCGCAGAGCCT
GCTGACCATGGAGGAGATCCAGTCGGTGGAGGAGACGCAGATCAAGGAGCGCAAGTGCCTGCTCCTCAAGATCCGCGGTGG
GAAACAGTTCATTTTGCAGTGCGATAGCGACCCTGAGCTGGTGCAGTGGAAGAAGGAGCTGCGCGACGCCTACCGCGAGGC
CCAGCAGCTGGTGCAGCGGGTGCCCAAGATGAAGAACAAGCCGCGCTCGCCCGTGGTGGAGCTGAGCAAGGTGCCGCTGGT
CCAGCGCGGCAGTGCCAACGGCCTC‌CACCATCACCATCACCAT‌TGA‌GGATCC‌GGCTGCTAACAAAGCCCGAAAGGAAGCTG
AGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGA
AAGGAGGAACTATATCCGGATATCCACAGGACGGGTGTGGTCGCCATGATCGCGTAGTCGATAGTGGCTCCAAGTAGCGAA
GCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGTG
```

ATG: Start codon

‌CATGA‌: BspHI restriction site, not reconstituted

‌XXX‌: hexahistidine tag

TGA: Stop codon

‌GGATCC‌: BamHI restriction site

```
Query  1    CGCTCCCTGGACTGGCAGATGGTCTTCTTGCAGAAGTACCCTCCCCCGCTGATCCCCCCA   60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1626 CGCTCCCTGGACTGGCAGATGGTCTTCTTGCAGAAGTACCCTCCCCCGCTGATCCCCCCA   1685
```

```
Query   61    CGAGGGGAGGTGAACGCGGCCGACGCCTTCGACATTGGCTCCTTCGATGAGGAGGACACA    120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1686  CGAGGGGAGGTGAACGCGGCCGACGCCTTCGACATTGGCTCCTTCGATGAGGAGGACACA    1745

Query   121   AAAGGAATCAAGTTACTGGACAGTGATCAGGAGCTCTACCGCAACTTCCCCCTCACCATC    180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1746  AAAGGAATCAAGTTACTGGACAGTGATCAGGAGCTCTACCGCAACTTCCCCCTCACCATC    1805

Query   181   TCGGAGCGGTGGCAGCAGGAGGTGGCAGAGACTGTCTTCGACACCATCAACGCTGAGACA    240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1806  TCGGAGCGGTGGCAGCAGGAGGTGGCAGAGACTGTCTTCGACACCATCAACGCTGAGACA    1865

Query   241   GACCGGCTGGAGGCTCGCAAGAAAGCCAAGAACAAGCAGCTGGGCCATGAGGAAGACTAC    300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1866  GACCGGCTGGAGGCTCGCAAGAAAGCCAAGAACAAGCAGCTGGGCCATGAGGAAGACTAC    1925

Query   301   GCCCTGGGCAAGGACTGCATCATGCATGGCTACATGTCCAAGATGGGCAACCCCTTCCTG    360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1926  GCCCTGGGCAAGGACTGCATCATGCATGGCTACATGTCCAAGATGGGCAACCCCTTCCTG    1985

Query   361   ACCCAGTGGCAGCGGCGGTACTTCTACCTGTTCCCCAACCGCCTCGAGTGGCGGGGCGAG    420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1986  ACCCAGTGGCAGCGGCGGTACTTCTACCTGTTCCCCAACCGCCTCGAGTGGCGGGGCGAG    2045

Query   421   GGCGAGGCCCCGCAGAGCCTGCTGACCATGGAGGAGATCCAGTCGGTGGAGGAGACGCAG    480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   2046  GGCGAGGCCCCGCAGAGCCTGCTGACCATGGAGGAGATCCAGTCGGTGGAGGAGACGCAG    2105

Query   481   ATCAAGGAGCGCAAGTGCCTGCTCCTCAAGATCCGCGGTGGGAAACAGTTCATTTTGCAG    540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   2106  ATCAAGGAGCGCAAGTGCCTGCTCCTCAAGATCCGCGGTGGGAAACAGTTCATTTTGCAG    2165

Query   541   TGCGATAGCGACCCTGAGCTGGTGCAGTGGAAGAAGGAGCTGCGCGACGCCTACCGCGAG    600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   2166  TGCGATAGCGACCCTGAGCTGGTGCAGTGGAAGAAGGAGCTGCGCGACGCCTACCGCGAG    2225

Query   601   GCCCAGCAGCTGGTGCAGCGGGTGCCCAAGATGAAGAACAAGCCGCGCTCGCCCGTGGTG    660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   2226  GCCCAGCAGCTGGTGCAGCGGGTGCCCAAGATGAAGAACAAGCCGCGCTCGCCCGTGGTG    2285

Query   661   GAGCTGAGCAAGGTGCCGCTGGTCCAGCGCGGCAGTGCCAACGGCCTC    708
              ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   2286  GAGCTGAGCAAGGTGCCGCTGGTCCAGCGCGGCAGTGCCAACGGCCTC    2333
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13.6 Sequencing of pcDNA3WD1

Sequencing of pcDNA3WD1 (MASTLIVGHWDGNMSLVDRRT)

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CTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTTGGTACCGAGCTCGGA
TCCATGGCCAGCACCCTGATCGTGGGCCACTGGGACGGCAACATGAGCCTGGTGGACAGGAGGACCTGACTCGAGCATGCA
TCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCC
ATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGA
AATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGA
AGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTA
TCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGC
CCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGG
GCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGG
GCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGG
AACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGA
GCTGATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCA
GCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGA
AGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCC
AGTTCCGCCCATTY
```

124

ATG: Start codon

GGATCC: BamHI restriction site

TGA: Stop codon

CTCGAG: XhoI restriction site

13.7 Sequencing of pcDNA3WD2

Sequencing of pcDNA3WD2 (MFLAEDASTLIVGHWDGNMSL)

CAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGC
TTGGTACCGAGCTCGGATCC**ATG**TTCCTGGCCGAGGACGCCAGCACCCTGATCGTGGGCCACTGGGACGGCAACATGAGCC
TG**TGA**CTCGAGCATGCATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTG
CCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTT
TCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGC
AAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGC
TGGGGCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACC
GCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGT
CAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGT
GATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGA
CTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCC
TATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAA
AGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGGAAAGTCCCCA
GGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCATTTAGTCAGCAACCATAGTCCCGCCCC

ATG: Start codon

GGATCC: BamHI restriction site

TGA: Stop codon

CTCGAG: XhoI restriction site

13.8 Sequencing of pcDNA3WD3

Sequencing of pcDNA3WD3 (MERSSFSSFDFLAEDASTLIVF)

ACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTTGGTACCGAGCTCGG
ATCC**ATG**GAGAGGAGCAGCTTCAGCAGCTTCGACTTCCTGGCYGAGGACGCCAGCACCCTGATCGTGTTC**TGA**CTCGAGCA
TGCATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCC
AGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATG
AGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATT
GGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGG
GGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCA
GCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATC
GGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTA
GTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAA
CTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAA
ATGAGCTGATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTC
CCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGGAAAGTCCCCAGGCTCCCCAGCAGG

CAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCTAAATCCG
GCCAGTTCC

ATG: Start codon

GGATCC: BamHI restriction site

TGA: Stop codon

CTCGAG: XhoI restriction site

13.9 Sequencing of pMHCWD3

Sequencing of pMHCWD3 (MERSSFSSFDFLAEDASTLIVF) clone 2

CTCCCCCATAAGAGTTTGAGTCGAC**ATG**GAGAGGAGCAGCTTCAGCAGCTTCGACTTCCTGGCCGAGGACGCCAGCACCCT
GATCGTGTTC**TGA**AAGCTTGATGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCC
AGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTG
GAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGCCTGCGGGGTCTATTGGGAACCAAGCTGGAG
TGCAGTGGCACAATCTTGGCTCACTGCAATCTCCGCCTCCTGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTTGTT
GGGATTCCAGGCATGCATGACCAGGCTCAGCTAATTTTTGTTTTTTTGGTAGAGACGGGGTTTCACCATATTGGCCAGGCT
GGTCTCCAACTCCTAATCTCAGGTGATCTACCCACCTTGGCCTCCCAAATTGCTGGGATTACAGGCGTGAACCACTGCTCC
CTTCCCTGTCCTTCTGATTTTAAAATAACTATACCAGCAGGAGGACGTCCAGACACAGCATAGGCTACCTGGCCATGCCCA
ACCGGTGGGACATTTGAGTTGCTTGCTTGGCACTGTCCTCTCATGCGTTGGGTCCACTCAGTAGATGCCTGTTGAATTCCT
GCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGTTTAAACTAGTATACACGTGGCGCCCGGGCGGCCGCCACCGCGGTG
GAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAA
TTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTA
ACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCA
ACGCSCGGGGAARAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGGTCA

ATG: Start codon

GTCGAC: SalI restriction site

TGA: Stop codon

AAGCTT: HindIII restriction site