

Non-G Protein-Coupled Receptor (GPCR) Substrates for GPCR Kinases (GRKs)

By

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Abstract

G protein-coupled receptors are one of the most enormous and important receptor families found in nature. They are 7-transmembrane receptors coupled to G proteins and detect a wide range of stimuli such as light, odors, taste, ions, and peptides. The activated GPCR is desensitized via two mechanisms: heterologous vs homologous desensitization. In homologous desensitization a class of mammalian kinase is responsible for serine/threonine phosphorylation of agonist-activated receptor, called GPCR kinases (GRKs). This class includes 7 kinases. It was first thought that GRKs could only phosphorylate ligand-activated GPCRs, but by discovering some of its non-GPCR targets we decide to collect data about them and the effects of their phosphorylation. By searching in pubmed database and studying a review article, we have found these non-GPCR substrates phosphorylated by GRKs: β -Arrestin 1, Dream, ENaCs, Nedd4 & Nedd4-2, Ezrin & Radixin, Tubulin, PDGFR β , EGFR, NHERF, Hip, IRS-1, PDE γ , p38 MAPKs, Phosducin & PhLP, R-Smads, synucleins, ribosomal protein P2, HDAC, NPM1, P53, APC, LRP6, NF κ B p105 & I κ B α . We investigate about the phosphorylation sites of these targets, the effects of their phosphorylation by GRKs on cell signaling, and the cultures or cells whose phosphorylations have been reported. Finally we discuss that the GRKs' role in homologous desensitization of GPCRs are not sufficient to explain all of GRKs functions, so we propose new roles for GRKs in cell processes by phosphorylating these non-GPCR substrates. We also discuss about difficulties accompanied finding these targets and suggest more in vivo studies for them.

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List of Abbreviations

A

ADH: Antidiuretic Hormone (Vasopressin)

AGC family: Protein Kinase A, G, and C family

AML: Acute Myeloid Leukemia

AP-2: Adaptor Protein 2

APC: Adenomatous Polyposis Coli

apoB: Apolipoprotein B

apoE: Apolipoprotein E

ARF: ADP-Ribosylation Factor

ARNO: ARF Nucleotide Binding Site Opener

ASEFs: APC-Stimulated Guanine Nucleotide Exchange Factors

ATM: Ataxia Telangiectasia-Mutated

ATP: Adenosine Triphosphate

AT1R: Angiotensin II Type 1 Receptor

B

B23: Nucleolar Phosphoprotein B23

BAD: Bcl-2-Associated Death Promoter

β2AR: β2-Adrenergic Receptor

β3AR: β3-Adrenergic Receptor

β-arr1: β-Arrestin 1

βARK: Beta(β)-Adrenergic Receptor Kinase

Bcl-2: B-cell lymphoma 2

Bcl-3: B-cell leukemia 3

Bcl-xL: B-cell lymphoma-extra Large

BMK1: ERK/Big MAP Kinase 1

BMPs: Bone Morphogenetic Proteins

βTrCP: β-Transducin repeat-Containing Protein

C

CamKII: Ca²⁺/Calmodulin-dependent protein Kinase II

cAMP: cyclic Adenosine Monophosphate

c-Cbl: Casitas B-lineage Lymphoma

cDNA: complementary DNA

CD43: Cluster of Differentiation 43

CD44: Cluster of Differentiation 44

Cdc42: Cell Division Control Protein 42

cGMP: cyclic Guanosine Monophosphate

CHK2: Checkpoint Kinase 2

ClC-5: Chloride channel-5

CK1: Casein Kinase 1

CK2: Casein Kinase 2

CK1ε: Casein Kinase 1 ε

CNS: Central Nervous System

Co-Smad: Common Smad

C-Rel: v-rel reticuloendotheliosis viral oncogene homolog (avian)

CRF: Corticotrophin-Releasing Factor

CRLR: Calcitonin-Receptor Like Receptor

c-Src: Cytoplasmic or Cellular Src (Sarcoma)

CSP: Calcium Sensor Protein

Cys: Cysteine

CXCL12: Chemokine (C-X-C motif) Ligand 12

CXCR2: Chemokine (C-X-C) Receptor 2

CXCR4: Chemokine (C-X-C) Receptor 4

D

DKK1: Dickkopf-1

DLG1: Mammalian Homolog Discs Large Binding Region

DOR: δ -opioid Receptor

DREAM: Downstream Regulatory Element Antagonist Modulator

DSB: DNA Double-Strand Breaks

E

EB1: Microtubule plus End-Binding Protein 1

EBP-50: ERM-Binding Phosphoprotein 50

EGFR: Epidermal Growth Factor Receptor

ENaCs: Epithelial Na⁺Channels

Eps15: Epidermal growth factor receptor Pathway Substrate 15

ErbB: Erythroblastic Leukemia Viral Oncogene

ERK: Extracellular-Signal-Regulated Kinase

ERM proteins: Ezrin-Radixin-Moesin proteins

ERMAD: ERM-Association-Domain

ET-1: Endothelin-1

ETA: Endothelin A

ETB: Endothelin B

Epac1: Exchange Protein Activated by cAMP 1

Epac2: Exchange Protein Activated by cAMP 2

F

FACS Analysis: Flow Cytometry and Fluorescence-activated Cell Sorting Analysis

FERM: 4.1 ERM

FSH: Follicle-Stimulating Hormone

Fz: Frizzled

G

Gab1: GRB2-associated Binding protein 1

GABA_B: γ -Aminobutyric Acid B

GAP: GTPase-Activation Proteins

G $\alpha_{q/11}$: G protein q/11 α subunit

GDP: Guanosine Diphosphate

GEF: Guanine nucleotide Exchange Factor

GGMP Domain: Glycine, Methionine, and Proline enriched domain

GIT-1: GRK Interactor 1

Gly: Glycine

GnRH: Gonadotropin Releasing Hormone

GnRHR: Gonadotropin Releasing Hormone Receptor

G proteins: Guanine nucleotide or GTP binding Proteins

GPCR: G Protein-Coupled Receptor

GRAFS: Glutamate, Rhodopsin, Adhesion, Frizzled/taste2, & Secretin

GRK: G protein-coupled Receptor Kinase

GS domain: Glycine & Serine rich domain

GSK3: Glycogen Synthase Kinase 3

GST: Glutathione S-Transferase

GTP: Guanosine Triphosphate

H

HDAC: Histone Deacetylases

HECT: Homologous to E6-AP, C-Terminus

HEK Cells: Human Embryonic Kidney Cells

HeLa cells: Henrietta Lacks-derived Cells

HH: Hypogonadotropic Hypogonadism

Hh: Hedgehog

Hip: Hsp70 interacting protein

Hsp: Heat shock protein

5-HT4: 5-Hydroxytryptamine Receptor 4

5-HT4R: 5-HT4 Receptor

I

IGF-1: Insulin-like Growth Factor-1

IκB: Inhibitor κB

IKK: IκB kinase

IL-1: Interleukin 1

Ile: Isoleucine

IMRAD: Introduction, Methods & materials, Results, And Discussion & conclusion

IQGAP: IQ-motif-containing GTPase-Activating Protein

IR: Ionizing Radiation

IRS: Insulin Receptor Substrates Complex

I-Smads: Inhibitory-Smads

Itch: Itchy E3 ubiquitin protein ligase

J

JNK: c-Jun N-terminal Kinase

JNK/SAPK: c-Jun N-terminal or Stress-Activated Protein Kinases

K

KAP3: Kinesin Associated Protein 3

KChIP: Potassium Channel Interacting Protein

KD: Kinase Domain

K_M: Michaelis constant in Michaelis–Menten kinetics

L

LB: Lewy Body

Lef-1/Tcf: Lymphoid enhancer factor-1/T-cell factor

LN: Lewy Neurite

LCD: Leucine-Charged residue-rich Domain

LDL: Low-Density Lipoprotein

LDLR: Low-Density Lipoprotein Receptor

LRP: Lipoprotein Receptor-related Protein

LPS: Lipopolysaccharide

M

MAPs: Microtubule-Associated Proteins

MAPK: Mitogen-Activated Protein Kinase

MDCK Cells: Madin Darby Canine Kidney epithelial Cells

MDM2: Murine Double Minute 2

MDMX: Murine Double Minute X

MEF2: Myocyte Enhancer Factor2

MEK: Mitogen-activated protein kinase / Extracellular signal-regulated kinase Kinase

mGluR: metabotropic Glutamate Receptor

MH1 domain: Mad Homology domain 1

MH2 domain: Mad Homology domain 2

MKK3: Mitogen-activated protein Kinase Kinase 3

MKK6: Mitogen-activated protein Kinase Kinase 6

m2 mAChR: m2 muscarinic Acetylcholine Receptor

MOR: μ -Opioid Receptor

μ ORs: μ -Opioid Receptors

N

NAC: Non-A β Component of Alzheimer's disease amyloid

NDI: Nephrogenic Diabetes Insipidus

Nedd4: Neural precursor cell-expressed developmentally downregulated gene 4

NEMO: NF κ B Essential Modulator

NES: Nuclear-Export Signal

NF κ B: Nuclear Factor kappa B

NHE1: Na⁺/H⁺ Exchanger 1

NHERF: Na⁺/H⁺ Exchanger Regulatory Factor

NLS: Nuclear Localization Sequence

NuLS: Nucleolar Localization Signal

NPM1: Nucleophosmin

NPM2: Nucleoplasmin

NSF: N-ethylmaleimide-Sensitive Factor

P

PACSIN: Protein kinase C and casein kinase Substrate in Neurons protein

PAR: Protease-Activated Receptor

PDE: cAMP Phosphodiesterase

PDGF: Platelet-Derived Growth Factor

PDGFR β : Platelet-Derived Growth Factor Receptor β

PDZ: PSD-95, Discs-large, and ZO-1

PEST domain: polypeptide domains rich in proline (P), glutamic acid (E), serine (S) and threonine (T)

PH: Pleckstrin Homology

PhLP: Phosducin-Like Protein

PI3K: Phosphatidylinositol (Phosphoinositide) 3 Kinase

PIP2: Phosphatidylinositol 4, 5-biphosphate

PKA: Protein Kinase A, cAMP-dependent protein kinase

PKC: Protein Kinase C

PKD: Protein Kinase D

PLC: Phospholipase C

PLD2: Phospholipase D2

PLK1: Polo-Like Kinase 1

PP1: Protein Phosphatase type 1

PP2A: Protein Phosphatase 2 A

PPSPxS domain: Proline-Proline-Proline-Serine-Proline-X-Serine domain

Pro: Proline

PY motif: Proline-rich motif

R

Rab: Ras-related GTP-Binding protein

Rac1: Ras-related C3 botulinum toxin substrate 1

RAMP: Receptor Activity-Modifying Protein

RGS: Regulator of G protein Signaling

RH: Regulator of G protein signaling (RGS) Homology

RKIP: Raf Kinase Inhibitor Protein

RP: Retinitis Pigmentosa

rRNA: ribosomal RNA

R-Smads: Receptor-regulated or Regulatory Smads

RTK: Receptor Tyrosine Kinase

S

SAMP: Serine-Alanine-Methionine-Proline repeat motif

SAPK: Stress-Activated Protein Kinase

Ser: Serine

Sgk1: Serum glucocorticoid-inducible kinase 1

SH2: Src Homology 2

SH3: Src Homology 3

Shc: SHC-adaptor protein

Shp2: Src homology 2 domain-containing protein-tyrosine phosphatase-2

SKK3: SAPK (Stress-Activated Protein Kinase) Kinase 3

SMC: Smooth Muscle Cell

Smo: Smoothed

Smurf1: SMAD specific E3 ubiquitin protein ligase 1

Smurf2: SMAD specific E3 ubiquitin protein ligase 2

SSXS motif: Serine-Serine-X-Serine motif

STAT: Signal Transducer and Activator of Transcription

T

T β R: TGF- β type I Receptor

TG: Triglycerides

TGF- β : Transforming Growth Factor- β

TGY: Threonine-Glycine-Tyrosine

Thr: Threonine

TL/IL-1: Toll-Like/Interleukin-1

TM: Transmembrane

TNF α : Tumour Necrosis Factor α

TPL2: Tumour Progression Locus-2

TPR domain: Tetrcopeptide Repeat domain

TSH: Thyroid-Stimulating Hormone

Tyr: Tyrosine

V-Z

VIP1: Vasoactive Intestinal Peptide type 1

VLDLR: Very Low-Density Lipoprotein Receptor

Wwp1: WW domain containing E3 ubiquitin protein ligase 1

Wwp2: WW domain containing E3 ubiquitin protein ligase 2

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Chapter 1: Introduction

The activity of every cell is regulated by a vast number of extracellular ligands and signals. These extracellular signals are transduced to intracellular environment by several families of cell membrane receptors. One the most important family of these receptors found in nature is the superfamily of G protein-coupled receptors (GPCRs) (Wess 1998). GPCRs are the heptahelical (7-transmembrane) receptors that detect a wide range of ligands such as ions, proteases, peptides, biogenic amines, lipids, glycoproteins, and nucleotides as well as light, taste and odors stimuli (Gether 2000). The GPCRs are coupled to the heterotrimeric guanine nucleotide-binding proteins (G proteins) that are involved in the regulation of effector protein activity, such as ion channels and enzymes (Offermanns 2003). At basal condition, G proteins include a GDP (Guanosine Diphosphate)-bound α subunit, a β subunit and a γ subunit. The β and γ subunits are associated to form an undissociable dimer. Upon activation of GPCRs by agonist exposure, the GDP-bound α subunits interact with activated GPCRs in order to replace its GDP with cytoplasmic GTP (Guanosine Triphosphate) that results in their dissociation from the $\beta\gamma$ dimers (Vauquelin and Van Liefde 2005). Then, both of GTP-bound α subunits and $\beta\gamma$ dimers associate with effectors to regulate their activity (Offermanns 2003).

GPCR kinases (GRKs) are a class of mammalian kinases with seven members, including GRK1 to GRK7, (Sterne-Marr et al. 2009) that are involved in homologous desensitization of GPCRs (Mushegian et al. 2012). GRKs phosphorylate the cytoplasmic domains and loops of stimulated GPCRs on serine (Ser) and threonine (Thr) residues. GRK-mediated phosphorylation of activated GPCRs increases the affinity of arrestins

for receptors and results in their dissociation from heterotrimeric G proteins, desensitization and endocytosis of the receptors (homologous desensitization (Lefkowitz 2007)) (Boguth et al. 2010).

It was first thought that GRKs only interact with and phosphorylate agonist-activated GPCRs. This specificity of substrate assumed to be their unique defining hallmark (Pitcher et al. 1998a). It is observed that activation of GPCRs by agonist exposure increases the kinase activity of GRKs. Therefore, upon binding of ligands to GPCRs, activated GRKs phosphorylate the receptor to promote its desensitization (Freeman et al. 2002).

GPCRs: Seven Transmembrane Receptors

The largest and most diverse superfamily of cell surface receptors in the mammalian genome is the G protein-coupled, or seven membrane-spanning receptors (GPCRs) (Luttrell 2006). They are encoded by ~1000 genes in the human genome (Lefkowitz 2004). Most of these genes encode 'orphans receptors', as their endogenous ligands and messengers are not discovered yet, and mostly include olfactory receptors (Lefkowitz 2004; Vauquelin and Van Liefde 2005). All GPCRs are composed of a single peptide including an extracellular N-terminus, seven hydrophobic transmembrane α -helices (TMI-VII), containing 20-25 amino acid residues, linked by three hydrophilic alternating intracellular (i1-3) and extracellular loops (e1-3), and an intracellular C-terminus (Figure 1) (Vauquelin and Van Liefde 2005; Luttrell 2006). The membrane-spanning α -helices of GPCRs have the most sequence conservation, while

intracellular and extracellular regions vary in size and complexity extensively. The transmembrane and extracellular domains participate in ligand binding, whereas the intracellular regions are involved in feedback modulation of receptor function and signal transduction via G protein activation. There are one or more sites for N-glycosylation within the N-terminal of the receptor, or less often, the extracellular loops. Most of the receptors usually contain two cysteine (Cys) residues forming a disulfide bridge between e1 and e2. This disulfide bridge is necessary for normal folding of the receptor. There is another Cys residue in the C-terminus, functioning as a site for palmitoylation, the process that palmitic acid binds via covalent binding. This lipid modification causes the formation of supposed fourth intracellular loop (Wess 1998; Luttrell 2006). There are also some phosphorylation sites within the C-terminus that participate in directing the desensitization and endocytosis of GPCRs (Perez and Karnik 2005).

Heptahelical receptors control nearly all of the physiological processes known in mammals (Lefkowitz 2004). GPCR signaling is involved in visual, olfactory, and gustatory sensations. They regulate hemodynamics, intermediary metabolism, growth, proliferation, differentiation and death of multiple cell types (Luttrell 2006) and play a role in direct neuroendocrine regulation of physiologic homeostasis and reproduction, and neurotransmission (Luttrell 2008). Due to these widespread roles and functions of GPCRs, it is not surprising that about 50% of all drugs clinically-used nowadays aim GPCRs (Luttrell 2008), function to block binding of endogenous ligand to the receptor, or mimic them (Luttrell 2006).

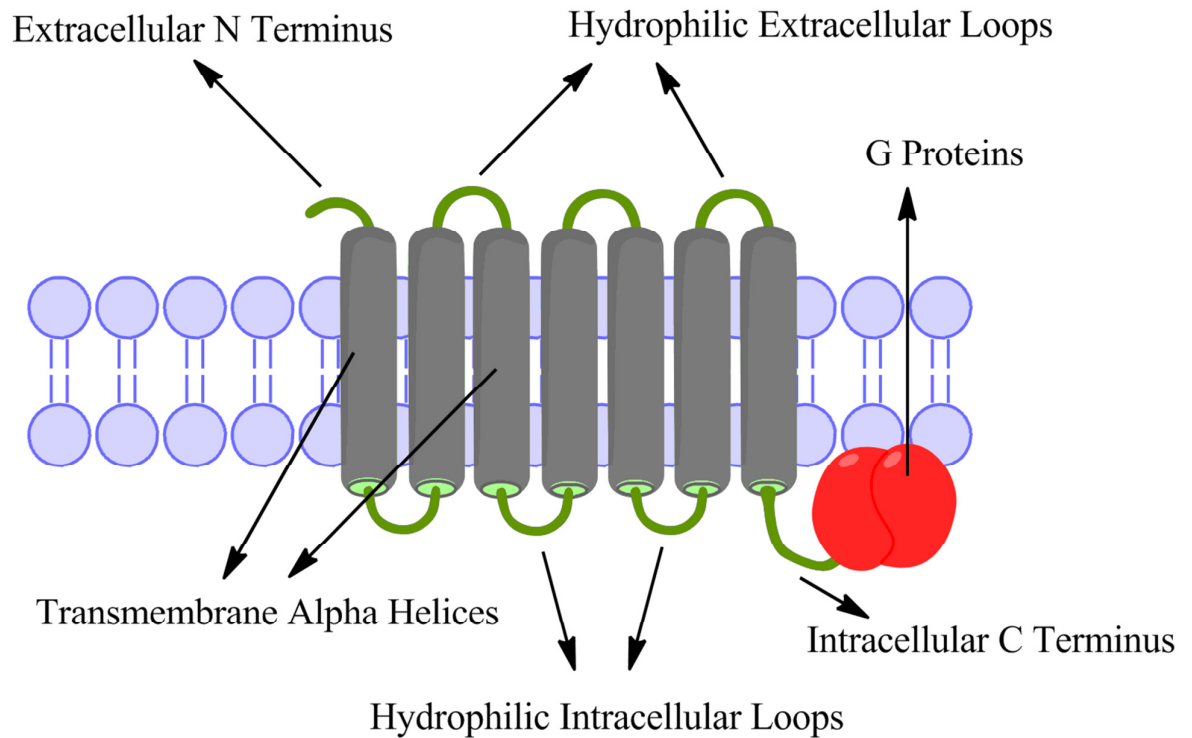


Figure 1: The schematic representation of GPCR structure.

GPCR Taxonomy

One of the classification systems for GPCRs is GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/taste2, & Secretin). In this system, receptors are categorized into five families: Glutamate, Rhodopsin, Adhesion, Frizzled/taste2, and Secretin. According to this comprehensive system, heptahelical receptors in this family have a common ancestor and they are evolved via exon shuffling and gene duplication (Luttrell 2006).

In another classification system, A through F classification system of Kolakowski (Kolakowski 1994), which is widely used, the GPCRs are grouped into six families, which three of them (Families A, B, and C) include most of identified human GPCRs:

- 1) Family A: the largest family, contains the rhodopsin-related GPCRs, protease-activated receptors, the receptors for glycoprotein hormones, biogenic amines and other small nonpeptide ligands, opioids, chemokines, and other small peptides (Luttrell 2008). The receptors for well-known ligands such as acetylcholine, adrenaline and noradrenaline, dopamine, Follicle-stimulating hormone and Luteinizing hormone are within this family (Foord et al. 2005). The arginine in the Asp-Arg-Tyr (DRY) sequence resided in the cytoplasmic part of 3rd membrane spanning α -helix (TMIII) is the only conserved amino acid among all the members of this family (Gether 2000). The DRY sequence and one Asp in 2nd membrane spanning α -helix are necessary for activation of these family receptors (Bockaert and Pin 1999).
- 2) Family B: the next largest family includes receptors that bind to higher-molecular-weight peptide hormones, such as parathyroid hormone, secretin, calcitonin, amylin, urocortins, adrenomedullin and glucagon (Foord et al. 2005; Luttrell 2008).
- 3) Family C: includes the calcium-sensing receptor, the γ -aminobutyric acid B (GABA_B) receptor, and the metabotropic glutamate receptors (Foord et al. 2005; Luttrell 2008).
- 4) Class D: includes fungal pheromone receptors. This class is exclusive to fungi (Davies et al. 2008).

- 5) Class E: includes cAMP receptors. This class is found only in *Dictyostelium* (Davies et al. 2008).
- 6) Class F: includes Frizzled/smoothened family receptors (Davies et al. 2008).

The Heterotrimeric G Proteins

The second component of GPCR signaling system is the heterotrimeric G proteins. The heterotrimeric G proteins are a family of GTPase that have a common multi-subunit structure. They are constituted of a 39 to 52 kDa α (or $G\alpha$) subunit (Luttrell 2006) that has GTP-binding and intrinsic GTPase activity to hydrolyze GTP to GDP and a tightly bound heterodimeric $\beta\gamma$ (or $G\beta\gamma$) subunit (Gainetdinov et al. 2004) that is linked to inactive GDP-bound α subunit noncovalently. Sixteen $G\alpha$ subunits and five $G\beta$ subunits are discovered in humans (Luttrell 2006; Denis et al. 2012), and most of the $G\alpha$ can bind to $G\beta$ subunits and form stable complexes in vitro (Luttrell 2006).

The $G\alpha$ subunits have two important domains: an α -helical domain inserted into the Ras-like domain and a GTP–GDP binding domain resembling a p21^{ras} in structure. None of them have membrane-spanning domains, but all are anchored to the plasma membrane. The N-terminus and C-terminus of $G\alpha$ subunits are involved in $G\beta\gamma$ subunit binding, and both receptors and effectors association (Luttrell 2008). All of the $G\beta$ subunits are 36 to 40 kDa in size (McIntire 2009), and have a shared three-dimensional structure with an N-terminal coiled-coil structure binding tightly to $G\gamma$ subunit. The remainder of the $G\beta$ protein is similar to a seven-bladed propeller, with each blade composed of interlocking β -sheets. All of $G\gamma$ subunits are 7–8 kDa and have

a C-terminal Cys-Ala-Ala-x motif, which is a site for prenylation. Prenylation is a lipid modification essential for membrane localization (Luttrell 2006). There are 12 identified G γ subunits in humans (Luttrell 2008; Denis et al. 2012).

The classification of G proteins is based on their α subunits. There are 16 identified α subunit genes in mammals that could express 20 to 23 distinct G α subunit proteins by alternative splicing (Luttrell 2008). They are classified, based on their sequence and functional similarities, into four subfamilies: G α s, G α i, G α q & G α 12 (Gainetdinov et al. 2004).

- 1) The subfamily of G α s includes the olfactory α subunit, G α olf, and the cyclase-stimulatory α subunit, G α s (Luttrell 2006).
- 2) The G α i subfamily contains G α o; two isoforms of the retinal α subunit, gustducin or G α gust; transducin or G α t; the taste α subunit; the adenylate cyclase-inhibitory α subunits, G α i1, G α i2, G α i3, and and G α z, an α subunit whose function is not well discovered (Luttrell 2006).
- 3) The subfamily of G α q contains two α subunits regulating PLC (Phospholipase C) activity, G α q and G α 11, as well as G α 14 and G α 15 (Luttrell 2006).
- 4) The G α 12 subfamily includes G α 12 and G α 13, that activate Rho family small GTPases to influence cellular growth, and regulate cytoskeletal assembly and rearrangement (Luttrell 2008).

These subfamilies have different and sometimes opposite roles and functions. For example, the activated G α 12 subunits turn on guanine nucleotide exchange factors for the small GTP-binding protein Rho, and activated G α q subunits stimulate

phospholipase C- β (PLC β), or activated G α s subunits stimulate adenylyl cyclases, but activated G α i subunits prevent adenylyl cyclases (Gainetdinov et al. 2004).

The N-terminal glycine (Gly) residue of G α i and G α o serves as a site for addition of the fatty acid myristate, necessary for membrane localization, and except G α t, all G α subunits are modified by palmitoylation of one or more N-terminal cysteine residues, that is critical for membrane association (Luttrell 2006).

The G Protein-Regulated Effector

The third component of the GPCR signaling system is the G protein-regulated effector. It is usually an ion channel or an enzyme, whose activity is regulated by both GTP-bound G α and G $\beta\gamma$ subunits (Luttrell 2006). The binding of ligand to the extracellular domains of GPCRs stimulates the binding of receptors to heterotrimeric G-protein via intracellular regions and function as the guanine nucleotide exchange factors (GEFs) to activate them. The activated G proteins become free from GPCRs to stimulate or prevent the activity of effectors (Figure 2) (Perez and Karnik 2005; Luttrell 2008). The effectors could be antiporters and ion channels such as Na⁺/H⁺ exchanger and the calcium and potassium channels, or enzymes such as phospholipases C and D, ERK (Extracellular-Signal-Regulated Kinase), p38 and JNK (c-Jun N-terminal Kinase) mitogen-activated protein kinases, and cyclic GMP phosphodiesterase (Bouvier 2001). Adenylate cyclases are one of the most known G protein-regulated effector enzymes. All of Adenylate cyclases are stimulated by G α s. Also, G $\beta\gamma$ subunits or calcium-calmodulin can stimulate some of adenylate cyclases only in the presence of activated G α s. Against the stimulatory effect of G α s, G $\beta\gamma$ and calcium-calmodulin; there is the

inhibitory effect of G α i, G α o and intracellular calcium. Some, but not all, adenylylate cyclase isoforms are inhibited by G α i, G α o and intracellular calcium. Another mechanism for modulation of some adenylylate cyclases activity is through phosphorylation via the second messenger-dependent protein kinases, protein kinase A (PKA; cAMP-dependent protein kinase) and protein kinase C (PKC). Adenylylate cyclases catalyze the synthesis of intracellular second messenger cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Then cAMP regulates the activity of certain cAMP-regulated guanine nucleotide exchange factors, e.g. Epac1 (Exchange Protein Activated by cAMP 1) and Epac2 (Exchange Protein Activated by cAMP 2), activating low-molecular weight GTPases. In addition to cAMP-regulated guanine nucleotide exchange factors, cAMP regulates the activity of cAMP-dependent protein kinase (PKA) (Luttrell 2006).

Another target group of G proteins includes the four isoforms of PLC β . This enzyme catalyzes the generation of two intracellular second messengers, inositol 1,4,5-trisphosphate (IP $_3$) and diacylglycerol (DAG), from membrane phosphatidylinositol hydrolysis. The roles of DAG and IP $_3$ are to control several isoforms of PKC by the help of calcium, and regulate the efflux of calcium from the endoplasmic reticulum, respectively (Luttrell 2006).

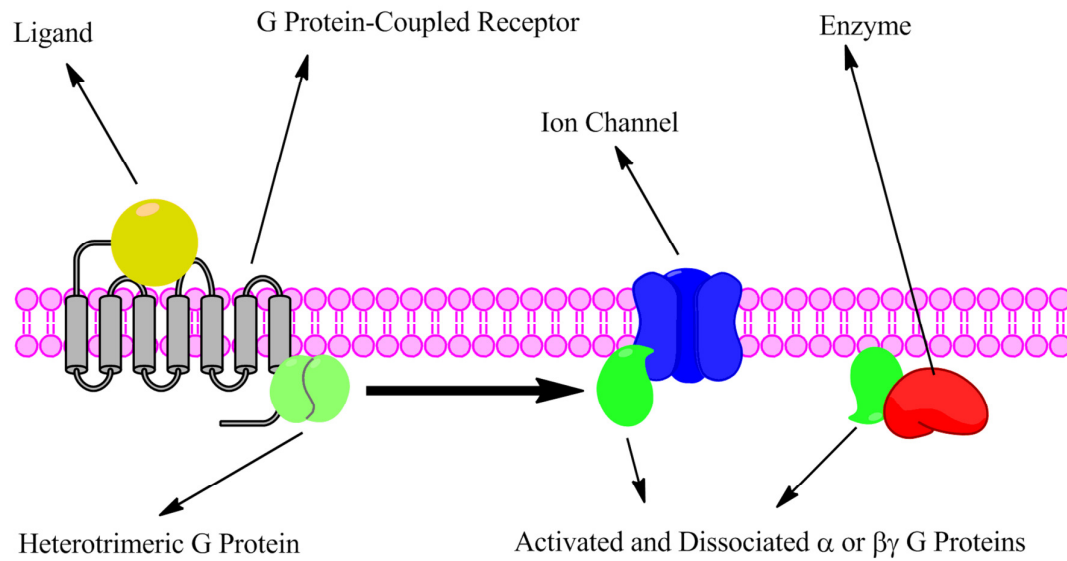


Figure 2: Components of GPCR signaling.

Both $G\alpha$ subunits of the G_q family and $G\beta\gamma$ subunits stimulate the activity of $PLC\beta_1$, $PLC\beta_2$ & $PLC\beta_3$ independently. $G\beta\gamma$ subunits also regulate the GRKs (e.g. GRK2 and GRK3) directly. The free $G\beta\gamma$ subunits of activated G proteins could bind to C-terminal regulatory domains of GRKs, permit the enzyme to translocate from the cytosol to the plasma membrane to phosphorylate GPCR in order to desensitize it (Luttrell 2006).

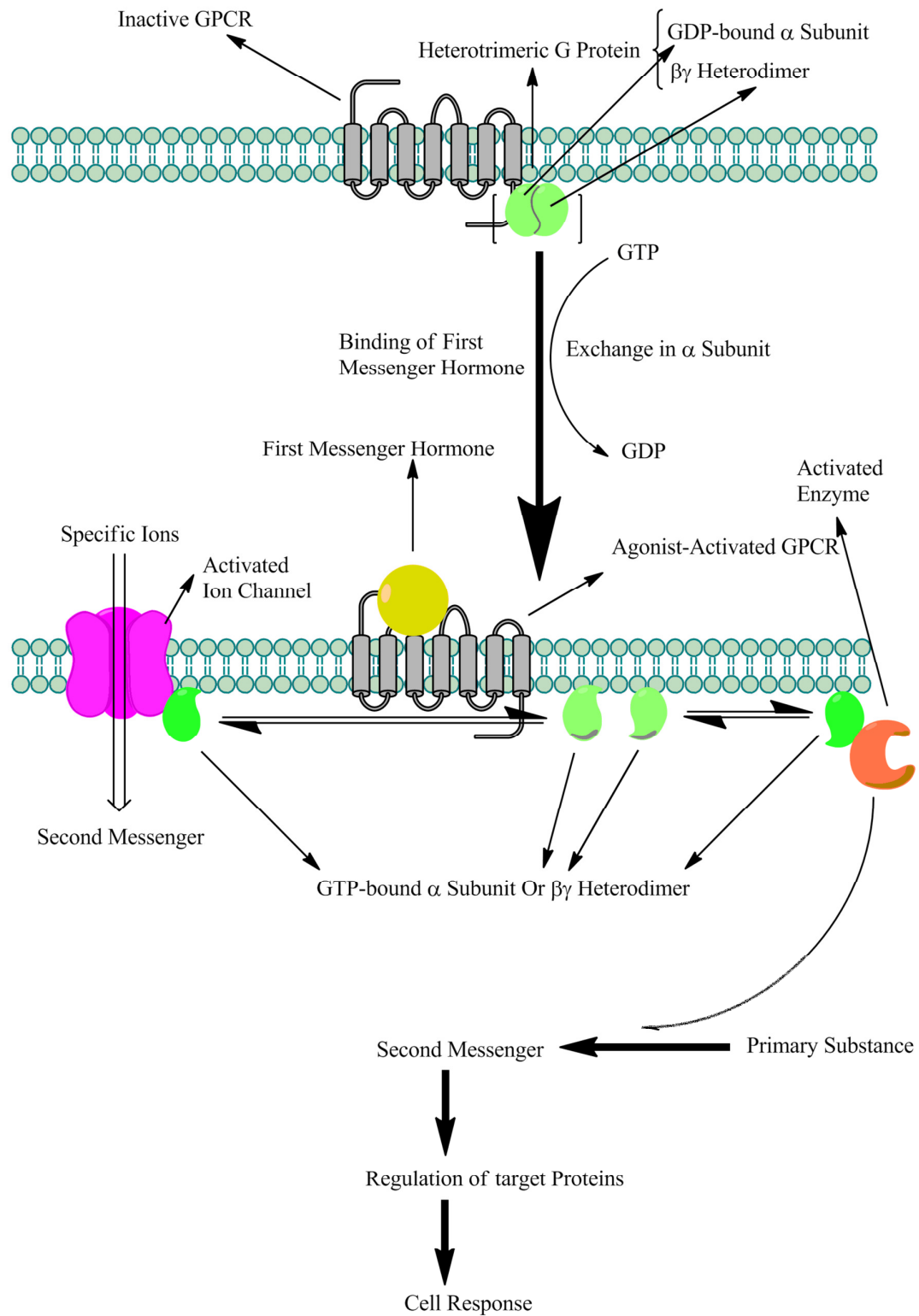
The Canonical Model for GPCR Signaling

GPCRs are guanine nucleotide exchange factors (GEFs) for heterotrimeric guanine nucleotide-binding (G) proteins (Gainetdinov et al. 2004). The GEF ability of GPCRs for G proteins is the basis of the canonical model of their signaling. According to this model, upon agonist exposure, GEF ability of GPCRs for G proteins is activated in

order to trigger intracellular cascades by stimulating effector enzymes or ion channels. By this property, one activated GPCR could activate multiple G proteins. For example, activated rhodopsin by a single light photon stimulates the activation of lots of transducin molecules in the rod outer segment. Then the free subunits of G protein activate ion channels or enzyme effectors to trigger the cascade of second messengers controlling downstream targets (Luttrell 2006). According to this model, in the inactive state, the α subunit is associated tightly to GDP and to $\beta\gamma$ complex to form a trimeric protein complex (Gainetdinov et al. 2004). When proper ligands bind to GPCRs and stimulate them, it causes the conformational changes of the intracellular regions of heptahelical receptors, to trigger the interaction of receptors with coupled G proteins (Hall et al. 1999a). By this interaction, G proteins undergo conformational changes to comfort release of GDP and binding of GTP to the α subunit (Gainetdinov et al. 2004) to dissociate it from $G\beta\gamma$ subunits. Then the activated subunits of G protein bind to trigger or inhibit intracellular cascades (Hall et al. 1999a).

Addition to this model, GPCRs function in several protein-protein interactions to transmit signals intracellularly even independent of G protein activation. Actually, it is proposed that some of non G protein-coupled heptahelical receptors, e.g. frizzled receptors, are the evolutionary branches of the phylogenetic tree of GPCRs (Luttrell 2006).

Figure 3: The Canonical Model for GPCR Signaling: According to this model, attachment of a ligand or "first-messenger" hormone to the extracellular or transmembrane domains of a GPCR causes conformational changes passed via the intracellular domains of receptor to its coupled heterotrimeric G proteins to stimulate catalyzing of GTP exchange for GDP on the α subunit. Then the GTP-bound α subunit dissociates from the $\beta\gamma$ heterodimer, in order to control the enzymes' activities such as phospholipase C (PLC) isoforms, adenylyl cyclases, and ion channels to produce "second messengers". Then, these second messengers regulate the activity of protein kinases participating in intermediary metabolism, until the hydrolysis of GTP to GDP by GTPase activity of α subunit and returning of G protein to the inactive heterotrimeric state (Luttrell 2006). (See next page)



Negative Regulation of GPCR Signaling

GPCRs are very dynamic cell components and their active and inactive forms are in balance with each other (Gether 2000). They could regulate their sensitivity and responsiveness to their ligands (Claing et al. 2002). Their agonist stimulation is dependent on concentration of the ligand. Thus, such as other receptors in body, the primary way for regulation of GPCR signaling is controlling the concentration of the ligand (Gainetdinov et al. 2004). Other specific ways involved in negative regulation of GPCR signaling are receptor desensitization, sequestration, and downregulation, degradation of second messengers by enzymes, and inactivation of G protein by GTPase-activation proteins (GAP). These serial actions control the responsiveness of GPCR, cause the GPCR removal from the plasma membrane, G protein uncoupling from GPCR, degradation or recycling of GPCR, and decrease the synthesis of new GPCRs due to decline in their transcription and translation (Ferguson 2001; Luttrell 2006). In the case of degradation of second messengers, for example cAMP phosphodiesterases (PDE), phosphatidylinositol phosphatases, and diacylglycerol kinases degrade the second messengers. Sometimes these enzymes are brought near the activated effector enzymes via binding to other GPCR regulatory proteins to degrade the second messengers easier. For example, β 2-adrenergic receptors stimulation causes the recruitment of PDE4D3 and PDE4D5 to the receptors by binding directly to β -arrestins, enhancing the degradation of cAMP to terminate the activity of membrane-associated PKA (Luttrell 2006).

GPCR Desensitization: Heterologous vs Homologous

Within seconds to minutes of binding of ligand to GPCR, desensitization begins by uncoupling of stimulated receptor from G proteins caused by its serine (Ser)/threonine (Thr) phosphorylation via GRKs promoting binding of β -arrestins to phosphorylated receptor, called homologous desensitization, or via second messenger-dependent protein kinases (PKA or PKC), called heterologous desensitization (Figure 4) (Bohm et al. 1997; Claing et al. 2002; Luttrell 2006). It is believed that some of C-terminal motifs of GPCRs are critically involved in controlling the fate of the receptor (Claing et al. 2002), and the cell environment and conformation of the receptor are the factors involved in the regulation of the level of desensitization (Ferguson 2001).

In heterologous desensitization, the Ser and Thr residues of the C-terminal tail domains and cytoplasmic loops of most of GPCRs are phosphorylated by second messenger-dependent protein kinases, impairing the physical coupling of G protein-GPCR. The second messenger-dependent protein kinases such as PKC and PKA are activated by second messengers such as diacylglycerol, Ca^{2+} , and cAMP and could transfer the γ -phosphate group of ATP to GPCRs. This type of desensitization is called heterologous desensitization, because activation of receptor by binding of ligand to it (agonist exposure) is not needed for receptor phosphorylation. Hence, the activated second messenger-dependent protein kinases can phosphorylate both agonist-bound and unbound GPCRs, containing other ligands' receptors (Ferguson 2001; Luttrell 2006). For example, adenylyl cyclase becomes active by G_s and catalyzes the synthesis of cAMP, the second messenger, after agonist-stimulation of β -adrenergic receptors. The increase in the concentration of cAMP results in the activation of PKA. Then PKA

could phosphorylate the β -adrenergic receptors as well as other GPCRs in order to promote their desensitization (Gainetdinov et al. 2004).

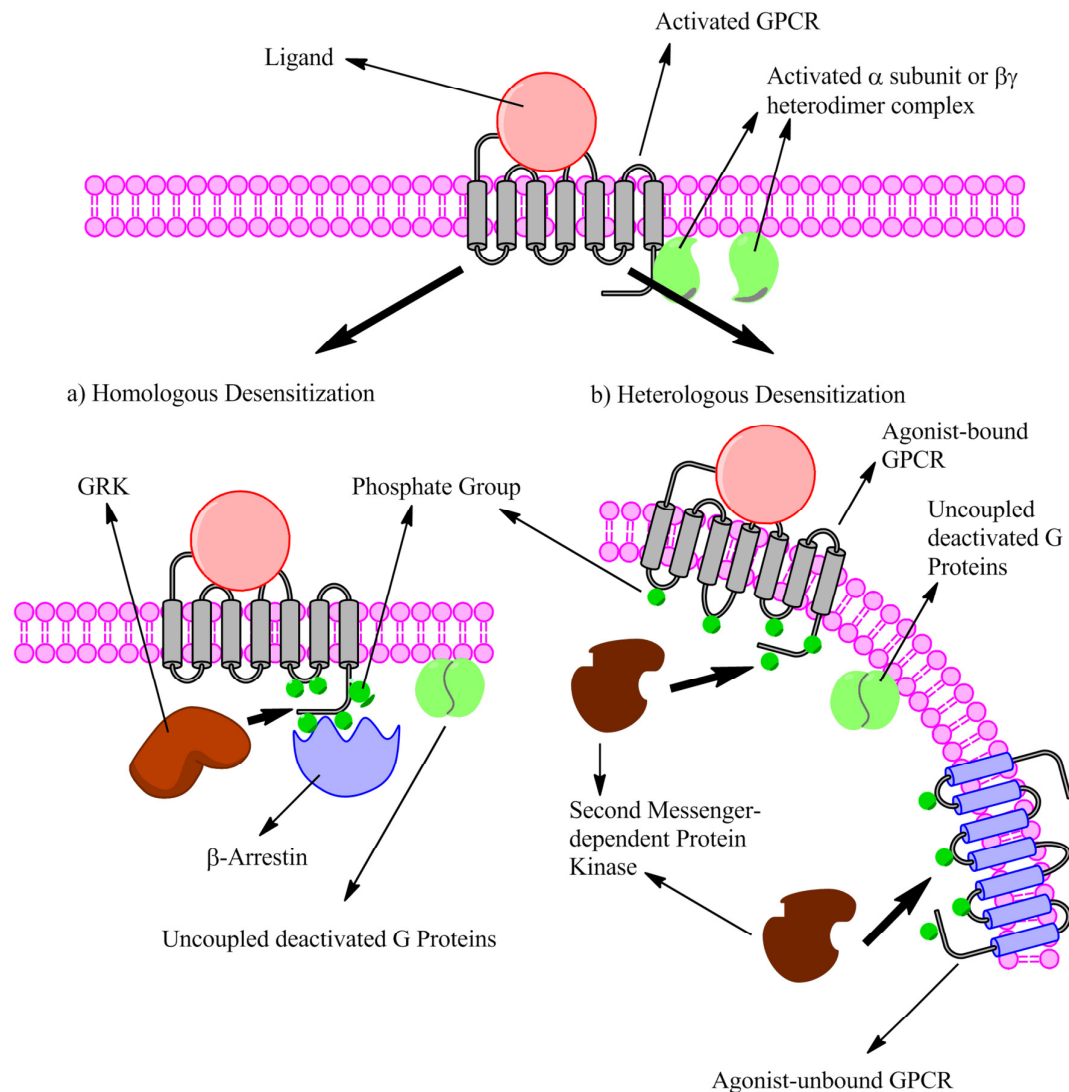


Figure 4: Two types of GPCR Desensitization: a) Homologous Desensitization, b) Heterologous Desensitization.

The homologous desensitization has two steps (Luttrell 2006). In the first step, GRKs (G protein-coupled Receptor Kinases) phosphorylate the GPCRs on Ser and Thr

residues in the i3 loop, such as m2 muscarinic acetylcholine receptor (m2 mAChR), and carboxy terminal tail, such as β 2-adrenergic receptor (β 2AR). This phosphorylation promotes the binding of a β -arrestin, or arrestin in the case of rhodopsin signaling (Ferguson 2001; Lefkowitz 2007), to GPCR in the second step. β -arrestins are proteins that play a role in physical uncoupling of G proteins from activated GPCRs. It is important to notify that GRK-mediated phosphorylation of GPCRs possesses little direct influence on receptor–G protein coupling and increase the affinity of arrestins to receptor (Luttrell 2006).

Sequestration

The endocytosis or internalization of GPCRs is called receptor sequestration, which lasts more than receptor desensitization. It occurs in most, but not all, GPCRs, and its mechanism could be blocked by dynamin inhibition for many of GPCRs. Dynamin is a large GTPase, and is required for the removal of clathrin-coated vesicles from the plasma membrane. As described in previous sections, homologous desensitization occurs through the phosphorylation of receptor by GRK and binding of β -arrestin. After binding of β -arrestin to phosphorylated GPCR, RxR and LIEF/L motifs near the C-terminal of β -arrestin associate with β 2 adaptin (β -subunit of the heterotetrameric clathrin adaptor protein 2 (AP-2) (Ferguson 2001; Claing et al. 2002)) and clathrin respectively, causing agonist-occupied GPCR to cluster in clathrin-coated pits to pinch off vesicles from the plasma membrane by the help of dynamin (Luttrell 2006). The heterotetrameric clathrin adaptor protein AP-2 has four subunits: α -adaptin with molecular weight of 100 kDa that could bind to dynamin, Eps15 (Epidermal

growth factor receptor Pathway Substrate 15) and clathrin; β 2-adaptin with molecular weight of 100 kDa that could associate with clathrin and dileucine motifs and is required for formation of clathrin coat; Finally, one μ 2- and one δ 2-subunit with molecular weights of 50 and 17 kDa, respectively (Ferguson 2001). In vitro studies have shown that only β -arrestin 1 & 2 could bind to clathrin. The C-terminal region of β -arrestin1, the residues 371-379, or more accurately 373-377 of β -arrestin2 and the residues 89-100 within the N-terminal globular region of the heavy chain of clathrin are critical for β -arrestin-clathrin association. Thus, β -arrestin has a dual function in GPCR desensitization and sequestration (Ferguson 2001; Claing et al. 2002).

GPCRs can be classified to two classes according to their pattern of interaction with the two isoforms of β -arrestin, β -arrestin 1 & β -arrestin 2 (Oakley et al. 2000; Luttrell 2006):

“Class A” GPCRs: contains the μ -opioid, endothelin A, dopamine D1A, and β 2 and α 1B adrenergic receptors. They bind to β -arrestin 2 with higher affinity than β -arrestin 1, however their interaction is transient. The complex of receptor- β -arrestin dissociates, after endocytosis of the GPCR. Then the sequestered receptor translocates to an endosomal pool, whereas the β -arrestin recycles to the plasma membrane (Luttrell 2006).

“Class B” GPCRs: includes the vasopressin V2, angiotensin AT1a, neurotensin 1, thyrotropin-releasing hormone and neurokinin NK-1 receptors. They bind to β -arrestin 1 and β -arrestin 2 with equal affinity. Their interaction is not transient. Therefore, they could form stable complexes with β -arrestin, leading the complex to endosomes (Luttrell 2006). Then some of GPCRs of this class are directed to lysosome to be

degraded, such as endothelin B and protease-activated receptors (PAR). The C-terminal regions of PAR are responsible for their directing to lysosomes (Ferguson 2001).

It is necessary to mention that under special conditions, β -arrestin-dependent desensitization of GPCR and receptor internalization are separate processes, because of the following evidences (Luttrell 2008):

- 1) Mutants of β 1-adrenergic receptor that are deficient in GRK phosphorylation sites show impaired β -arrestin recruitment and clathrin-mediated internalization, but could sequester through PKA-mediated phosphorylation and caveolae-dependent endocytosis (Luttrell 2008).
- 2) GRK-mediated phosphorylation and β -arrestin 1 binding are required for desensitization of PAR1 receptor, but not for clathrin-mediated internalization (Luttrell 2008).
- 3) At the exposure of β 2-adrenergic receptors to ligand, both of β -arrestin 2 and the receptor are transiently and rapidly ubiquitinated (Luttrell 2008). The E3 ubiquitin ligase, MDM2 (MURINE DOUBLE MINUTE 2), binds to β -arrestin directly and catalyzes its ubiquitination. This ubiquitination is critical for internalization of β 2 receptor. But the β 2 receptor ubiquitination, catalyzed by unidentified ubiquitin ligase, is required for degradation of the receptor, not its endocytosis (Luttrell 2006).

Arrestins

The first arrestin, termed S antigen, was identified as a 48 kDa protein involved in a degenerative eye disease (Krupnick and Benovic 1998). In vertebrate, there are two known arrestins in the retina, called visual arrestin (S antigen or arrestin 1) and cone arrestin (C-arrestin, X-arrestin, or arrestin 4 (Gainetdinov et al. 2004)), whose role is to control function of photoreceptors (Luttrell 2008). They also are expressed in the pineal glands at lower levels than the retina (Ferguson 2001). There are two known non-visual arrestins, called β -arrestin 1 (arrestin 2) and β -arrestin 2 (arrestin 3), whose function is to control most of the other GPCRs' activity in the genome (Luttrell 2006). The structures of β -arrestins are highly homologous: 78% of their amino acid residues are shared with each other (Claing et al. 2002). By cloning cDNA (complementary DNA) of β -arrestin 1, it was discovered that this protein is composed of 418 amino acids (Krupnick and Benovic 1998).

The visual arrestins are made up of two major functional domains, the N domain and C domain, which each constitutes a seven-stranded β sandwich and are located between residues 8-180, and residues 188-362 respectively (Ferguson 2001). The non-visual arrestins also have the similar molecular structure that the two major domains are joined together by a short hinge. The N-terminal residues of Lys14 and Lys15 are supposed to direct the phosphorylation recognition domain of arrestins toward the phosphate groups bound to the GPCR (Moore et al. 2007).

Generally, the β -arrestins are expressed in all tissues, and they are highly expressed in the spleen and brain (Luttrell and Lefkowitz 2002). β -arrestin 1 is less widely expressed than β -arrestin 2 in the brain of rat. Although, the expression level of

β -arrestin 2 is more in the hypothalamus, hippocampus and striatum, but the expression level of β -arrestin 1 is higher in the olfactory bulb and tract (Pierce and Lefkowitz 2001). All four arrestins associate specifically to agonist-occupied, GRK-phosphorylated GPCRs to uncouple the G protein from receptor (Luttrell 2006). The N-terminal half of all arrestins are responsible for recognizing these GPCRs (Krupnick and Benovic 1998). In addition to this role, β -arrestins, not the visual arrestins, have binding motifs for clathrin and the β 2-adaptin subunit of the AP-2 complex in their C-terminal tail that cause to target GPCRs to clathrin-coated pits for receptor sequestration. These additional interactions are the reason to determine the two subfamilies of arrestin (Luttrell 2006). The binding motifs for clathrin and the β 2-adaptin subunit of the AP-2 complex are an LIEF sequence, resided between residues 374 and 377, and an RxR sequence, located between arginine residues R394 and R396 of β -arrestin 2 (Claing et al. 2002; Luttrell and Lefkowitz 2002). These arginine residues (R394 & R396) are also available in β -arrestin 1 and are involved in association of β -arrestin 1 with β subunit of the AP-2 complex. It should be noticed that β -arrestins interact with clathrin and AP-2 independently (Claing et al. 2002). Moreover, β -arrestins participate in translocation of cytosolic protein such as c-Src (Cytoplasmic or Cellular Src (Sarcoma)) and PDEs to the activated-GPCR in order to facilitate its desensitization. These enzymes function to phosphorylate GRK2 and degrade cAMP, respectively to increase the activity of GRK2 and suppress the GPCR-triggered signaling (Kohout and Lefkowitz 2003). It is important to mention that there are other β -arrestin- & clathrin-independent mechanisms for endocytosis of GPCRs, containing Caveolae (Pierce and Lefkowitz 2001).

Likewise, β -arrestins participate in internalization and regulation of cell membrane receptors other than classical GPCRs, such as growth factor and lipid receptors, Notch, Smoothed (Smo), Frizzled (Fz), low-density lipoprotein (LDL) receptors, Toll-like/interleukin-1 (TL/IL-1) receptors, insulin-like growth factor-1 (IGF-1) receptor, and transforming growth factor- β (TGF- β) receptors (Moore et al. 2007). Besides, β -arrestins are involved in signaling cascade of GPCRs. For example, they can make complexes with Src family tyrosine kinase to regulate internalization of GPCRs. The proline (Pro)-rich PXXP domains of β -arrestin 1, resided at N-terminal residues 88-91 and 121-124, are responsible for interaction with Src homology 3 (SH3) motif of Src kinase (Luttrell and Lefkowitz 2002). Additionally, arrestins could function as the adaptors for MDM2, ARNO (ARF Nucleotide Binding Site Opener) and NSF (N-ethylmaleimide-Sensitive Factor) (Gainetdinov et al. 2004). They could also serve as the scaffolds for two Mitogen-activated protein kinases (MAPK) pathways: extracellular-signal-regulated kinase (ERK) cascades (by both of β -arrestin 1 & 2) and c-Jun N-terminal Kinase type 3 (JNK3) cascade (only by β -arrestin 2) (Pierce and Lefkowitz 2001). Moreover, β -arrestins could interact with numerous E3 ligases, such as Nedd4-1. For example, β -arrestin 1 and Nedd4-1 are involved in regulation of the Na⁺/H⁺ exchanger 1 (NHE1) ubiquitination, internalization and function (Simonin and Fuster 2010). Another E3 ubiquitin ligase that β -arrestins (here β -arrestin 2) could interact with to become ubiquitinated is MDM2. This ubiquitination is critical for rapid endocytosis of β 2-adrenergic receptor (Bockaert et al. 2004). The functions of β -arrestins are not restricted to the cytoplasm, but also into the nucleus: The other function of β -arrestins is the regulation of gene transcription. For example, β -arrestin 1 has been observed in nucleus. It is suggested that β -arrestin 1 regulates expression of

specific genes (Moore et al. 2007). It is involved in some congenital diseases as well: As an example, in Oguchi disease, a congenital form of night blindness, the arrestin gene has mostly lost 1 bp of its sequence homozygously (Bohm et al. 1997).

β-Arrestin-independent Internalization Mechanisms

Most of the GPCRs are internalized via a β-Arrestin- and dynamin-dependent internalization mechanism. Some of these GPCRs are α2-adrenergic, β2-adrenergic, μ-opioid, chemokine CXCR4, V2 vasopressin, and somatostatin, and thyroid-releasing receptors (Tan et al. 2004). In addition to this mechanism, there are other internalization pathways for GPCRs:

1) β-Arrestin-independent, Dynamin-dependent endocytosis pathway:

There are some receptors, such as VIP1 (Vasoactive Intestinal Peptide type 1) and the two types of endothelin receptors including Endothelin A (ETA) & Endothelin B (ETB), which might be internalized from the plasma membrane without the β-Arrestin involvement, but by participation of caveolae. Caveolae participate in receptor endocytosis and signal transduction and bind to dynamin via its neck (Claing et al. 2002). It is important to mention that there are two machineries for endocytosis of GPCRs: caveolae and clathrin-coated pits. The phosphorylation of GPCRs on specific sites chooses which one of these machineries becomes active. For example, in the case of β1-AR receptor, GRK-mediated phosphorylation leads to internalization of receptor via clathrin-coated pits, whereas PKA-mediated phosphorylation results in activation of caveolae internalization machinery (Tan et al. 2004).

2) β -Arrestin-independent, Dynamin-independent endocytosis pathway:

Some of the GPCRs such as the bradykinin type 2, the *N*-formyl peptide, and the M2 muscarinic receptors that are sequestered from cell membrane with unidentified mechanisms independent of β -arrestin and dynamin (Claing et al. 2002).

Resensitization, Recycling, and Downregulation

There are 4 pathways for desensitization and resensitization of GPCRs:

1) GPCRs such as β 3-adrenergic receptor (β 3AR) that are not sequestered and internalized upon ligand exposure (Ferguson 2001).

2) GPCRs such as β 2AR that are endocytosed in a β -arrestin-dependent manner, but they dissociate from β -arrestin soon after formation of endocytotic vesicles. Therefore, they are internalized without β -arrestin bound, and are dephosphorylated and returned to plasma membrane (Anborgh et al. 2000; Ferguson 2001).

3) GPCRs such as neurokinin 1 receptor that are sequestered with β -arrestin bound, but after the dissociation of β -arrestin from the receptors in endosomes, they are dephosphorylated and returned to plasma membrane rapidly (Ferguson 2001).

4) GPCRs such as vasopressin V2 receptors that are sequestered with arrestin bound, similar to third group, but they are directed to lysosomes for degradation or they are recycled back to plasma membrane very slowly (Ferguson 2001; Luttrell 2006).

As it was briefly explained above, pathways 3 & 4 are two pathways for receptor recycling in the β -arrestin dependent manner: rapid and slow recycling. In

“rapid recycling” the receptor returns to the plasma membrane after endocytosis. But in “slow recycling” the receptor undergoes slow recycling or degradation. The major factor that determines the recycling pathway of internalized GPCR is the stability of the β -arrestin-receptor complex. For example, “Class A” GPCRs such as β 2AR separates from β -arrestin during endocytosis and goes to acidified endosomes that are full of GPCR-specific protein phosphatase PP2A (Protein Phosphatase 2A). In these endosomes, the β 2AR becomes dephosphorylated, dissociates from ligand and goes back to the plasma membrane by recycling endosomes. However, in “Class B” GPCRs, such as V2 receptors, they prefer to be degraded, because they form stable complex with β -arrestin. It is good to know that the C-terminal tails of the receptors determine its recycling pathway, as changing the C-terminal tails of V2 and β 2 adrenergic receptors with each other, reverses their dephosphorylation and recycling patterns (Luttrell 2006).

Another component of GPCR internalization machinery is the Rab (Ras-related GTP-Binding protein) family of small Ras-like GTPases. The Rab GTPases control the budding, transport, docking, and fusion of intracellular vesicles. The endocytosis of GPCR and their transit between early, late, and recycling endosomes and lysosomes are controlled by several members of this family, containing Rab4, Rab5, Rab7, and Rab11 (Luttrell 2006).

Positive Regulation of GPCR Signaling

The signaling of GPCRs could be augmented by some protein-protein interactions that affect binding of ligand, G proteins and effectors to the receptor. These protein-protein interactions contain the association of different scaffolding proteins to the C-terminal and i3 loop of GPCR, RAMPs (Receptor Activity-Modifying Proteins)-GPCR interaction, and GPCR dimerization/oligomerization (Figure 5) (Luttrell 2006).

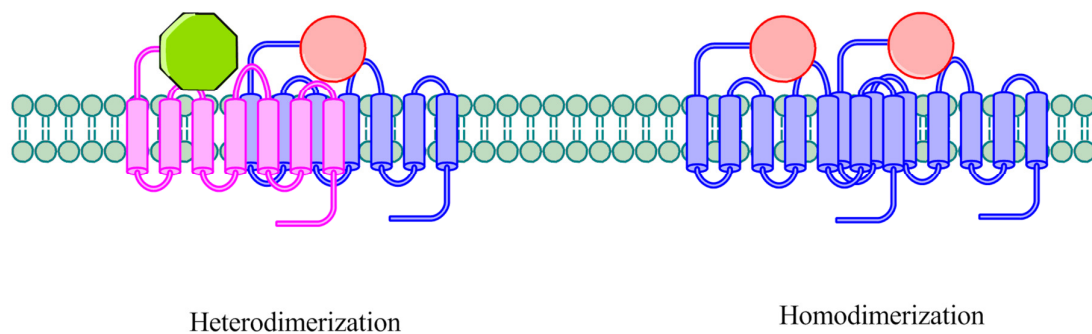


Figure 5: Homodimerization vs Heterodimerization of GPCR.

Receptor Activity Modifying Proteins (RAMPs)

Receptor Activity Modifying Proteins (RAMPs) are 148- to 174-amino acid glycoproteins and are the members of a family of single membrane-spanning proteins. There are three RAMP proteins identified yet, called RAMP-1, -2, & -3. Their intracellular domains are short, but their extracellular tails are long. The RAMPs are critically involved in regulating the function, trafficking and the ligand specificity of calcitonin receptor, the calcitonin-receptor like receptor (CRLR) and perhaps other

GPCRs. They also enhance maturation and cell membrane delivery of the receptors (Bouvier 2001; Luttrell 2006; Jacob et al. 2012).

GPCR Oligomerization

Many GPCRs could bind to each other and form homodimers, heterodimers (Figure 5), or higher order multimers (oligomers) (Luttrell 2006) leading to different signaling cascades (Gainetdinov et al. 2004). For example, dimerization of the GABA_B receptor is the prerequisite for trafficking of nascent receptor to the plasma membrane (Luttrell 2006). GPCR dimerization and oligomerization also play important roles in coupling and stimulation of G proteins by the GPCR and targeting of the receptors to plasma membrane (Milligan 2007).

GPCR-related Diseases

As described before genes encoding GPCRs consist ~3% of the human genome (Insel et al. 2007). Additionally, GPCRs are the largest superfamily of plasma membrane receptors, and 50% of all therapeutic agents target them, it is not surprising that they could be related to vast and diverse diseases (Luttrell 2008). GPCR agonists and antagonists are used in the treatment of diseases of respiratory, central nervous system (CNS), metabolic, urogenital, and cardiovascular systems (Insel et al. 2007). For example, GABA_B receptors are involved in mood disorders or corticotrophin-releasing factor (CRF) receptors exhibit antidepressant and anxiolytic activity (Catapano and Manji 2007).

Different reasons exist for disorders related to GPCRs:

- 1) Mislocalization of GPCRs: As the correct localization of every cell surface receptor is critically involved in its ligand binding and signaling, GPCRs mislocalization could results in some disorders such as hypogonadotropic hypogonadism (HH), developed by accumulation of the mutant gonadotropin releasing hormone (GnRH) receptor in the cell, X-linked nephrogenic diabetes insipidus (NDI), resulting from intracellular accumulation of mutant V2 vasopressin receptor, and retinitis pigmentosa (RP), caused by mutations of ~36 RP genes and the accumulation of mutant rhodopsin in photoreceptors(Tan et al. 2004).
- 2) Unregulated endocytosis of GPCRs: In some cases, the unregulated constitutive endocytosis of a particular GPCR leads to disease. For example, Arg137His mutation within the DRY motif of vasopressin receptor results in constitutive desensitization and endocytosis of the receptor in an arrestin-dependent manner and is associated with familial nephrogenic diabetes insipidus (Perez and Karnik 2005).
- 3) Generation of monoclonal or polyclonal antibodies against GPCR: For example, in Graves disease, the thyroid-stimulating hormone (TSH) receptors are stimulated by immune cells-generated antibodies. It results in a type of hyperthyroidism (Insel et al. 2007).
- 4) GPCR mutation: As explained above, it could be the primary reason of some of the aforementioned reasons and diseases. There is one type of hypogonadotropic hypogonadism occurred by mutations in the gene of GnRH

receptor (GnRHR) (Tan et al. 2004). In other examples, NDI, female infertility and Hirschsprung's disease are the results of multiple mutation within genes of V2 vasopressin receptor, follicle-stimulating hormone (FSH) receptor, and endothelin receptor B, respectively (Insel et al. 2007).

GRKs

Around 1980, Lefkowitz and his colleagues discovered for the first time that the phosphorylation of β -adrenergic receptors is associated with their desensitization (Stadel et al. 1983; Sibley et al. 1984; Sibley et al. 1985; Lefkowitz 2007). A few years later, the kinase responsible for this phosphorylation was discovered and termed the β -adrenergic receptor kinase (β ARK), nowadays named GRK2 (Benovic et al. 1986). The GRK2 was purified from bovine brain and its gene was cloned by Jeff Benovic, a graduate student (Benovic et al. 1989; Lefkowitz 2007). Approximately at the same period of time, another kinase named rhodopsin kinase was identified that involved in inhibiting rhodopsin signaling. As the chemical structure of rhodopsin kinase, today called GRK1 (Luttrell 2006), was very alike with the structure of β ARK, it was suggested that they belong to a family of proteins termed G protein coupled receptor (GPCR) kinases (GRKs) (Lefkowitz 2007), because they phosphorylate GPCRs at both threonine and serine residues residing in their C-terminal region or the 3rd intracellular loop (Delom and Fessart 2011).

Seven GRKs are identified in mammalian cells. GRK1 (rhodopsin kinase) and GRK7 are expressed in rods and cones of retina, respectively (Lefkowitz 2007) and

pinealocytes (Gurevich et al. 2012) and participate in the control of rhodopsin photoreceptors, while GRK2-GRK6 are expressed ubiquitously (Luttrell 2006), except GRK4 that is expressed highly in testis (Ruiz-Gomez et al. 2007) as well as uterus myometrium, brain (Gurevich et al. 2012), kidney and cerebellum, but in lesser extent (Ribas et al. 2007).

Based on sequence similarities and differences, GRKs are categorized in three subfamilies (Ribas et al. 2007): 1) The visual GRK or rhodopsin kinase subfamily including GRK1 and GRK7, 2) The β ARK subfamily containing GRK2 and GRK3 (also termed β ARK1 & β ARK2 respectively (Bohm et al. 1997)), which have approximately 84% sequence homology (Pitcher et al. 1998a), and can be found in endoplasmic reticulum of hepatocytes in addition to cell membrane and cytosol (Bohm et al. 1997), 3) The GRK4 subfamily including GRK4 (also called IT11-A (Bohm et al. 1997)), GRK5, and GRK6, which have approximately 70% sequence homology (Pitcher et al. 1998a). The last subfamily members, such as GRK5 and GRK6, could be found in the nucleus (Gurevich et al. 2012), because they contain a functional nuclear localization sequence (NLS) (Martini et al. 2008). The NLS region of GRK5 gives it the ability to bind to DNA, too (Liu et al. 2010; Gurevich et al. 2012).

GRKs contain three shared domains in their structures (Figure 6): a centrally located ~270 amino acid catalytic domain containing the glycine-X-glycine-X-X-glycine-X-lysine sequence important in binding of GRKs to ATP and the catalytic Asp-Leu-Gly motif, both common among protein kinases, a ~190 amino acid domain located at N-terminal tail which is involved in GRK regulation, recognition of GPCRs and anchoring to intracellular cell surface, and a 105-233 amino acid C-terminal domain which

participates in phospholipid association and is highly variable in structure and length (Bohm et al. 1997; Pitcher et al. 1998a; Pronin et al. 2000; Ribas et al. 2007). For example, the length of C-terminal tail in GRK1, and subfamilies of GRK2 and GRK4 are approximately 100, 230 and 130 amino acids, respectively (Pitcher et al. 1998a). GRK5 C-terminal domain interacts with phospholipids, protein kinase C and calmodulin to regulate GRK5, and contains lots of basic residues and autophosphorylation sites, conserved in GRK5 found in mouse, bovine and human (Chen et al. 2010a). But there are short prenylation sequences in C-terminal tail of GRK1 and GRK7, lipid-binding positively charged regions and palmitoylation sites in GRK4 and GRK6, and pleckstrin homology (PH) motifs in GRK2 and GRK3 associates with G $\beta\gamma$ subunits (Gurevich et al. 2012) and phospholipids to target the kinase to cell surface and contains one C-terminal α -helix and seven β -strands. There is also a ~120 amino acid regulator of G protein signaling (RGS) homology (RH) domain in the N-terminal domain of GRKs with weak homology (~27%) (Ribas et al. 2007). The RGS domain has GTPase activity, hence could decrease the activity of GTP-bound G α subunit (Delom and Fessart 2011). For example, via this RGS domain, GRK2 and GRK3 could bind and sequester the activated GTP-bound G α_q , in order to reduce the signaling of Gq-coupled receptor (Kohout and Lefkowitz 2003). This ability of GRK2 is critical in phosphorylation independent desensitization (Pao and Benovic 2002). This RH domain is followed by a Ser/Thr protein kinase domain (KD) which belongs to the AGC kinase family (Gurevich et al. 2012). The RH domain in GRK2 includes nine α -helices similar to other RGS domains and two extra α -helices originated in amino acids 513-547. The RH domain could associate with both the PH domain and the GRK to participate in regulation of the enzyme activity (Ribas et al. 2007).

The substrates of GRKs do not reveal any vivid consensus sequence, but it has been observed that GRK1 and GRK2 phosphorylate mostly serine and threonine residues close to acidic residues, while GRK5 and GRK6 phosphorylate serine residues adjacent to basic amino acids on the amino terminal side (Pitcher et al. 1998a). GRK2 also could phosphorylate clusters of serine and threonine in α 2A-adrenergic receptors, M2 receptors, M3 receptors, and phosducin (Yoshida et al. 2003).

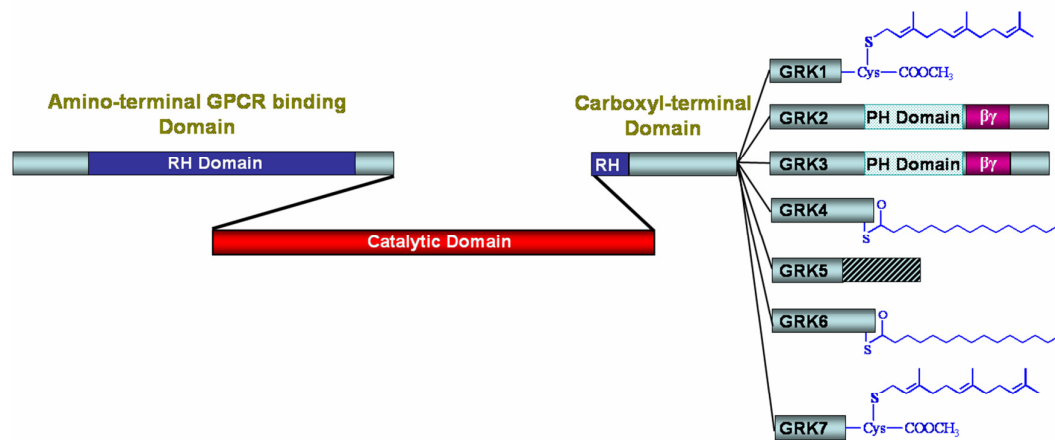


Figure 6: Domains of GRKs: All of GRKs have three main domains including 1) an Amino-terminal tail (N-terminal, ~190 residues) important for GPCR binding and recognition, regulation of GRK and anchors to intracellular cell surface, 2) a catalytic domain (central, ~270 residues) binds to ATP and phosphorylates GPCRs on Ser/Thr, and 3) a carboxy-terminal domain (~105-233 residues) that associates with phospholipids. The other domains are not common for all GRKs.

Splice Variants of GRKs

In human, the genes of visual GRK subfamily have 4 exons, the genes of β ARK subfamily have 21 exons, and the genes of GRK4 subfamily have 16 exons. Alternative splicing does not occur in GRK subfamilies, except GRK4 subfamily. Human GRK4 has 4 splice variants: 578-amino acid GRK4 α composing all 16 exons, 546-amino acid GRK4 β deficient in-frame exon XV, 532 amino acid GRK4 γ deficient in-frame exon II, and 500 amino acid GRK4 δ deficient both exon II & exon XV. Another member of this subfamily, human GRK6 has 3 isoforms: 576 amino acid GRK6A, 589 amino acid GRK6B, and 560 amino acid GRK6C (Gurevich et al. 2012).

Membrane Targeting of GRKs

It is vital for GRK function to target to membrane, and it is done by a domain in the C-terminus. GRK1 and GRK7 have a Cys-Ala-Ala-x motif in their C-terminus. The farnesylation of this motif helps the GRK1 to be translocated from the cytosol to the plasma membrane by the light (Luttrell 2006), while geranylgeranylation of this motif has this result in the case of GRK7. Thus, prenylation of this C-terminal motif in both visual GRKs is necessary for their membrane binding (Gurevich et al. 2012). The membrane targeting of GRK2 and GRK3 are not executed by their isoprenylation (Ferguson 2001). Instead, they possess C-terminal G $\beta\gamma$ subunit-binding and pleckstrin-homology domains, interacting with free G $\beta\gamma$ subunits and inositol phospholipids to translocate kinase to the plasma membrane (Luttrell 2006). The binding of GRK2 RGS domain and a C-terminal 5-amino-acid clathrin-binding sequence to caveolin and clathrin is other ways of membrane localization of this GRK (Pao and Benovic 2002).

GRK4 and GRK6 have cysteine residues in C-terminus, serving as sites for palmitoylation. This lipid modification causes the kinase to localize to plasma membrane (Luttrell 2006). There are numerous C-terminal domains enhancing or preventing translocation of GRK6A to the membrane such as a lipid-binding amphipathic helix, palmitoylation sites, and a string of acidic residues. The later suppresses the membrane localization of GRK6A. It is interesting to mention that GRK6B and GRK6C do not have palmitoylation sites, but they can translocate to the membrane, because they lack GRK6A C-terminus and its elements, especially the region rich in acidic residues inhibiting membrane localization (Gurevich et al. 2012). GRK5 has a highly basic 46 residue C-terminal domain that interacts with membrane phospholipids electrostatically to translocate the kinase to plasma membrane (Ferguson 2001; Luttrell 2006). The C-terminal basic residues of GRK5 have positive charges, hence could be attracted to membrane lipids whose heads have negative charges (Kohout and Lefkowitz 2003).

Regulation of GRK Activity

Protein-protein interactions and phosphorylation are two mechanisms to regulate the activity of GRKs, as well as other kinases. For protein-protein interaction, G $\beta\gamma$ subunits interact with PH domains and enhance the activity of GRK2 and GRK3 in vitro, but do not stimulate the activity of GRK5 (Gurevich et al. 2012). Also, the binding of G $\beta\gamma$ subunits to N-terminal region of GRK2 facilitates its translocation to the cell membrane (Ribas et al. 2007). Anionic phospholipids like phosphoinositides activate GRK2 and GRK3 (DeBurman et al. 1996), but phospholipids such as

phosphatidylinositol 4, 5-bisphosphate (PIP₂) activate the members of GRK4 subfamily. The regions of GRKs associating with lipids are different: for example GRK4 subfamily members bind to lipids such as PIP₂ through their N-terminus, while GRK2 and GRK3 interact with the lipids via their PH domains (Gurevich et al. 2012). Recoverin is a calcium sensor protein (CSP) that regulates only the GRK1 activity (Ferguson 2001). CSPs are activated by exposure to intracellular or extracellular calcium. Recoverin inhibits the GRK1-mediated phosphorylation of rhodopsin via binding to GRK1 (Kohout and Lefkowitz 2003).

Actin, caveolin-1 and calmodulin are other proteins that interact with GRKs directly to regulate their activity. For example, calmodulin could associate with all isoforms of GRKs, especially GRK5 (Gurevich et al. 2012). Interaction of calmodulin with N-terminal region of GRK5 activates autophosphorylation of GRK5 at residues except Ser484 and Thr485 (phosphorylation of GRK5 at both these sites stimulates its kinase activity), and decreases its interaction with lipids, which inhibits the activity of GRK5, its membrane localization and its association with GPCRs (Ferguson 2001; Gurevich et al. 2012). Calmodulin and caveolin could also bind to GRK2 in order to modulate its function (Ruiz-Gomez et al. 2000).

GRK activity is regulated by phosphorylation. GRK1 autophosphorylates itself at Ser488 and Thr489 to decrease its kinase activity towards phosphorylated light-activated rhodopsin. It has been shown in cultured cells and in vitro that PKA could phosphorylate GRK1 at Ser21 and GRK7 at Ser23 and Ser26 to diminish their function towards rhodopsin (Gurevich et al. 2012).

In the case of GRK2, Erk1/2 MAPK phosphorylates it at Ser670 residing in its C-terminal to inhibit its interaction with G $\beta\gamma$ subunits and reduce its kinase activity toward GPCRs (Ferguson 2001; Kohout and Lefkowitz 2003; Ribas et al. 2007), while PKA phosphorylates GRK2 at Ser685 that accelerates its interaction with G $\beta\gamma$ subunits and promotes its membrane localization (Kohout and Lefkowitz 2003; Ribas et al. 2007). Cytoplasmic Src (c-Src) and PKC are other kinases phosphorylating GRK2. C-Src tyrosine phosphorylates this GRK to stimulate its interaction with G α_q and its kinase activity (Ferguson 2001; Gurevich et al. 2012) as well as its degradation by ubiquitin/proteasome mechanism (Kohout and Lefkowitz 2003). PKC serine phosphorylates GRK2 to increase its kinase activity and membrane targeting (Ferguson 2001). Unlike GRK5, phosphorylation of GRK2 by PKC increases its activity toward rhodopsin (Gurevich et al. 2012).

An important and interesting relationship between GPCRs and GRK2 is reported by Haga and colleagues (Haga et al. 1998) that activated GPCRs could have two different roles and interaction sites with GRK2: activates GRK2 and be phosphorylated by GRK2. For example, stimulated muscarinic acetylcholine m2 receptor binds to GRK2 ligand exposure at two different sites: Phosphorylation sites, where the GRK2 phosphorylates the receptor, are established within the central part of the third intracellular loop, and the activation sites are located in the intracellular regions near the transmembrane α -helices III, VI, V and VII. The interactions of both activation sites of the stimulated receptor and free G $\beta\gamma$ with GRK2 cause the kinase to be localized to the cell membrane (Haga et al. 1998).

As described above, GRK5 autophosphorylates at Ser484 and Thr485 residing at its C-terminus. Phosphorylation of these residues increases the kinase activity of GRK5

towards GPCRs (Gurevich et al. 2012). In addition, Phosphorylation of C-terminal tail of GRK5 by PKC also decreases its activity, unlike GRK2 (Ferguson 2001; Kohout and Lefkowitz 2003).

There are also other mechanisms than phosphorylation and protein-protein interactions to adjust activity of GRKs. For example, upon activation of many GPCRs, nitric oxide synthase and S-nitrosothiols S-nitrosylate GRK2 at Cys340 to inhibit phosphorylation of β AR, which suppresses subsequent processes such as endocytosis and downregulation of agonist-activated GPCR (Whalen et al. 2007).

In the case of GRK4 and GRK6, as we said in previous section, their palmitoylation at C-terminal cysteine residues results in their membrane localization. This lipid modification also potentiates the activity of them, as it has been observed that the kinase activity of palmitoylated GRK6 toward β 2AR is 10-times more than that of the non-palmitoylated GRK6 (Ferguson 2001).

Phosphorylation-independent GRK-mediated desensitization

Another desensitization mechanism for some of GPCRs, e.g. the mGluR1a metabotropic glutamate receptor, which may be mediated by GRKs, is through phosphorylation-independent desensitization. GRK2 has an N-terminal RGS homology domain and C-terminal $G\beta\gamma$ binding domain. The RGS homology domain has the ability to bind to free $G\alpha_{q/11}$ (G protein q/11 α subunit) subunits directly, although it possesses little or no GAP activity. As these domains of GRK2 and its kinase domain located at three different regions of the enzyme, it is supposed that GRK2 can bind to

the receptor, G $\beta\gamma$ subunits, and free G $\alpha_{q/11}$ simultaneously. Therefore, GRK2, which is bound to the mGluR1a metabotropic glutamate receptor, could inhibit reassociation of GDP-bound G $\alpha_{q/11}$ with G $\beta\gamma$, after sequestration of free G $\beta\gamma$ by GRK2 and GTP has been hydrolyzed (Luttrell 2006).

Diseases Related to GRKs

First, we investigate the effects and phenotypes of GRKs gene knockout in mice: Knockout of GRK1 gene associated with stimulation of rhodopsin in mice, results in degeneration of retina in a light-dependent manner, and prolonged light-response of photoreceptors (Kohout and Lefkowitz 2003). It has been observed that knockout of GRK2 gene in mice is lethal for 9-12 day embryo, whereas knockout of GRK3 gene does not inhibit normal embryogenesis and postnatal development of mice (Pitcher et al. 1998a; Penela et al. 2010), but results in supersensitivity of olfactory epithelium (Kohout and Lefkowitz 2003). The knockout of GRK4 gene in mice does not express any apparent phenotypes without any corruption in the activity of sperm. Deficiency of GRK5 increases regulation of lung and central M2 muscarinic receptor, but does not have any effect on adjustment of this GPCR in heart. Also, defects or lack of GRK5 or GRK2 may be involved in onset and early phases of Alzheimer's and Parkinson's disease, as both of these kinases phosphorylate α - and β -synucleins, proteins involved in the progress of both diseases (Premont and Gainetdinov 2007). The knockout of GRK6 gene, weakens the chemotaxis of lymphocytes (Kohout and Lefkowitz 2003), but enhances the chemotaxis of neutrophils (Premont and Gainetdinov 2007).

It is suggested that GRKs may be involved in some of human diseases such as hypertension, chronic heart failure, opiate addiction, myocardial ischemia and retinitis pigmentosa. In the case of retinitis pigmentosa, it has been observed that there are multiple mutations in rhodopsin, especially GRK1-mediated phosphorylation sites at its C-terminal region (Pitcher et al. 1998a). Elevated vascular GRK2 is proposed to be important for progression of hypertension, as GRK2 stimulates the activity of the ENaCs (Epithelial Na⁺ Channels), which are linked to the hypertension development. High activity of cardiac GRK2 also results in abnormalities in heart (Penela et al. 2010).

Objectives

In introduction, we briefly reviewed the principles of GPCR signaling including their activation and their homologues desensitization by GRKs. We realized that the definition of GRKs is based on specificity of their kinase activity for GPCRs. It was assumed for a long time that GPCR superfamily is the only substrate for GRKs (Pitcher et al. 1998a; Pitcher et al. 1998b). But today, it is obvious that this classic view about substrates for GRKs is not true. Scientists have realized that GRKs do not show fidelity to GPCRs (Yoshida et al. 2003). The first clue disproving this classic view emerged from discovering of phosphorylation of tubulin by GRK2 in 1998. It was the first non-GPCR substrate for GRKs (Carman et al. 1998; Haga et al. 1998; Pitcher et al. 1998b). The next non-GPCR substrate was Na⁺/H⁺ exchanger regulatory factor (NHERF), which was discovered in 1999 (Hall et al. 1999b), and the list of non-GPCR substrates of GRKs have been expanding until now (Appendix) containing non-GPCR receptors, adapter proteins, cytoskeletal proteins, channels, and transcription factors. By discovery of

each of these novel substrates, new functions are proposed for GRKs in different processes of cell such as development, proliferation, growth, motility, tumorigenesis and apoptosis (Gurevich et al. 2012). For example, GRK5 phosphorylates p53 in vivo (Chen et al. 2010a). It also could translocate into the nucleus. Thus, scientists suggest that GRK5 could play a role in the regulation of gene transcription and genome stability (Martini et al. 2008; Sorriento et al. 2008; Chen et al. 2010a). Therefore, in this thesis we are going to do an extensive literature review to identify and introduce all of non-GPCR substrates for GRKs, their site(s) of phosphorylation, and the effects of their GRK-mediated phosphorylation in cell processes such as signaling, development, proliferation, immunity, gene expression and other cellular mechanisms (Gurevich et al. 2012). I should mention that there is a debate for considering one of GRK substrates as GPCR: Smoothed (Smo). First, we added Smoothed, which is phosphorylated by GRK2 (Meloni et al. 2006; Molnar et al. 2007), to our non-GPCR substrates list, but as this heptahelical receptor is considered as a distant group of GPCR by some of recent articles (Gurevich et al. 2012), we decided to include it in "introduction" section instead of our results. The other substrate we argue about is adenomatous polyposis coli (APC). It has been shown that GRK2 could interact with APC, but its phosphorylation by this kinase is not fully proved (Wang et al. 2009).

Smoothed, a Far-related GPCR Phosphorylated by GRKs

Smoothed (Smo) is a seven-transmembrane (7TM) signaling protein, the same as G protein-coupled receptors and functions during animal development

(Molnar et al. 2007) and plays a role as the transducer of Hedgehog signaling. The activity of Smo in vertebrates and invertebrates is tightly related to its localization in cells. For example, movement and localization of Smo to the primary cilia is required for its activity in vertebrates (Philipp et al. 2008). In mammals, the activity of Smo is regulated by three various ligands hedgehog (Hh) including Sonic hedgehog, Desert and Indian, through their binding to Patched, the inhibitor of Smo (Meloni et al. 2006). Patched (Ptc) is a 12-transmembrane transporter-like protein functioning as the receptor for the ligand Hedgehog (Meloni et al. 2006) by the help of Ihog, a single-spanning transmembrane protein as its coreceptor (Chen et al. 2010b). The transcription of Ptc-1 is directly regulated by Hh pathway (Philipp et al. 2008).

According to proposed pathway for Hh signaling, the Smo is catalytically inhibited by Ptc in the absence of Hh (Philipp et al. 2008). After the physical interaction of Hh with Ptc and Ihog, Ptc-repressed Smo is released to activate the latent Zn finger transcription factors Gli (Chen et al. 2010b), termed Cubitus interruptus (Ci) in flies (Philipp et al. 2008). Then the transcription factor Gli participates in regulating cellular proliferation and differentiation (Meloni et al. 2006).

In *Drosophila*, multiple sites in C-terminal tail of Smo are phosphorylated by PKA and Casein Kinase 1 (CK1), after Hh signaling. It has been observed that these phosphorylations are required for activity and cell surface accumulation of Smo, and correlate with its activity level (Chen et al. 2010b). Also, it has been demonstrated that Gprk2, a G protein-coupled receptor kinase in *Drosophila* and homolog of mammalian GRK5 (Philipp et al. 2008), functions in activation (Molnar et al. 2007) and regulation of Smo conformation, highly promoting Hh signaling. After PKA- and CK1-mediated phosphorylation of serine residues at C-terminus of Smo, Gprk2 phosphorylates

Ser741/Thr742 resided in the C-terminal tail of Smo to activate it. Gprk2 also could be dimerized/oligomerized to associate to C-terminal of Smo and maintain the active conformation of Smo and increase its dimerization/oligomerization in a kinase-independent manner. By both of these mechanisms, kinase-dependent and kinase-independent, Gprk2 elevates the Smo to its maximal activity. Therefore, Gprk2 function is required for high levels of Hh signaling (Chen et al. 2010b). It also has been shown that the only one β ARK-like kinase in zebrafish, termed the *zebrafish* GRK2/3 (zGRK2/3), could directly phosphorylate Smo to increase its activity and elevate Hh signaling in cells (Philipp et al. 2008). In mammals, it is observed that GRK2 can interact with and phosphorylate Smo in Human Embryonic Kidney (HEK) cells. GRK2 facilitates the binding of β -arrestin 2 to active Smo at the membrane to promote Gli-dependent signaling (Meloni et al. 2006). It is discovered that the association of β -arrestin 2 to GRK2-mediated phosphorylated Smo promotes its internalization in clathrin-coated pits (Molnar et al. 2007).

Chapter 2: Methodology

In order to identify non-GPCR substrates for GRKs, we performed an extensive search in PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>) using a combination of keywords including "G protein-coupled receptor kinases", "substrates", "non-GPCR substrates" and "non-receptor substrates". Papers showing phosphorylation of non-GPCR substrates by different isoforms of GRKs were selected and following information was extracted for each substrate: GRK isoform responsible for phosphorylation, phosphorylation site(s), consequences of GRK-mediated phosphorylation and methods used to detect phosphorylation.

Chapter 3: Results

Our investigation led to identification of 31 non-GPCR substrates for GRKs. Those substrates include a diverse range of proteins such as transcription factors, signaling proteins, ribosomal proteins and cytoskeletal proteins, etc. All the substrates will be discussed in the following section.

Non-GPCR Substrates for GRKs

β-Arrestin 1

As described before, β-Arrestin 1 (β-arr1) is one of the two isoforms of β-Arrestins (Luttrell 2006) and is composed of 418 amino acids (Figure 7) (Krupnick and Benovic 1998). β-Arrestins are vitally involved in GRK-mediated internalization and desensitization of GPCRs (Pierce and Lefkowitz 2001). For example, using transfected cellular systems it has been shown, β-Arrestin 1 participates in regulating the desensitization of GPCRs such as μ-opioid receptors (μORs) and D1 and D2 dopamine receptors (Gainetdinov et al. 2004).

It has been shown that the in vitro ERK1- & ERK2-mediated phosphorylation of β-Arrestin 1 at Ser412 regulates its role in receptor internalization (Luttrell and Lefkowitz 2002). This Serine residue is resided in β-arr1 R2 region that includes RXR and LIEF binding domains for AP2 and clathrin respectively (Barthet et al. 2009). Also, there is an ERK consensus sequence in the vicinity of Ser412 of β-Arrestin 1. The dephosphorylation of this residue is critical for β-arr1 interaction with clathrin and Src (Hupfeld et al. 2005). Upon activation of β2AR, phospho-Ser412-β-arr1 binds to the receptor and is dephosphorylated rapidly to be able to bind to clathrin and promote

the endocytosis of β 2-AR- β -arr1 complex (Barthet et al. 2009). The other kinase that could phosphorylate β -arr1 is Src. Src Kinases phosphorylate β -arr1 at the Tyr54 within its N-terminal domain (Delom and Fessart 2011).

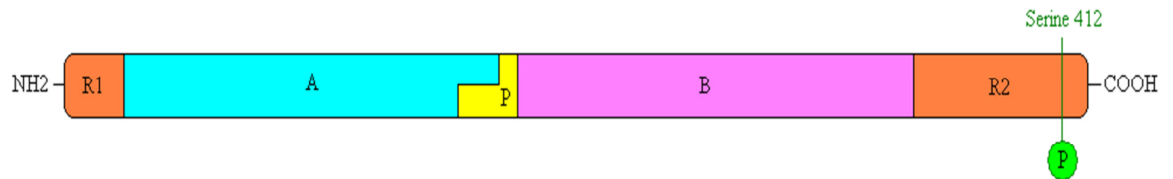


Figure 7: Domains of β -Arrestin 1 and their canonical roles (Ferguson 2001; Luttrell and Lefkowitz 2002).

R1 Domain: N-terminal regulatory domain

A Domain: responsible for activated-GPCR recognition

P Domain: Phosphate sensor region, it shares most of its N-terminal residues with A domain

B Domain: responsible for secondary binding to receptors

R2 Domain: contains LIEF & RXR binding motifs as well as GRK5-mediated phosphorylation site, Ser412.

The main form of β -Arrestin 1 in cytoplasm is phospho-Ser412- β -arr1. It was shown for the first time that GRK5 could phosphorylate β -Arrestin 1 at its Ser412 in colliculi neurons and human embryonic kidney 293 (HEK293) cells to suppress the 5-Hydroxytryptamine Receptor 4 (5-HT₄R)-activated Src/ERK cascade in a G protein-

independent manner. It is observed that GRK2 could also phosphorylate purified β -Arrestin 1 but in lesser extent. 5-HT₄R is a GPCR that triggers two G protein-dependent Gs/cAMP/PKA cascade and G protein-independent Src/ERK pathway, desensitized by GRK2 and GRK5 respectively. GRK5 prevents the signaling pathway of 5-HT₄R-mediated Src/ERK by phosphorylating the receptor at its C-terminal serine-threonine cluster. Then, the β -Arrestin 1 interacts with the phosphorylated receptor and is subsequently phosphorylated by GRK5 at its Ser412. The receptor-joined phospho-Ser412- β -arr1 inhibits the activation of 5-HT₄ receptor-bound Src (Barthet et al. 2009).

Downstream regulatory element antagonist modulator (Dream)

Downstream regulatory element antagonist modulator (DREAM)/potassium channel interacting protein (KChIP3) is a 221-residue multifunctional protein of the neuronal calcium sensor subfamily of EF-hand Ca^{2+} -binding proteins that has particular roles in and out of nucleus. Out of nucleus, DREAM (KChIP3) interacts with potassium channels of the Kv4 class leading their transit to and inside the cell membrane and controlling the gating properties of the channel through a Ca^{2+} -dependent pathway. For example, in neurons and cardiac myocytes, the Kv4.2 channel forms primary subunit underlying transient outward A-type K^+ currents. DREAM/KChIP3 requires to be palmitoylated at the cysteines in positions 45 and 46, to regulate Kv4 trafficking in the plasma membrane. In the nucleus, DREAM/KChIP3 functions as a Ca^{2+} -dependent transcriptional repressor. In the absence of calcium stimulation, DREAM joins to specific sites in the DNA. It also interacts with various nucleoproteins, cAMP-response

element-binding protein and cAMP-responsive element modulator, to influence their transcriptional properties in a Ca^{2+} -dependent fashion (Ruiz-Gomez et al. 2007).

Studies of Ruiz-Gomez and colleagues (Ruiz-Gomez et al. 2007) on HEK293 cells have indicated that Leucine-charged residue-rich domains (LCDs) in DREAM are important for the interaction with GRK2 and GRK6 (Figure 8). They also have shown that GRK1 can interact with the retinal protein recoverin, a member of family of small neuronal calcium sensors that includes DREAM, to be inhibited. Thus, these data suggest that DREAM is a good substrate for GRK2 & 6. But in vitro experiments showed that GRK2 is a much better kinase for DREAM. DREAM could be phosphorylated at Ser95. This position and an acidic residue in its vicinity are conserved in this protein from numerous mammalian species. The negative charge of Phospho-Ser95 is required for the translocation of the Kv4 channels from the endoplasmic reticulum at some steps, but should be removed directly by calcineurin in later steps for correct expression of the DREAM-channel complex in the plasma membrane. Therefore, the GRK2-mediated phosphorylation of DREAM at Ser95 decreases the membrane expression of Kv4 potassium channels by inhibiting their trafficking to and localization on plasma membrane, but does not affect their tetramerization. Also, this phosphorylation does not have any effects on the transcriptional repressor function of DREAM. Instead, it is indicated that a kinase downstream from phosphoinositide 3-kinase/Akt could phosphorylate DREAM to control its binding to DNA and its repressor function of the *hrk* gene expression (Ruiz-Gomez et al. 2007).

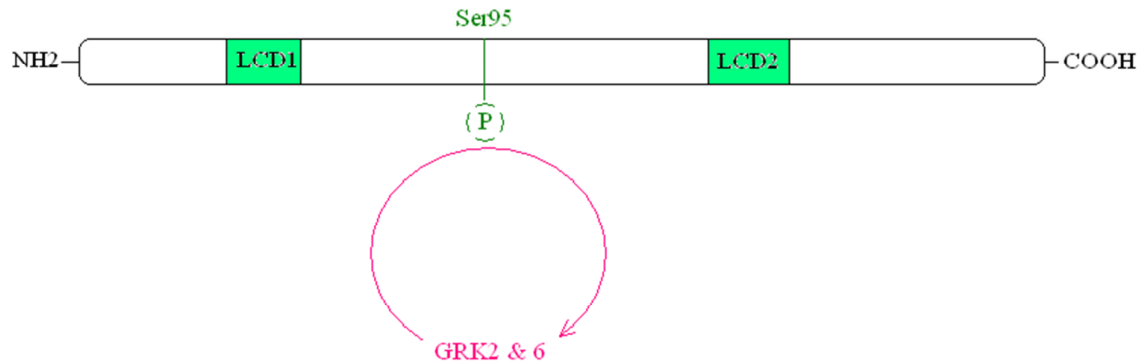


Figure 8: DREAM Leucine-charged residue-rich domains (LCDs) and GRK-mediated Phosphorylation site.

LCDs: Leucine-charged residue-rich Domains, important for interaction with GRK2, 6 & α - or ϵ cAMP-responsive element modulator.

LCD1: Located between residues 47-52.

LCD2: Located between residues 155-159.

Epithelial Na⁺ Channels (ENaCs)

Epithelial Na⁺ channels (ENaCs), which are expressed in the salt-scavenging epithelia, such as the distal nephron, distal colon, lung and the excretory ducts of salivary and sweat glands, function in the regulation of normal blood pressure and extracellular fluid volume (Dinudom et al. 2004) by mediating sodium transport across these epithelia (Sanchez-Perez et al. 2007). Their function is stimulated by glucocorticoids in the lung, and vasopressin (Antidiuretic Hormone (ADH)) and aldosterone in the kidney (Rotin and Staub 2012). ENaCs are made of three

homologous subunits in epithelia: α -ENaC, β -ENaC, and γ -ENaC, which each has two transmembrane domains and cytosolic N and C termini (Dinudom et al. 2004). There is a PPxY motif (PY motif or proline-rich motif) in the C terminal domain of each subunit that can interact with E3 ubiquitin protein ligases Nedd4 and Nedd4-2 WW domains (Sanchez-Perez et al. 2007), causing the channels to be ubiquitinated by Nedd4 and Nedd4-2, when the intracellular Na^+ increases. The ubiquitination of ENaC results in their endocytosis and destruction. This is the pathway to downregulate the ENaC activity. But there is also a mechanism for upregulation of its function through the action of kinases: CK2 (Casein Kinase 2) and GRK2. Experiments in stably transfected Madin–Darby canine kidney (MDCK) cells have indicated that the C termini of β -ENaC and γ -ENaC, specially the C-terminal 20 residues of the β subunit, are phosphorylated in exposure to aldosterone and insulin (Dinudom et al. 2004). Also, it is suggested that activating ENaC hormones may cause the Nedd4-2 to be phosphorylated, preventing its interaction with the channels (Dinudom et al. 2004; Sanchez-Perez et al. 2007). It is realized by in vivo studies that a conserved acidic sequence in the C-terminal 10 aa of the β -ENaC is phosphorylated by CK2. The Ser633 in the C terminus of β -ENaC is also the site of phosphorylation by GRK2 during in vitro experiments. This phosphorylation makes the ENaC insensitive to the ubiquitin protein ligase Nedd4-2 through an unknown mechanism, and increases their activity, because Nedd4 and Nedd4-2 could interact just with the dephosphorylated-ENaC. Hence, it was suggested that increased activity of GRK2, associated with hypertension in animal models and humans, may increase activity of ENaC by interrupting their Na^+ feedback inhibitory system to absorb Na^+ properly in distal nephron, and results in symptoms and consequences similar to Liddle’s syndrome caused by mutations in PPxY motifs (PY motifs) in the C

termini of β - and γ -ENaC, inhibiting its interaction with Nedd4-2. These symptoms could be treated by PP1 (Protein Phosphatase type 1) that dephosphorylates phospho-ENaC and sensitizes them to ubiquitin protein ligases (Figure 9) (Dinudom et al. 2004).

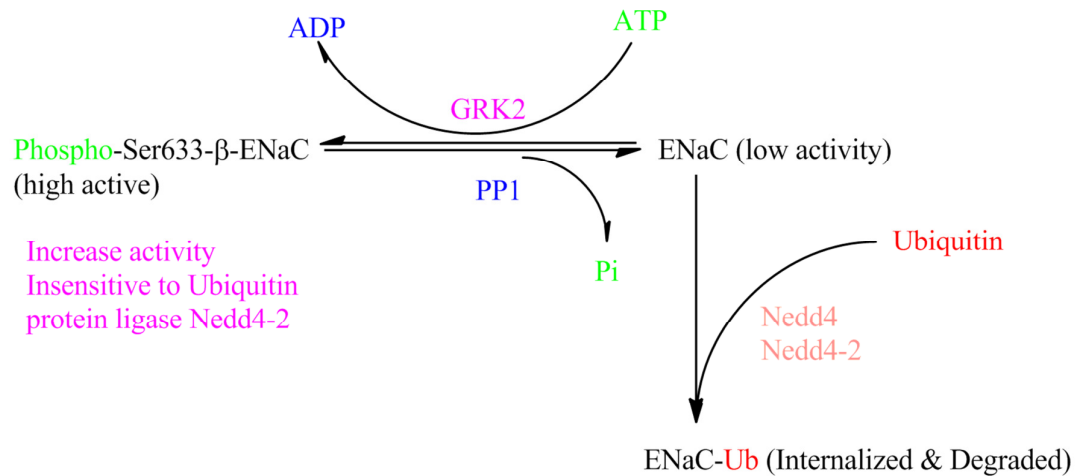


Figure 9: The GRK2-mediated phosphorylation of β -ENaC increases the ENaC function and inhibits its downregulation by Nedd4-mediated ubiquitination.

Neural precursor cell-expressed developmentally downregulated gene 4 (Nedd4) and Nedd4-2

As discussed in the previous section, Nedd4 (Neural precursor cell-expressed developmentally downregulated gene 4) and Nedd4-2 (Nedd4-like protein), members of a family of E3 ubiquitin-protein ligases called Nedd4 family, ubiquitinate ENaC to regulate its surface expression and target it to degradation (ENaC was the first identified substrate of Nedd4 (Yang and Kumar 2010)) (Sanchez-Perez et al. 2007). They trigger the endocytosis and inhibition of the channels, when the intracellular Na^+ increases. Other membrane transporters reported to be regulated by Nedd4 family

contains voltage-gated Na⁺ channels and the Cl⁻ channel, ClC-5 (Chloride channel-5) (Dinudom et al. 2004).

In human, Nedd4 family includes nine E3 ubiquitin-protein ligases, called Nedd4, Nedd4-2 (Nedd4L), Itch (Itchy E3 ubiquitin protein ligase), Smurf1 (SMAD specific E3 ubiquitin protein ligase 1), Smurf2 (SMAD specific E3 ubiquitin protein ligase 2), Nedl1, Nedl2, Wwp1 (WW domain containing E3 ubiquitin protein ligase 1), and Wwp2 (WW domain containing E3 ubiquitin protein ligase 2). The genes of Nedd4 and Nedd4-2 are also available in all vertebrates (Yang and Kumar 2010). All the family members share three similar domains: a catalytic HECT (homologous to E6-AP, C-terminus) domain, 2-4 WW domains, and an N-terminal C2 (calcium/lipid binding) domain, which have role or are important in binding of ubiquitin to substrate proteins, interaction of the ubiquitin protein ligases to PPxY (PY) motifs, and localization, respectively. There are six different domains in Nedd4 & Nedd4-2 (Figure 10). In mouse & rat, the Nedd4 has 3, and in human the Nedd4 has 4 WW domains (Arthur et al. 2006; Sanchez-Perez et al. 2007; Yang and Kumar 2010).

Similar to β subunit of ENaC, Nedd4 and Nedd4-2 are substrate for GRK2-mediated phosphorylation. It has been reported that GRK2 immunoprecipitates with Nedd4 and Nedd4-2 from mouse tissues. Thus, Nedd4 and Nedd4-2 interact with GRK2 in vivo. It has been shown that WW1 and WW2 domains of Nedd4-2 can be phosphorylated by GRK2 in vitro, but its WW3, WW4 and HECTc domains cannot. It is interesting to know that the WW1 & 2 domains do not bind to ENaC, but the WW3 & 4 domain do. The WW3 domain of Nedd4 is a phosphorylation substrate of GRK2, whereas it has similar sequences with WW4 domain of Nedd4-2. The Thr466 within

this WW3 domain of Nedd4, which binds to PY motif of ENaC subunits, is the GRK2-mediated phosphorylation site. The phosphorylation of this position disrupts the conformation of Nedd4 WW3 domain, resulting in the reduction of its affinity to interact with PY motifs of ENaC subunits. Also, threonine residue at position 304 and the serine residues at positions 253, 259, 261, 306, 337, and 418 in Nedd4-2 were selected as the best potential sites for GRK2-mediated phosphorylation via using computer programs such as PREDIKIN and NetPhos 2.0 (Sanchez-Perez et al. 2007).

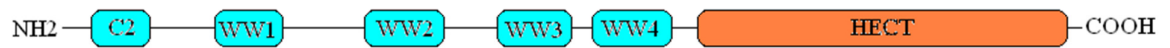


Figure 10: Domains of Nedd4 & Nedd4-2 in human.

C2 Domain: residues 35-137 (in Nedd4-2) (Arthur et al. 2006)

WW1 Domain: residues 221-254 (in Nedd4-2) (Arthur et al. 2006)

WW2 Domain: residues 414-447 (in Nedd4-2) (Arthur et al. 2006)

WW3 Domain: residues 526-559 (in Nedd4-2) (Arthur et al. 2006)

WW4 Domain: residues 577-610 (in Nedd4-2) (Arthur et al. 2006)

HECT Domain: residues 669-1003 (in Nedd4-2) (Arthur et al. 2006)

Finally, we could conclude that GRK2 can regulate sodium transport across the epithelia in gut, lungs, and kidney (Sanchez-Perez et al. 2007) by two phosphorylating mechanisms: First by phosphorylating the Ser633 in the C terminus of β -ENaC to activate it and prevent its interaction with E3 ubiquitine protein ligases such as Nedd4 and Nedd4-2 (Dinudom et al. 2004), and second by phosphorylating WW domains of

Nedd4 to modulate its interaction with transporter proteins such as ENaC in order to regulate them. The phosphorylation of Nedd4-2 does not seem to have any effects on its binding to ENaC, as both of its WW3 & WW4 domains are not phosphorylated, but it is thought that its phosphorylation might affect its association with other proteins (Dinudom et al. 2004; Sanchez-Perez et al. 2007).

Cytoskeletal Proteins

ERM Family of Proteins: Ezrin & Radixin

Two members of ezrin-radixin-moesin family proteins (ERM) are substrates of GRKs for phosphorylation: Ezrin and Radixin. ERM proteins function in cell signaling (Kahsai et al. 2010) and bind cortical actin to the cell membrane-associated proteins to trigger changes in actin cytoskeleton for forming of membrane structures including membrane ruffles and microvilli (Cant and Pitcher 2005). They also play important roles for formation of diverse actin rich cell-surface structures and may participate in tumor metastasis (Kahsai et al. 2010).

The ERM proteins have FERM (4.1 ERM) domain in their N-terminus, which associates with phosphatidylinositol 4,5-bisphosphate (PIP2) and cell membrane associated proteins (Kahsai et al. 2010), and a domain in their C-terminus interacting with actin (Cant and Pitcher 2005). In the cytosol, ERM proteins exist in an inactive and closed state wherein the N-terminal FERM region and C-terminal actin binding domain interact with each other in a head-to-tail manner to inhibit binding to membrane-associated proteins and actin, respectively. Unmasking of both of these sites activates

the ERM proteins, and needs the interaction of PIP2 with the N-terminal FERM domain and subsequent phosphorylation of a regulatory threonine residue at C-terminus (Thr567 of ezrin, Thr564 of radixin and Thr558 of moesin) (Cant and Pitcher 2005; Kahsai et al. 2010). After activation, ERM proteins could bind actin filaments to the integral and peripheral membrane proteins, such as CD43 (Cluster of Differentiation 43), CD44 (Cluster of Differentiation 44), and Na⁺/H⁺ exchanger regulatory factor 1 (NHERF, also called ERM-binding phosphoprotein 50 or EBP-50). ERM proteins are able to activate and be activated by Rho family GTPases. Binding of Rho GDP dissociation inhibitor to ERM proteins activates the Rho proteins. The activated Rho proteins stimulate the association of ERM proteins with CD44, which stimulates Rac1 (Ras-related C3 botulinum toxin substrate 1) signaling through interaction with guanine nucleotide exchange factor Tiam1 (Kahsai et al. 2010).

It has been shown that the regulatory threonine residues at C-terminus of ERM proteins could be phosphorylated by numerous kinases, such as protein kinase C α , protein kinase C θ , Nck-interacting kinase, Rho kinase, myotonic dystrophy kinase-related Cdc42 (Cell Division Control Protein 42)-binding kinase, and GRK2 (Kahsai et al. 2010). For example, both of in vitro and in vivo studies have shown that Ezrin phosphorylation at Thr567 could be done by protein kinase C (PKC) and Rho kinase to trigger activation of ERM proteins (Cant and Pitcher 2005). It has been shown that the ERM proteins phosphorylation on C-terminal Thr residues increases within the migratory stage of wound closure (Kahsai et al. 2010).

In vitro and cultured mammalian cells studies by Cant & Pitcher (Cant and Pitcher 2005) have shown that ezrin could be phosphorylated by GRK2 at Thr567, in

the carboxy terminus of the protein. They observed that GRK2 phosphorylated ezrin in the presence of phosphatidylcholine, PIP2 and G $\beta\gamma$. PIP2 and G $\beta\gamma$ could bind to PH (pleckstrin-homology) domain of GRK2 at its C-terminus to translocate it to the cell membrane. PIP2 also could interact with N-terminal FERM domain of ezrin. Therefore, these interactions are needed for colocalization of both GRK2 and ezrin to the plasma membrane in order to phosphorylate Thr567 of ezrin required for its activation and active-form stabilization. They also discovered that phosphorylated ezrin at Thr567 by GRK2, but not Na⁺/H⁺ exchanger regulatory factor (NHERF), is required for the internalization of β 2AR after receptor activation by ligand binding in mammalian cell cultures (Figure 11). However, this phosphorylated ezrin is not adequate for biogenesis of membrane ruffles by triggering changes in actin cytoskeleton. They also suggested GRK6 may play an important role in reorganization of actin cytoskeleton needed for cell migration by observing disrupted chemotactic movements of neutrophils and splenocytes in GRK6-deficient mice. Therefore, it is suggested that phosphorylated ezrin may be involved in endocytosis of GPCRs which need GRK2 and β -arrestin for their internalization (Cant and Pitcher 2005).

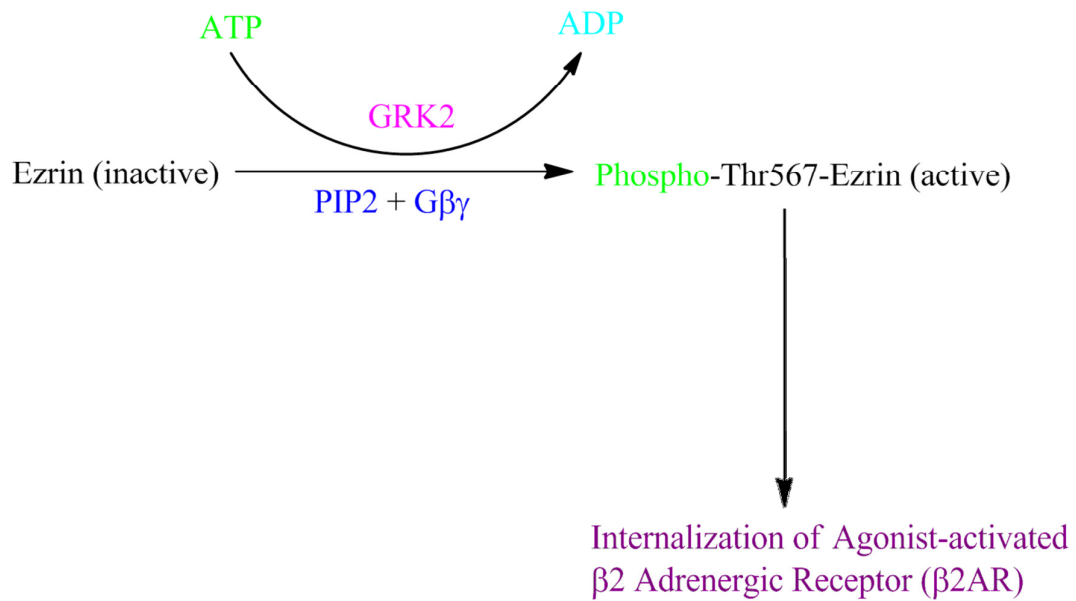


Figure 11: Phosphorylation of Ezrin by GRK2 and its results.

Radixin could interact with CD44 and actin by its N-terminal and C-terminal domains, respectively. In vitro studies by Kahsai et al. (Kahsai et al. 2010) have shown that Thr564 near the C-terminal end of radixin is the exclusive phosphorylated site for GRK2. This phosphorylation unmasks the membrane protein- and actin-binding regions of radixin by altering its conformation, and is involved in cell migration into wound and extension of membrane outgrowths. Activation of radixin via its phosphorylation also increases the activity of Rac1. The same as ezrin, GRK2 phosphorylates radixin in the presence of PIP2 and Gβγ. PIP2 opens the closed conformation of radixin and activates it (Kahsai et al. 2010).

Tubulin

Tubulin consists approximately 5% of whole brain proteins. It is the first discovered non-GPCR substrate for GRK2 (Pitcher et al. 1998b) and GRK5 (Yoshida et al. 2003). Tubulin has two main forms in the cell, cycling between each other: tubulin heterodimers that are soluble in the cytoplasm and include different isotypes of α - and β -tubulin ($\alpha\beta$ dimers), and polymerized tubulin subunits that form microtubules and are not soluble in cytoplasm. It has been shown that $G\alpha$ and $G\beta\gamma$ could interact with tubulin directly (Carman et al. 1998).

It is reported that $G\beta\gamma$ and tubulin could associate with distinct domains of the C-terminus of GRK2 independently (Haga et al. 1998). $G\beta\gamma$ binds only to GRK2, not GRK1 (Yoshida et al. 2003). It also could bind to microtubules directly (Carman et al. 1998). The interaction of tubulin with GRK2 inhibits the interaction of kinase with m2 and rhodopsin receptors to phosphorylate them competitively, whereas tubulin binding could not sequester GRK2 catalytic activity. Tubulin also could bind to catalytic domain of GRK2. Thus, it is proposed that tubulin may function as both activator and substrate of GRK2 (Haga et al. 1998). The principal binding site for tubulin is the central catalytic domain of GRK2 (residues 185–467), which has been conserved among GRKs in the highest degree. It is because of this region that several GRKs could phosphorylate tubulin (Carman et al. 1998).

It is reported by Eiper (Eipper 1972) that a serine residue of the β subunit of tubulin derived from brain is phosphorylated by an unknown kinase. Also, in vitro studies have indicated that tubulin could be phosphorylated by tyrosine kinases, cAMP-dependent protein kinase, Ca^{2+} /calmodulin-dependent protein kinase II (CamKII)

(Haga et al. 1998), CK1 and CK2 (Pitcher et al. 1998b). This phosphorylation causes the cAMP-dependent and Ca^{2+} /calmodulin-dependent kinases and tyrosine kinases, such as the insulin receptor and c-Src, to control the assembly and function of microtubules (Carman et al. 1998). Additionally, it has been observed that agonist exposure of GPCRs and $\text{G}\beta\gamma$ potentiate GRK2 to phosphorylate tubulin in intact cells (Pitcher et al. 1998b). GRK2 could phosphorylate polymerized tubulin even more than unpolymerized form. The study has shown that unpolymerized phosphorylated tubulin could form microtubules by their polymerization (Haga et al. 1998). Even it is observed that a small amount of tubulin polymerizes into microtubules during its phosphorylation by GRKs preferentially. This phosphorylation does not stop as microtubules assemble (Carman et al. 1998). GRK2 has phosphorylated microtubule-associated tubulin endogenously and copurified with microtubules (Pitcher et al. 1998b), but it does not have kinase activity for microtubule-associated proteins (MAPs) (Haga et al. 1998). MAPs and motor proteins bind to C-terminal tails of α - and β -tubulin, thus it is suggested that GRK2-mediated phosphorylation of β -tubulin's C-terminus might affect the binding of MAPs and motor proteins to β -tubulin for regulating the assembly of microtubules in mitosis and organelle transport. Also, GRK2-mediated regulation of microtubule dynamics may be involved in internalization of some GPCRs such as β -Adrenergic receptor (Yoshida et al. 2003). The difference between kinase activity of GRK2 and CK2 is that CK2 utilizes both GTP and ATP, but GRK2 uses ATP as the phosphate source exclusively (Pitcher et al. 1998b). Similar to CK2, GRKs also could bind and phosphorylate Taxol-stabilized microtubules directly (Carman et al. 1998).

Tubulin could be phosphorylated by GRK1 and GRK5 with the stoichiometries of ~0.45 mol of Pi/mol and ~1.6 mol/mol of tubulin dimers, respectively. Therefore, GRK1 is a weak kinase for tubulin. GRK2 and GRK5 could phosphorylate at least two sites of tubulin dimer. As the mobility of GRK5-phosphorylated tubulin dimers is lower than the other one, it seems that the nature of this phosphorylation is distinct. Also, it is identified by in vitro studies that both GRK2 and GRK5 prefer to phosphorylate β -tubulin isotypes, especially β III-isotype under specified circumstances (Carman et al. 1998). Subsequently, it was reported that GRK2 specifically phosphorylates β -tubulin, not α -tubulin. There are 4 isotypes of β -tubulin: β I, β II, β III and β IV. The GRK2-mediated phosphorylation sites are Thr409 and Ser420 resided within the C-terminus of β -tubulin (Figure 12). As there are conserved sequences around these phosphorylation sites in all 4 isotypes of β -tubulin, it seems that all of them could be phosphorylated by GRK2. For example, both of these residues are the only GRK2-mediated phosphorylation sites in β I-tubulin (Yoshida et al. 2003). Additionally, in vivo experiments have shown that β III-isotype of tubulin derived from brain is the main tubulin isotype phosphorylated in brain at Ser444. This phosphorylation may be done by CK2 (Carman et al. 1998), and GRK2 may be more responsible for phosphorylation of Thr409 and Ser420, as for other β -tubulin isotypes (Yoshida et al. 2003).

Why are Thr409 and Ser420 the GRK2-mediated phosphorylation sites of tubulin dimers and microtubules? It is realized that the 408-423 residues of β -tubulin C-terminus shapes an α -helix so that the Thr409 and Ser420 reside on the outermost surface of microtubules. The C-terminal regions of β III-isotype containing Ser444 are situated on the outermost surface of microtubules, too. Thus, the microtubules, as

well as tubulin dimers, could be phosphorylated by GRK2. Also, tubulin dimers could polymerize and assemble microtubules (Yoshida et al. 2003). According to tertiary structure of tubulin, its association with GRK2 and proposed physiological roles of GRK2-mediated phosphorylation of tubulin, it is suggested that GRK2 is able to phosphorylate tubulin in vivo, too (Pitcher et al. 1998b). Moreover, GRKs could interact with microtubules in vivo (Carman et al. 1998).

GRK-mediated phosphorylation of tubulin enables it to associate with membranes, and causes the microtubule to interact with actin filaments. The interaction of tubulin and GRKs could control the function, and localization of GRKs in the cytoplasm and membrane, as well as function and stability of cytoskeleton. Therefore, their interaction is a new identified bridge between cytoskeleton and GPCR cell signaling (Carman et al. 1998).

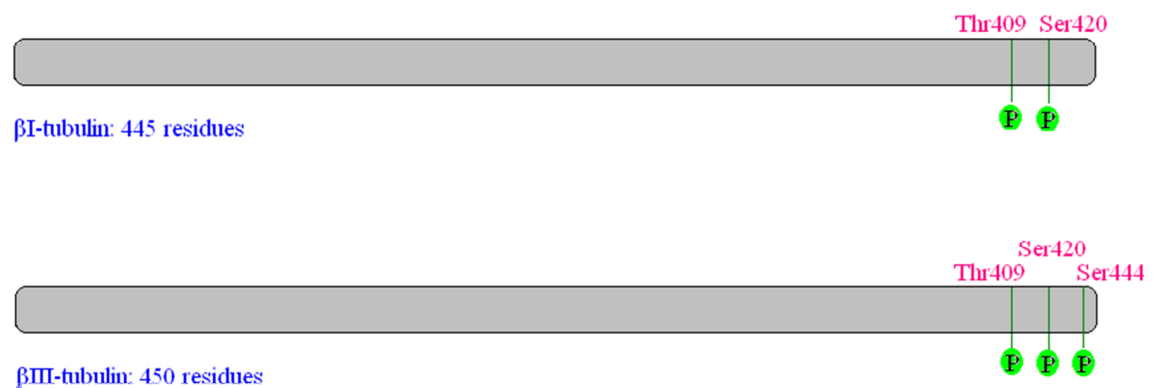


Figure 12: GRK2-mediated phosphorylation sites of β I-tubulin & β III-tubulin.

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are a family of receptors for extracellular ligands that trigger intracellular cascades via tyrosine phosphorylation. Their structure is made of an extracellular ligand binding region, a transmembrane domain and an intracellular domain with tyrosine kinase activity (Wang and Hung 2012). The desensitization of RTKs may be performed by serine/threonine kinases. Two members of this family are substrates for GRKs (Freedman et al. 2002):

1) Platelet-derived growth factor receptor β (PDGFR β)

The platelet-derived growth factor receptor β (PDGFR β) is a type of RTK (Freedman et al. 2002) and functions as the receptor for the platelet-derived growth factor (Mendelson et al. 2010) and participates in proliferation, migration and maintenance of mesenchymal cells (Hildreth et al. 2004), and is also involved in generation of malignant neoplasia and atherosclerosis (Wu et al. 2005), blood vessel development (Mendelson et al. 2010), wound healing and development of fetus (Cai et al. 2009).

PDGFR β , such as most of RTKs, exists as an inactive monomeric receptor. PDGF binding to the receptor results in activation of PDGFR β by its dimerization (Freedman et al. 2002) to phosphorylate PDGFR β and its substrates on tyrosine residues and trigger intracellular signaling cascades (Wu et al. 2006). The Tyr857 resided in the kinase activation loop is the autophosphorylated site of PDGFR β (Wu et al. 2005). The dimerization and autophosphorylation of PDGFR β makes phosphotyrosine docking sites for substrates that have phosphotyrosine-binding and SH2 (Src Homology 2)

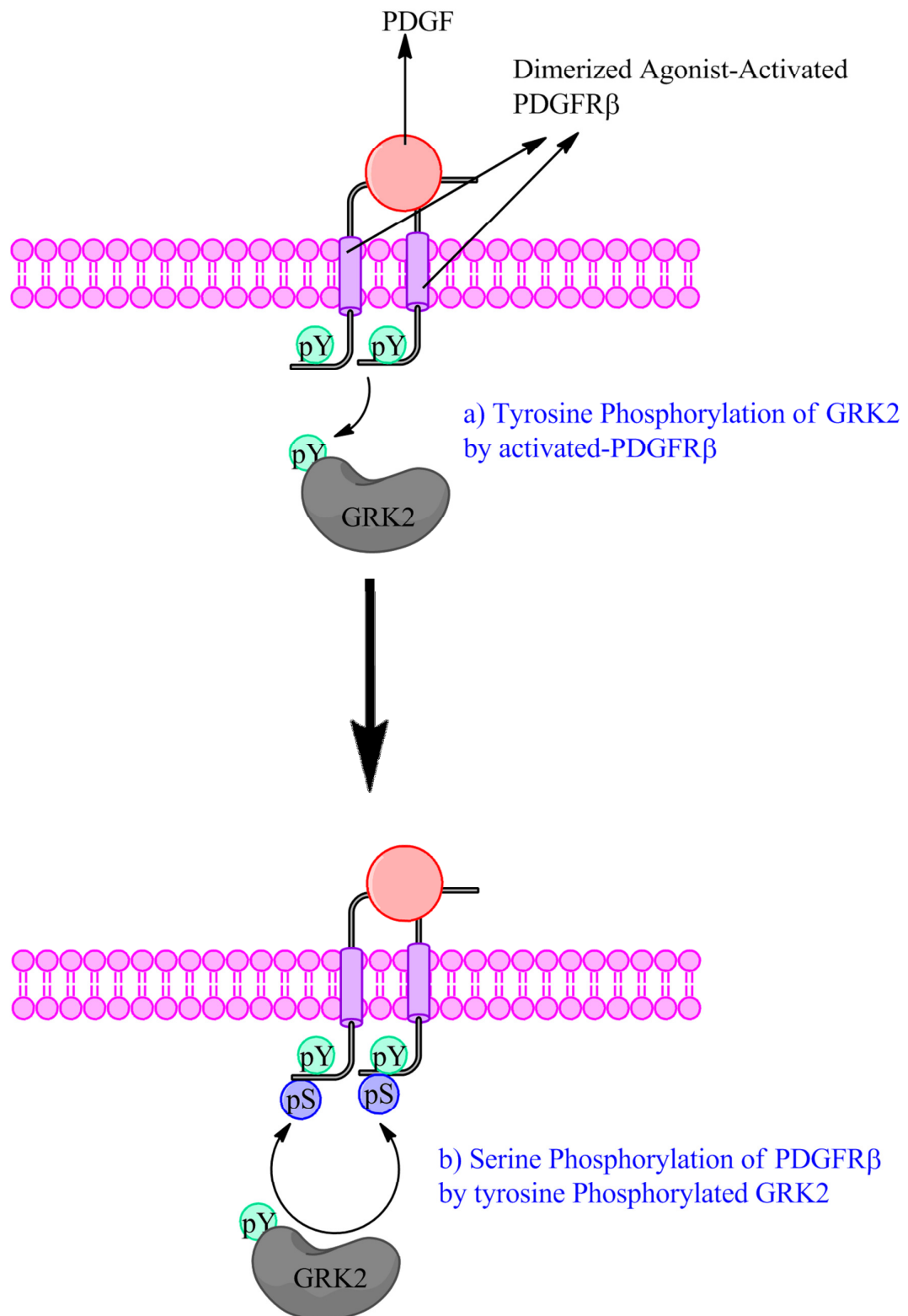
domains (Cai et al. 2009). The activated PDGFR β s keep on signaling, until they are degraded in the lysosome (Wu et al. 2006). Hence, the regulation of PDGFR β s is very important. There are multiple mechanisms to inactivate PDGFR β signaling, such as Tyr dephosphorylation and Ser phosphorylation of PDGFR β (Freedman et al. 2002) induced by agonist. It is suggested that in fibroblast, the serine phosphorylation of PDGFR β could be done mostly by GRK2 (Wu et al. 2005). The dephosphorylation of activated PDGFR β is mediated by phosphatase Shp2 (the Src homology 2 domain-containing protein-tyrosine phosphatase-2). This phosphatase is activated by PDGFR β directly to deactivate this RTK (Cai et al. 2009).

The PDGFR β is the first identified RTK phosphorylated and desensitized by a GRK, GRK2 (Figure 13) (Freedman et al. 2002). GRK2 could interact with PDGFR β in HEK293 and smooth muscle cells (SMC) and phosphorylate it (Freedman et al. 2002). It is realized that in cells expressing GRK2, the agonist-stimulated PDGFR β s activate c-Src, and c-Src activates GRK2 subsequently (Freedman et al. 2002) by phosphorylating tyrosine residues resided in N-terminal region of GRK2. It is interesting to know that these tyrosine residues are phosphorylated by agonist-stimulated PDGFR β , too. Therefore, the activated-PDGFR β could also activate GRK2 (Wu et al. 2005). Then, phosphorylated-GRK2 phosphorylates PDGFR β s on serine residues. The tyrosine kinase activity of PDGFR β s is essential to be recognized by GRK2 as a substrate. The serine phosphorylation of the receptor results in its ubiquitination by c-Cbl (Casitas B-lineage Lymphoma) or other potential ubiquitin E3 ligases (Freedman et al. 2002). The Ser1104 is the residue that is

phosphorylated by GRK2. This serine residue is located in the C-terminal tetrapeptide Asp-Ser1104-Phe-Leu of PDGFR β . This region is involved in interaction of the receptor with NHERF, enhancing the dimerization and signaling of PDGFR β . Thus, the phosphorylation of Ser1104 reduces the interaction of NHERF with PDGFR β (Hildreth et al. 2004). GRK2 also could bind to caveolin and phosphoinositide 3-kinase- β , essential element for endocytosis of PDGFR β (Freedman et al. 2002).

Another GRK that could phosphorylate PDGFR β is GRK5 (Figure 14). It is discovered that GRK5, but not GRK2, is involved in serine phosphorylation and desensitization of agonist-activated PDGFR β in SMC. It is thought that Ser1005, Ser1006, or both are possible GRK5-mediated phosphorylation sites of PDGFR β . Phosphorylation of these residues potentiates the phosphorylation of Tyr1009 by the PDGFR β Tyr kinase. As Tyr1009 is the primary docking site of PDGFR β for Shp2, its phosphorylation enhances the binding of Shp2 to the receptor in order to dephosphorylate and desensitize it (Wu et al. 2006). Such as GRK2, GRK5 could be activated by PDGFR β -mediated tyrosine phosphorylation. The 3 residues of Tyr13, Tyr86, and Tyr90 located in N-terminal of GRK2 are the sites phosphorylated by agonist-induced PDGFR β . Of these tyrosine residues, the third (Tyr90) is only conserved in GRK5 and phosphorylated by activated PDGFR β (Cai et al. 2009).

Figure 13: The interaction of GRK2 & PDGFR β : a) Phosphorylation of GRK2 at its N-terminal tyrosine residues by dimerized agonist activated-PDGFR β . b) Phosphorylation of PDGFR β at its Ser1104 by tyrosine phosphorylated GRK2. pS=Phospho-Serine, pY=Phospho-Tyrosine. (See next page)



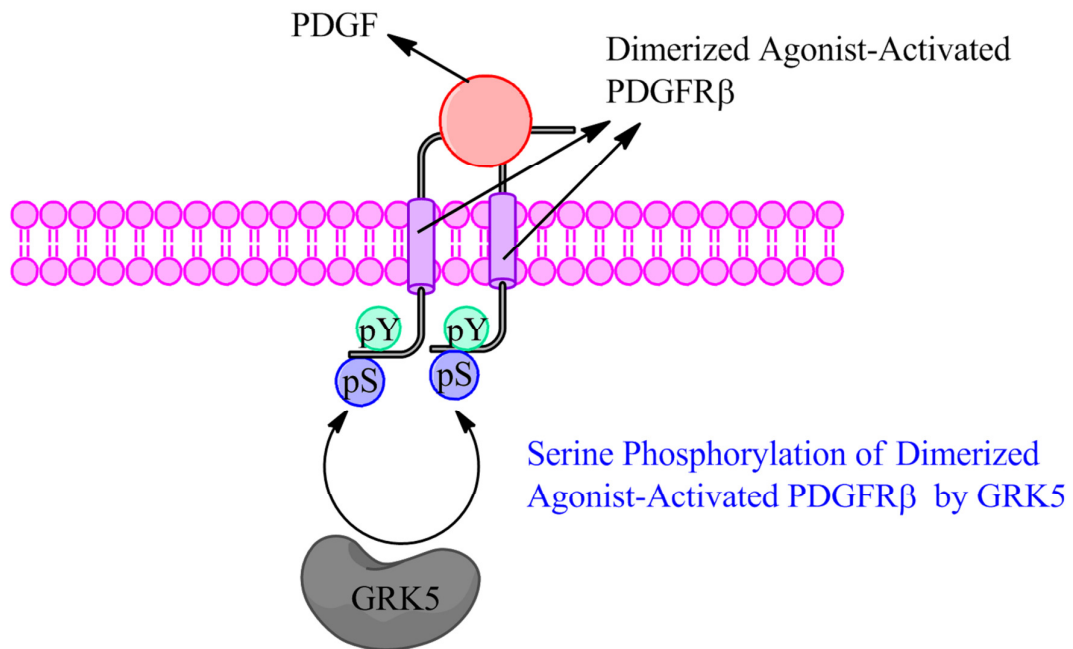


Figure 14: Phosphorylation of dimerized agonist-activated PDGFR β at its Ser1005 or Ser1006 by GRK5. pS=Phospho-Serine, pY=Phospho-Tyrosine.

2) Epidermal growth factor receptor (EGFR)

Another important member of transmembrane RTKs family is epidermal growth factor receptor (EGFR) which is involved in motility, growth, differentiation, maintenance (Chen et al. 2008), division, apoptosis, adhesion (Huang and Powers 2012) and death of cells (Hamdy et al. 2011) as well as progression and growth of different cancers (von der Heyde and Beissbarth 2012) such as non-small cell lung cancer (Huang and Powers 2012). EGFR also could be internalized and transported to nucleus to function as transcriptional factor and regulate replication and repair of DNA and development of tumors. In addition to nucleus, internalized EGFR could be translocated to other

intracellular organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus (Wang and Hung 2012).

There are four members identified for EGFR family: EGFR/ErbB-1 (Erythroblastic Leukemia Viral Oncogene)/HER-1, ErbB-2/HER-2/neu, ErbB-3/HER-3, and ErbB-4/HER-4. All of these members have tyrosine kinase activity except ErbB-3 (Wang and Hung 2012). The first member of family, EGFR/ErbB-1/HER-1, participates in generation of numerous pathophysiological processes such as atherosclerosis (Hamdy et al. 2011), and except ErbB-4, all of the members are expressed in human tumors of epithelial origin (Wang and Hung 2012).

Similar to PDGFR β , EGFR dimerizes upon binding of its ligand, such as EGF, to stimulate its tyrosine kinase activity and trigger multiple signaling cascades including STAT (signal transducer and activator of transcription), MAPK (mitogen-activated protein kinase), PI3K (phosphatidylinositol-3 kinase), and phospholipase C (Wang and Hung 2012). The GRB2 and Shc (SHC-adaptor protein) are the principal adaptors for EGFR to trigger MAPK pathway (von der Heyde and Beissbarth 2012). Gab1 (GRB2-associated Binding protein 1) is another adaptor protein for EGFR. This protein is phosphorylated by EGFR to trigger EGFR signaling cascades, but it is dephosphorylated by the action of phosphatase Shp2. Unlike PDGFR β , EGFR could stimulate Shp2 indirectly (Cai et al. 2009).

EGFR, as well as PDGFR β , could interact with and be serine phosphorylated by GRK2 both in purified protein systems and in cells in an agonist-dependent manner, but in contrast with PDGFR β , EGFR is not

desensitized or ubiquitinated by GRK2-mediated serine phosphorylation (Freedman et al. 2002; Hildreth et al. 2004). It is interesting to mention that EGF-activated EGFR, like activated PDGFR β , could phosphorylate GRK2 at the N-terminal tyrosine residues that PDGFR β and c-Src phosphorylate, to activate GRK2 and regulate the endocytosis of δ -opioid receptor (DOR) and μ -opioid receptor (MOR) (Chen et al. 2008).

Na⁺/H⁺ exchanger regulatory factor (NHERF)

The Na⁺/H⁺ exchanger regulatory factor (NHERF) (also called ERM-binding phosphoprotein 50 or EBP-50), with a molecular mass of 50 kDa, functions as a protein cofactor for PKA to inhibit the action of Na⁺/H⁺ exchanger 3 (NHE3) in a cAMP-dependent manner. NHE3 is a type of the Na⁺/H⁺ exchanger (NHE) isoforms that regulate sodium concentration and pH in most cell types, and their activity is controlled by intracellular concentrations of cAMP. The inhibition of NHE3 is performed by PKA-mediated phosphorylation of both NHE3 and its cofactor, NHERF. NHERF has two PDZ (PSD-95, Discs-large, and ZO-1) domains (Figure 15) (Hall et al. 1999b). By these two PDZ domains, NHERF could bind to C-termini of specific agonist-activated receptors such as β 2AR (Cao et al. 1999; Hall et al. 1999b), or interact with ERM-association-domain (ERMAD) of ERM proteins (Reczek et al. 1997). It has been shown that NHERF controls the sorting of the parathyroid hormone receptor (Sneddon et al. 2003) and human κ opioid receptor (Li et al. 2002), and plays a role in trafficking of β 2AR between the recycling endosome and lysosome after endocytosis (Cao et al. 1999).

In vitro studies have shown that NHERF is a substrate of GRK6A. The PDZ domains of NHERF bind to the C-terminus of GRK6A. Other variants of GRK6, including GRK6B & GRK6C, could not interact and phosphorylate NHERF, they differ in their C-terminus with GRK6A, and only GRK6A has the motif T-R-L at the end of its C-terminus that has the affinity for NHERF binding. It is observed that GRK6A phosphorylates NHERF at its Ser289 in HEK293 cells. In vitro analysis has shown that this serine phosphorylation, along with phosphorylation of Ser287 & 290, increase the inhibitory effect of NHERF on NEH3 (Hall et al. 1999b).

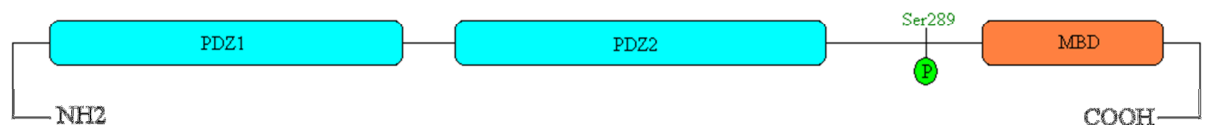


Figure 15: Domains of NHERF: Two PDZ & one MERM (Merlin, Ezrin, Radixin, & Moesin homology)-binding domain (MBD). The Serine289 phosphorylated by GRK6A is located between PDZ2 and MBD regions.

Hsp70 interacting protein (Hip)

Hsp70 (Heat shock protein 70) interacting protein (Hip) is a class of co-chaperones interacting with ATPase region of Hsc70, Hsp70 and Hsp90 and stabilizing their complex with ADP to assist them in protein folding, or protein transport. The Hip itself could associate with unfolded proteins to inhibit their accumulation (Howarth et

al. 2009; Barker and Benovic 2011). The Hip is composed of five domains (Barker and Benovic 2011): 1) an N-terminal domain, 2) a tetratricopeptide repeat (TPR) domain having three copies of TPR motif (Barker and Benovic 2011) and interacting with growth hormone receptor (Slotman et al. 2012), agonist-bound chemokine receptors CXCR2 (Chemokine (C-X-C) Receptor 2) and CXCR4 (Chemokine (C-X-C) Receptor 4), a GPCR for CXCL12 (Chemokine (C-X-C motif) Ligand 12) 3) a highly charged domain, 4) glycine, methionine, and proline enriched domain (GGMP), and 5) a C-terminal region (Barker and Benovic 2011).

Another interacting protein with Hip is GRK5. It has been shown that the C-terminal region of Hip between residues 303 and 319 interacts with GRK5. GRK5 could also phosphorylate Hip primarily at Ser346 in vitro and in HEK293 cells. This phosphorylation positively regulates the Hip-mediated endocytosis of CXCR4 after binding of its ligand: CXCL12 (Figure 16). Additionally, GRK2 could phosphorylate Hip in low levels, and it is observed that knockdown of GRK3 and GRK6 influenced the Hip phosphorylation somehow. Also, it is suggested that there may be novel kinases that phosphorylate other residues of Hip to regulate its function (Barker and Benovic 2011).

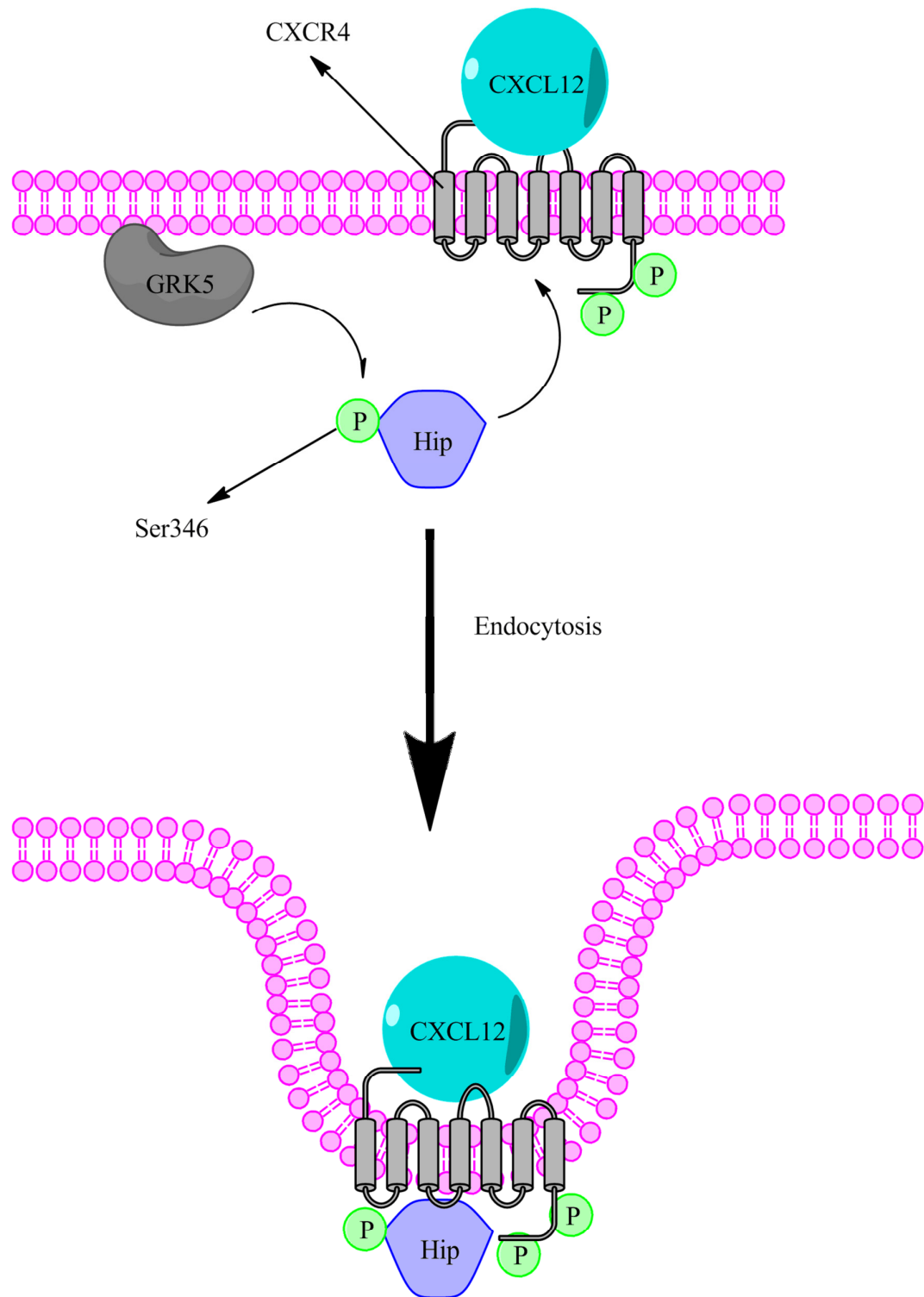


Figure 16: Phosphorylation of Hip by GRK5 at Ser346 results in increase of Hip-mediated endocytosis of agonist-activated CXCR4.

Insulin Receptor Substrates 1 (IRS-1)

Insulin Receptor Substrates complex (IRS) including IRS1-4 are the downstream substrates of insulin receptor. The IRS-1 is a 180 kDa insulin receptor intracellular substrate that has a major function in signaling of both insulin and insulin-like growth factor (IGF-1). It has several serine, threonine, and tyrosine residues which could be phosphorylated by different kinases. Its tyrosine phosphorylation leads to increase of the insulin/IGF-1 signaling cascade, whereas its serine and threonine phosphorylation reduces the insulin/IGF-1 signaling and results in insulin resistance observed in patients with diabetes. IRS-1 could regulate the members of the Bcl-2 (B-cell lymphoma 2) family, such as Bcl-xL (B-cell lymphoma-extra Large), by unknown mechanisms to induce antiapoptosis (Walker et al. 2012).

Insulin receptor is a receptor with tyrosine kinase activity that phosphorylates numerous intracellular substrates, such as Shc, G protein q/11 α subunit ($G\alpha_{q/11}$), IRS-1, and IRS-2, to trigger its signaling cascade. The stimulation of insulin receptor by insulin-exposure causes cdc42 activation and interaction with phosphatidylinositol 3 (PI3)-kinase, as well as elevation in tyrosine phosphorylation of $G\alpha_{q/11}$ C-terminus. The insulin-mediated increase in activity of $G\alpha_{q/11}$ results in stimulation of glucose transport. This insulin-stimulated glucose transport is inhibited by direct interaction of GRK2 with active $G\alpha_{q/11}$ via its RGS domain and independent of its kinase activity. GRK2 also could prevent the function of cdc42. Thus, the GRK2-mediated inhibition of active $G\alpha_{q/11}$ and cdc42 results in prevention of $G\alpha_{q/11}$ /cdc42/PI3-kinase pathway occurred in glucose transport, and causes insulin resistance (Usui et al. 2005).

Insulin resistance exists in patients with increased plasma levels of Endothelin-1 (ET-1). This vascular polypeptide is secreted by endothelial cells and binds to heptahelical endothelin type A receptor to induce its action and enhance the kinase activity of GRK2 resulting in phosphorylation of the endothelin type A (ETA) receptor. The signaling induced by ET-1 is facilitated by GRK2. This kinase also negatively regulates the insulin signaling. It is observed that chronic exposure of ET-1 to its receptor results in insulin resistance, as well as reduction of tyrosine phosphorylation of $G\alpha_{q/11}$ and IRS-1, leading to IRS-1 degradation and reducing its interaction with p85. These results for IRS-1 would also results from its serine phosphorylation, too. Thus, it has been shown by in vitro studies that GRK2 potentiates IRS-1 degradation by phosphorylating Ser612/636 (Figure 17). Whereas IRS-1 is phosphorylated at Ser307 for being degraded after chronic ET-1 treatment, but phosphorylation of this position is performed by another serine kinase (Usui et al. 2005). Recently, it has been discovered that phosphorylation of IRS-1 at Ser307 is executed by $TNF\alpha$ preferentially. This phosphorylation has inhibitory effect on insulin/IGF-1 receptor signaling enhancing apoptosis in different tissues all over the body, especially in diabetic retina (Walker et al. 2012). The other kinases that are able to phosphorylate IRS proteins at serine and threonine residues are Janus kinase, I κ B (Inhibitor κ B) kinase, MAPK, glycogen synthase kinase 3 (GSK3), protein kinase C, and mammalian target of rapamycin (Usui et al. 2005).

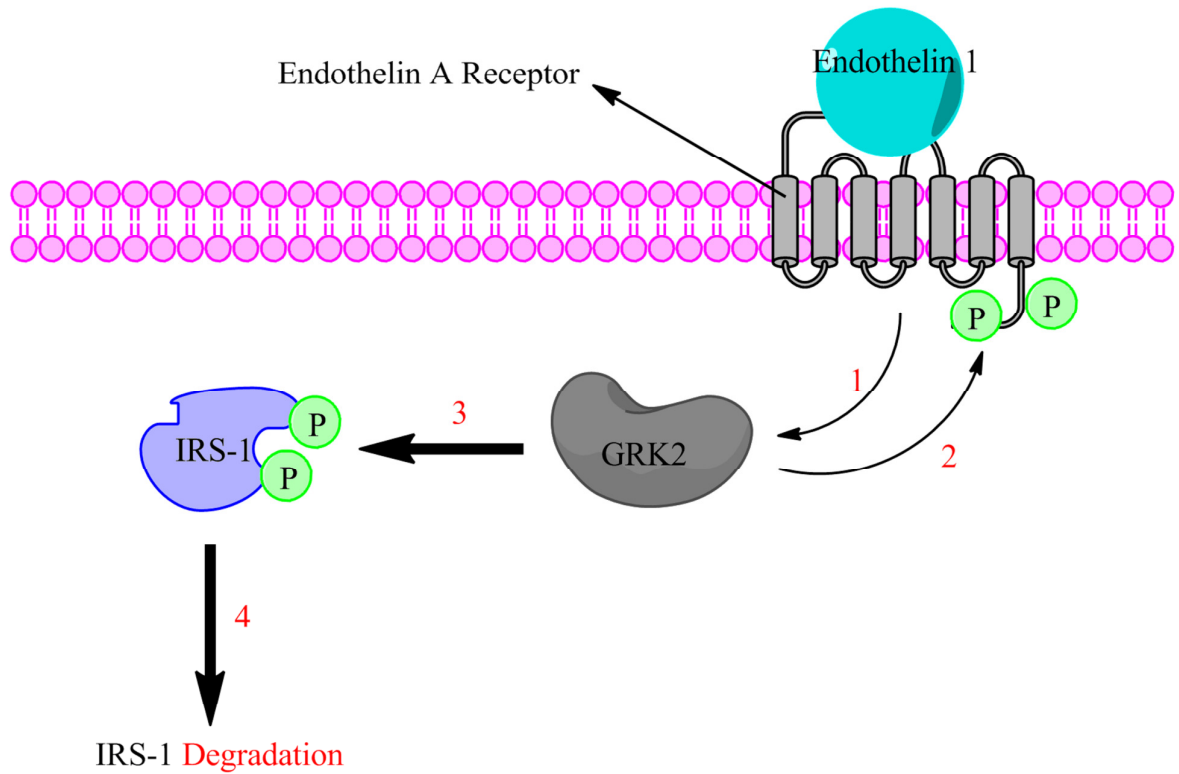


Figure 17: Endothelin 1 signaling results in IRS-1 phosphorylation and degradation: Agonist activation of Endothelin A receptor by Endothelin 1 increases the kinase activity of GRK2 (Stage 1). Stimulated GRK2 phosphorylates the agonist-bound Endothelin A receptor (Stage 2) and IRS-1 at Serine612/636 (Stage 3). The phosphorylation of IRS-1 leads to its degradation (Stage 4).

Inhibitory γ subunit of PDE6 (PDE γ)

The tetrameric retinal rod and cone photoreceptor type 6 retinal cyclic guanosine monophosphate phosphodiesterase (called PDE6) participates in the cascade of phototransduction (Wan et al. 2001) and is made of two identical inhibitory γ subunits (termed PDE γ) and two $\alpha\beta$ subunits (termed PDE $\alpha\beta$) which are catalytically

similar. PDE γ is a small protein with 87 amino acids and has a central region with positive charge (residues Gly19-Gly49) and a hydrophobic C-terminal region with negative charge (Thr62-Ile87 (Isoleucine87)) (Figure 18). The function of PDE γ is to inactivate PDE6 in the dark and control the turn on and turn off of visual signaling after photoresponse. It is also suggested that PDE γ could be involved in endocytosis in cone or rod cells through interaction of the proline rich domain located at its N-terminus with PACSIN (Protein kinase C and casein kinase Substrate in Neurons protein) (Chu et al. 2010). There is only an SH3 consensus binding site in rod and cone PDE γ isoforms that could interact with an SH3 domain in c-Src and dynamin II. The two PDE γ isoforms expressed in rod and cone cells are different with each other in their N-terminal domains. But their C-terminal regions, important for association with transducin and PDE6, are mostly the same. The expressions of the PDE γ isoforms are not restricted to retinal tissue. For example, it is observed that the rod PDE γ is expressed in testes, kidney, heart, lung, airway and pulmonary smooth muscle (Wan et al. 2003).

In vitro studies have shown that GRK2 could interact and phosphorylate rod PDE γ in HEK293 cells after stimulation of the p42/p44 mitogen-activated protein kinase (p42/p44 MAPK) in the presence of EGF and thrombin. The Thr62 is the GRK2-mediated phosphorylation site of rod PDE γ . This residue is conserved in cone PDE γ , another good substrate of GRK2 (Wan et al. 2001) and its phosphorylation is necessary for the interaction of rod PDE γ with dynamin II (Wan et al. 2003). The activation of p42/p44 MAPK pathway is involved in initiation of cell proliferation after agonist exposure of cell surface receptors such as tyrosine kinase receptors, GPCRs and growth factor receptor (Wan et al. 2001). The EGF-dependent activation of p42/p44 MAPK is

regulated by PDE γ , GRK2 and c-Src. It is interesting to know that the activated p42/p44 MAPK may phosphorylate Thr22 of PDE γ to inhibit the activity of PDE γ - c-Src/GRK2 complex (Wan et al. 2003).

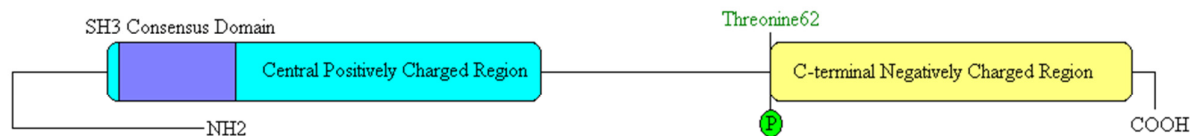


Figure 18: The PDE γ domains and its GRK2-mediated phosphorylation site.

Central Positively Charged Region: residues between Gly19–Gly49, containing SH3 Consensus binding domain.

SH3 Consensus Domain: residues between Pro20-Pro28, interacting with SH3 domain of Dynamin II & c-Src.

C-terminal Negatively Charged Region: residues between Thr62 – Ile87 (the last residue), involved in interaction with Transducin & PDE6.

The Thr62, the first residue of C-terminal Negatively Charged Region is the GRK2-mediated phosphorylation site within the protein.

p38 Mitogen-Activated Protein Kinases(p38MAPKs)

Mitogen-Activated Protein Kinases (MAPKs) are protein kinases that phosphorylate both nuclear and cytoplasmic substrates at serine/threonine residues (Nebreda and Porras 2000). They contain four subgroups: (1) extracellular signal-regulated kinases (ERKs) (Zarubin and Han 2005) that phosphorylate specific residues

of GRK2 to inhibit its interaction with G $\beta\gamma$ and results in reduction of GPCR phosphorylation (Peregrin et al. 2006), (2) c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), (3) ERK/big MAP kinase 1 (BMK1), and (4) the p38 MAPKs. These isoforms of p38 MAPK family share a threonine-glycine-tyrosine (TGY) dual phosphorylation motif: (1) p38 α , expressed ubiquitously and known as p38 (Zarubin and Han 2005) and SAPK2a (Nebreda and Porras 2000), identified with the molecular mass of 38 kDa and could be quickly phosphorylated and activated at its tyrosine residue in a Lipopolysaccharide (LPS)-dependent manner. p38 activation also depends on cell type as well as mitogenic stimulus. (2) p38 β , known as SAPK2b, is expressed ubiquitously, too. (3) p38 γ , known as ERK6 and SAPK3, is expressed in some specific tissues. (4) p38 δ , known as SAPK4, is expressed in some specific tissues, too (Zarubin and Han 2005). All of these p38 isoforms are usually phosphorylated and activated by two principal MAPK kinases: MKK6, known as SKK3 (SAPK Kinase 3) (Nebreda and Porras 2000) and MKK3 (Figure 19). Additionally, there are other mechanisms to activate p38 MAPKs independent of MAPK kinases in different cells under several physiological and pathological circumstances (Zarubin and Han 2005). The p38 MAPKs play role to mediate neuronal differentiation. The pro-inflammatory cytokines such as tumour necrosis factor α (TNF α) and interleukin 1 (IL-1) could intensely activate p38 MAPKs to regulate inflammatory response (Nebreda and Porras 2000).

p38 MAPKs could be phosphorylated by GRK2 at Thr123. In vitro studies have shown that the Thr123 of murine p38 α is phosphorylated by GRK2 directly. This residue is located at the entrance of docking groove of p38 and highly conserved as serine or threonine in p38 of most mammalian species. There is a main difference

between GRK2- and MKK-mediated phosphorylation of p38. In contrast to MKK, GRK2 phosphorylates p38 to inactivate it. It is observed that the activity or protein levels of GRK2 correlate with p38 activity and phosphorylation of its Thr123. Thus, GRK2 could be considered as an “inactivating MAPKK” (Peregrin et al. 2006).

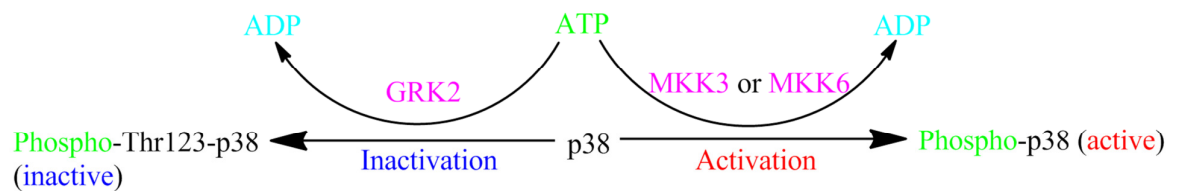


Figure 19: p38 MAPK could be phosphorylated by MKK3 or MKK6 to become fully activated or phosphorylated by GRK2 at Thr123 to become inactivated.

Phosducin and Phosducin-like Protein

Phosducin and phosducin-like protein (PhLP) are cytosolic proteins that associate with G $\beta\gamma$ subunits to disrupt G $\beta\gamma$ re-association with G α GDP and regulate signaling of G-proteins. Phosducin is a phosphoprotein with molecular mass of 33 kDa and is expressed ubiquitously. It shares 65% amino acid homology with PhLP and both could inhibit activation and translocation of GRK2 mediated by G $\beta\gamma$ (Ruiz-Gomez et al. 2000).

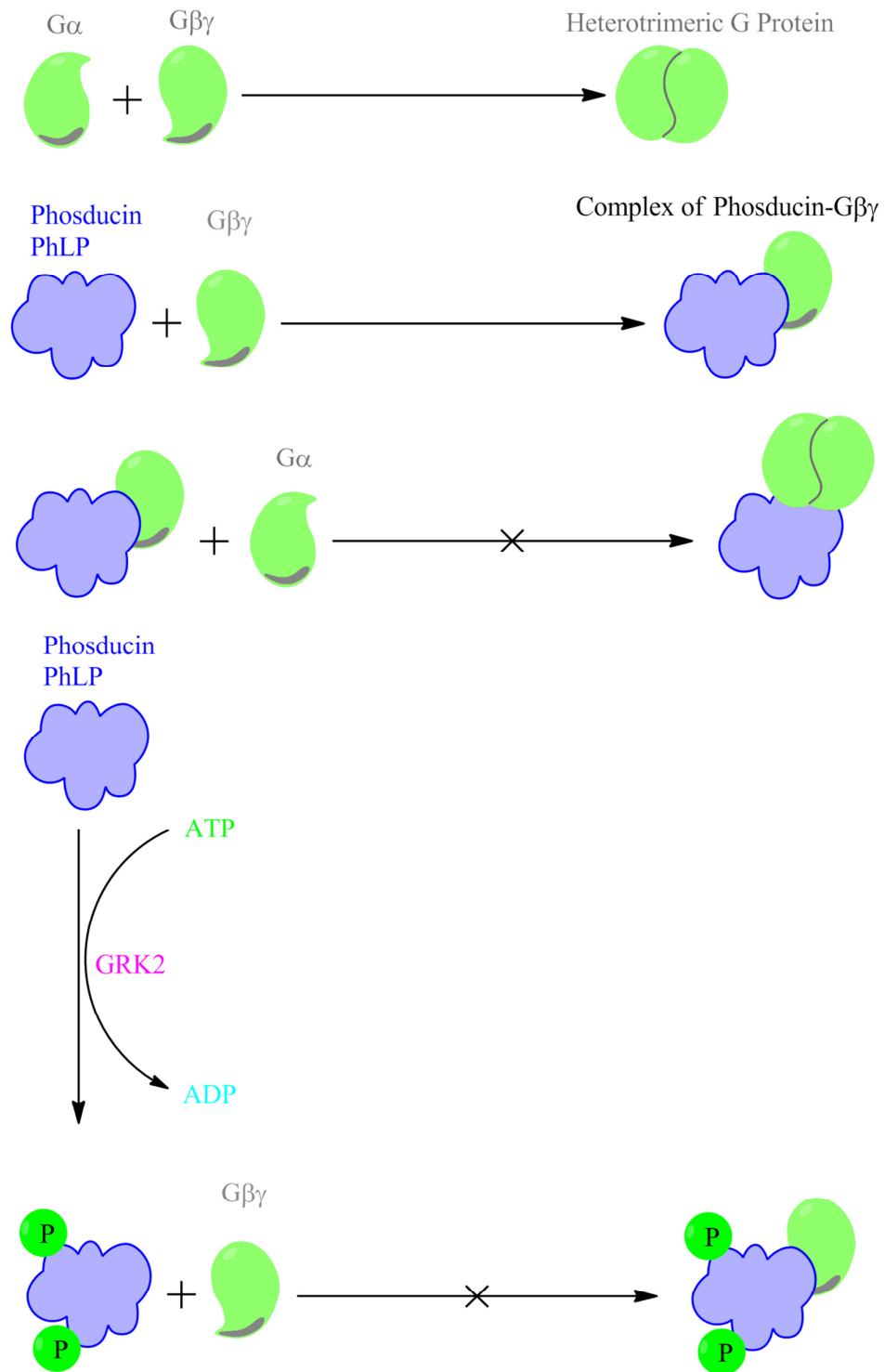
In vitro analysis has shown that phosducin and PhLP are high-affinity substrates for GRK2. The C-terminal region of both phosducin, residues 204-245, and PhLP, residues 195-218, are the vital domains for phosphorylation by GRK2 as well as binding

to Gβγ subunits. There are some serine and threonine residues within their C-terminal regions, surrounded by negatively-charged amino acids. GRK2 phosphorylates these residues to reduce the affinity of phosducin and PhLP to Gβγ, and inhibits their interaction (Figure 20) (Ruiz-Gomez et al. 2000).

Phosducin has also another kinase: PKA. Although PKA could phosphorylate phosducin too, but it is different with GRK2-mediated phosphorylation in the following ways (Ruiz-Gomez et al. 2000):

- 1) GRK2 could phosphorylate both phosducin and PhLP, but PKA could only phosphorylate phosducin.
- 2) The GRK2-mediated phosphorylation of phosducin has more inhibitory effect on phosducin than the phosphorylation by PKA.
- 3) GRK2 and PKA phosphorylate phosducin independently and on distinct residues.

Figure 20: The comparison of phosducin and GRK2-mediated phosphorylated phosducin interaction with Gβγ: The interaction of phosducin & PhLP with Gβα inhibits its reassociation with Gα, but the phosphorylation of phosducin & PhLP by GRK2 decreases their affinity to Gβγ. Thus, they cannot bind to Gβγ. (See next page)



Receptor-regulated or regulatory Smads (R-Smads)

Receptor-regulated or regulatory Smads (R-Smads) containing Smads 1, 2, 3, 5 and 8, are one of the three categories of Smad family. The members of Smad family are involved in converting the signals of transforming growth factor- β (TGF- β) superfamily ligands. TGF- β superfamily regulates cell growth, differentiation and apoptosis (Singh et al. 2011) and includes TGF- β s, bone morphogenetic proteins (BMPs) and activins (Ho et al. 2005). Ligands of TGF- β family bind to and activate transmembrane serine/threonine kinase receptors, called TGF- β type I receptor (T β RI) and TGF- β type II receptor (T β RII), by heterodimerizing T β RII with T β RI to activate it via transphosphorylating GS domain (Gly & Ser rich domain) of T β RI. Then, the activated T β RI phosphorylates two of the three serine residues in a C-terminal SSXS motif of R-Smads, such as Smad2 or Smad3, to activate them. The activated Smad2 or Smad3 heterodimerize with Smad4 and enter into the nucleus to control expression of specific genes (Figure 21). It is observed that Smad1, Smad5 and Smad8 are responsible for mediating the signals of BMPs, whereas Smad2 and Smad3 involved in transducing the activin and TGF- β signals (Singh et al. 2011).

There are 8 members of Smad family in mammalian cells which are categorized into three classes: 1) R-Smad, which we talked about their function briefly. 2) Common Smad (Co-Smad) including Smad4, and 3) Anti- or inhibitory-Smads (I-Smads) containing Smad 6 & 7, which regulate TGF- β pathway negatively through inhibiting the interaction and phosphorylation of R-Smads by T β RI. General structure of Smad family members is composed of two conserved domains that have mutually inhibitory effect on each other and can be relieved by T β RI-mediated phosphorylation: The N-

terminal Mad Homology domain1 (MH1 domain) which has DNA-binding activity and the C-terminal Mad Homology domain 2 (MH2 domain) which possesses transcriptional activity. These two domains are linked together by a linker region that has lots of regulatory peptide motifs, such as potential phosphorylation sites for CamKII, MAPKs, protein kinase C (PKC), and Erk-family MAP kinases. The phosphorylation of Smads with these kinases modulates the activity of them, as well as activating them. It also causes the Smads to be integrated into other signaling pathways that result in regulation of TGF- β signaling (Singh et al. 2011).

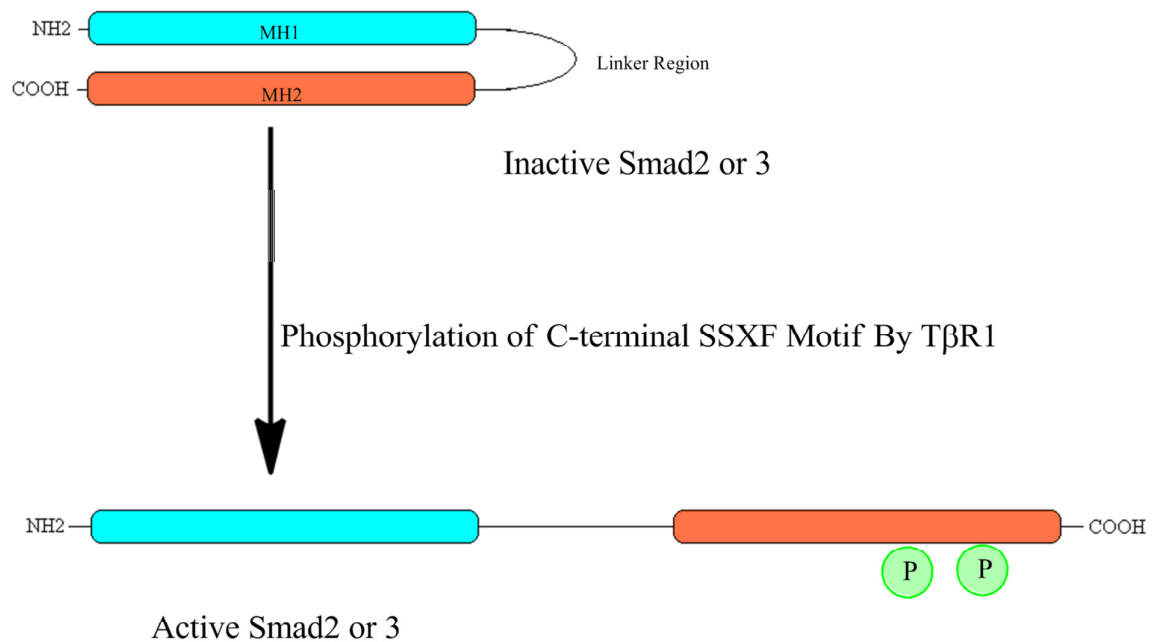


Figure 21: Serine Phosphorylation of C-terminal SSXF motif by activated T β R1 results in its heterodimerization with Smad4 and entry into the nucleus.

A novel kinase for R-Smads has been discovered by in vitro studies and in hepatocarcinoma cells of human: GRK2. This kinase could associate and phosphorylate

Smad2 and Smad3 at their Glutathione S-Transferase (GST)-linker domain following TGF- β stimulation. The Thr197 of Smad2 is known to be mainly phosphorylated by GRK2. It has been shown that GRK2-mediated phosphorylation of R-Smads at their linker region, inhibits type I activin receptor-mediated phosphorylation of their C-terminus, and results in block of nuclear translocation of Smad complex (Figure 22). Therefore, GRK2 prevents the signaling cascades of activin/TGF- β by phosphorylating R-Smads. It is interesting to mention that GRK2 expression is increased in response to activin/TGF- β signaling, but GRK2 negatively regulates their signaling in normal and cancer liver cells (Ho et al. 2005).

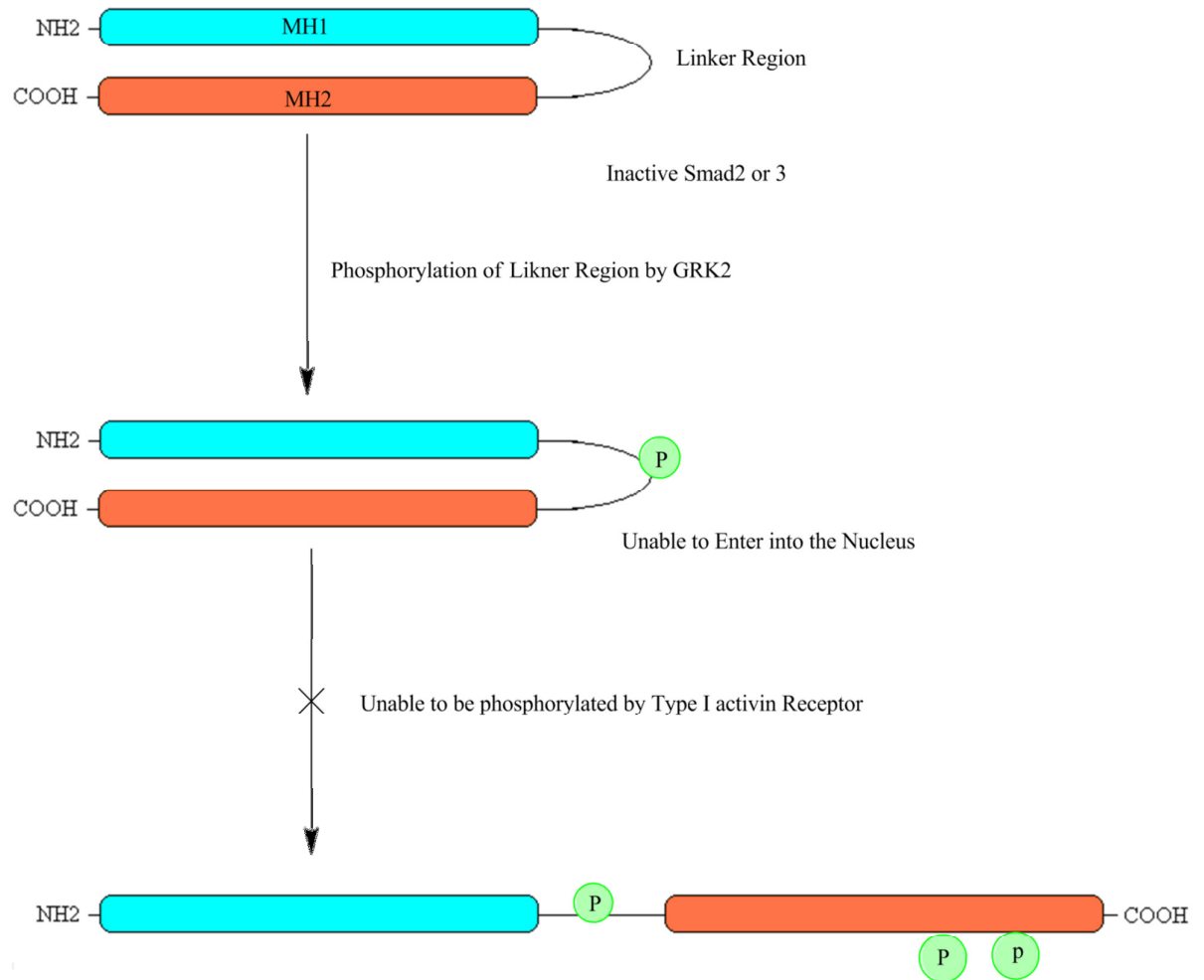


Figure 22: Phosphorylation of R-Smads, Smad2 or 3, by GRK2 inhibits their phosphorylation at C-terminus by Type I activin Receptor which activates the R-Smads.

Synucleins

Synucleins are small proteins (127-140 amino acids) with the molecular mass of 14 kDa and may participate in regulation of intracellular vesicular trafficking and development of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases. They are expressed in brain at high levels, but also could be found in many

tissues and cell lines such as COS and HEK293 in lower levels. There are 4 isoforms of synucleins: α , β , γ , and synoretin (Pronin et al. 2000). α -Synuclein is found all over the brain, but usually restricted to presynaptic terminals of dopaminergic neurons (Fiske et al. 2011). It composes majority of fibrillar aggregates in Lewy bodies (LBs) and Lewy neurites (LNs), intracytoplasmic inclusions in living neurons (Machiya et al. 2010), which have principal role in the pathogenesis of “Synucleinopathies”, a type of neurodegenerative disorder, such as Parkinson’s disease (Khandelwal et al. 2010). α -Synuclein is made up of three domains: (1) The N-terminal domain includes amino acids 1-60 and makes an amphipathic α -helix to interact with lipids. (2) The NAC (Non-A β Component of Alzheimer's disease amyloid) domain contains amino acids 61-95 that is amyloidogenic in high levels and is required for fibril aggregation and formation. (3) The C-terminal domain includes amino acids 96-140 resists against fibril aggregation and is usually shortened in LB (Figure 23) (Fiske et al. 2011). The γ -synuclein, the 3rd member of synuclein family, may participate in the etiology of breast cancer. It is observed that synucleins can associate with protein kinase C, Erk, BAD (Bcl-2-Associated Death Promoter), and 14-3-3 proteins and they can associate with phospholipids and inhibit the activity of phospholipase D2 (PLD2) in vitro. Thus, their phosphorylation results in releasing the inhibition of PLD2 in order to arrange actin cytoskeleton and formation of vesicles for recycling and internalizing the receptor (Pronin et al. 2000).

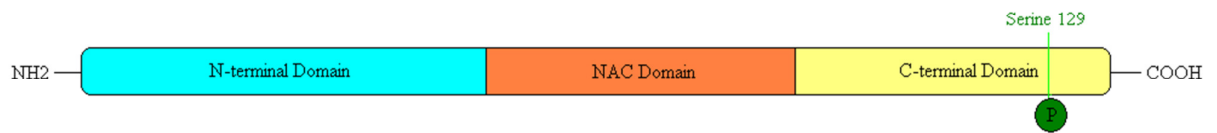


Figure 23: The domains of α -Synuclein and the site of GRK-mediated phosphorylation.

The N-terminal Domain: residues between 1-60, Amphipatic α -helix associates with Lipids.

The NAC Domain: residues 61-95, needed for aggregation and formation of fibrils.

The C-terminal Domain: residues 96-140, containing the GRK-mediated phosphorylation site: Ser129.

All isoforms of synucleins are in vitro substrates for different kinases, especially GRKs. It is observed that GRK2 & 5 could phosphorylate α -synuclein in COS-1 cells. There are four serine residues in α -synuclein. In vitro studies have shown that α -synuclein could be phosphorylated at its Ser129 by numerous kinases including GRK1, 2, 5, & 6, CK1 & CK2, and the polo-like kinases. The α -synuclein within the Lewy bodies, observed in Parkinson's disease, is highly phosphorylated at Ser87 and Ser129. It is not well known that which kinases are responsible for serine phosphorylation in LBs by in vivo experiments (Pronin et al. 2000; Machiya et al. 2010; Fiske et al. 2011), but it is suggested that as GRK5 accumulates in LBs, it may phosphorylate α -synuclein at Ser129 (Liu et al. 2010). This residue's phosphorylation along with amino acid Alanine76 plays a vital role in regulation of α -synuclein aggregation and membrane association (Fiske et al. 2011). It is also discovered that the phosphorylation of Ser129

leads the α -synuclein to be dephosphorylated and degraded by the proteasome pathway and independent of ubiquitin (Machiya et al. 2010). The other isoform of synucleins closely related to α -synuclein, called β -synuclein, is poorly phosphorylated at its Ser118 by CamKII, CK1, CK2, and more efficiently by GRK5, but highly phosphorylated by GRK2. The other isoform of synuclein, γ -synuclein, can be phosphorylated by GRKs more slowly than α -synuclein. It also could be phosphorylated by CamKII and CK2 more efficiently than β -synuclein. The Ser124 is the phosphorylation site of the γ -synuclein executed by GRK2, CK2 and CamKII, but GRK5 phosphorylates γ -synuclein on at least two different sites and not Ser124 in a phospholipid dependent manner. The last isoform of synuclein closely related to γ -synuclein, synoretin, is phosphorylated by GRK2 & 5 at its serine residue similar to the phosphorylation of γ -synuclein, but very poorly phosphorylated by CamKII and CK2. Such as γ -synuclein, synoretin is phosphorylated by GRK5 on at least two distinct sites. Thus, it is supposed that the serine residue phosphorylated by GRKs is slightly conserved in all isoforms of synuclein, while their C-terminal domains, containing the GRK-mediated phosphorylation serine, differ with each other. As mentioned above, synucleins could interact and sequester the action of PLD2 and phospholipids, so their serine phosphorylation by GRKs could prevent their association with PLD2 and phospholipids. PLD is critically involved in internalization and recycling of receptors through the rearrangement of actin cytoskeleton at cell cortex and positive regulation of vesicle formation (Pronin et al. 2000).

P2

P2 is a 60S ribosomal protein with molecular mass of approximately 17-18 kDa and belongs to ribosomal P-protein family. The ribosomal P-protein family members including P0 to P2, have highly homologous regions in their C-terminus that could be phosphorylated in vivo, and also a part of the elongation factor-binding site, which interacts with GTPase center in the 60S ribosomal subunit. The phosphorylation of P2 at these C-terminal regions is essential for its translational function, although the kinase(s) that perform(s) this phosphorylation in vivo are not well known. It is known that the phosphorylation of P2 C-terminal domains regulates protein synthesis in vitro (Freeman et al. 2002).

It has been reported that GRK2 phosphorylated P1 and P2 in vitro at the K_M s (Michaelis constant in Michaelis–Menten kinetics) of 1.0 ± 0.1 and 0.9 ± 0.0 μ M, respectively, near the K_M for phosphorylation of another non-GPCR substrate of GRKs, Tubulin, with K_M of approximately 1.0 μ M. Thus, such as Tubulin, P1 and P2 are good substrates for GRK2. Ser102 and Ser105 of P2 C-terminus are phosphorylated by CK2 in vivo and in vitro. As there is a C-terminal domain in P proteins that serine residues are flanked by acidic residues, and CK2 and GRK2 want to phosphorylate serine residues enclosed by acidic residues, hence GRK2 phosphorylates P2 at the same sites that CK2 phosphorylates: Ser102 and Ser105. It has been shown that GRK2 could only phosphorylate serine residues at P2 C-terminus. It is also identified that GRK2-mediated phosphorylation of P2 is stimulated by agonist-activation of β 2AR in HEK293 cells. Moreover, it has been revealed that GRK2 activated by β 2AR stimulation could phosphorylate P2 in vivo. It is interesting to note that C-terminal phosphorylation of P1

leads to degradation of this protein and has important function in yeast (Freeman et al. 2002).

Histone deacetylases (HDAC)

Histone deacetylases (HDAC) are a family of proteins that deacetylate histones to condense chromosome and repress transcription. Under normal circumstances in heart, the class II HDACs interacts with the MEF2 (Myocyte Enhancer Factor 2), a transcription factor activating expression of hypertrophic gene, and represses it (Figure 24. A). Under hypertrophic stimulated conditions, HDACs are phosphorylated by protein kinases that are activated by GPCRs. The phosphorylated HDACs are then dissociated from MEF2 and translocated out of the nucleus. Therefore, the free MEF2 could activate and increase genes related to hypertrophy. One of the most important members of Class II HDACs mediating the activity of MEF2 under hypertrophic conditions is HDAC5 (Martini et al. 2008).

The HDACs could be phosphorylated by kinases such as protein kinase D (PKD), CamKII and PKC. It has also been shown that in cardiomyocytes GRK5 could interact with HDACs, especially HDAC5, and phosphorylates them both in vitro and in vivo (Martini et al. 2008; Zhang et al. 2011). The activation of GPCR, for example angiotensin II type 1 receptor (AT1R), leads to the activation of Gq signaling cascade to induce hypertrophy of heart via MEF2-mediated expression of hypertrophic genes. Activation of GPCR bound to Gq induces the transport of the GRK5 into the nucleus. GRK5, such as other members of the GRK4 subfamily, has a functional nuclear

localization sequence (NLS) to signal the kinase to translocate in to the nucleus. It is also the only cardiomyocyte-expressed GRK that can translocate into the nucleus. After entering the GRK5 into the nucleus, it phosphorylates HDAC5 at least on one site, Ser259, to separate and release its inhibition on MEF2 (Figure 24. B) (Martini et al. 2008; Zhang et al. 2011). The HDAC kinase activity of GRK5 is not restricted to cardiomyocytes. It has been shown that GRK5 has HDAC kinase activity in the neuroblastoma cells (Liu et al. 2010).

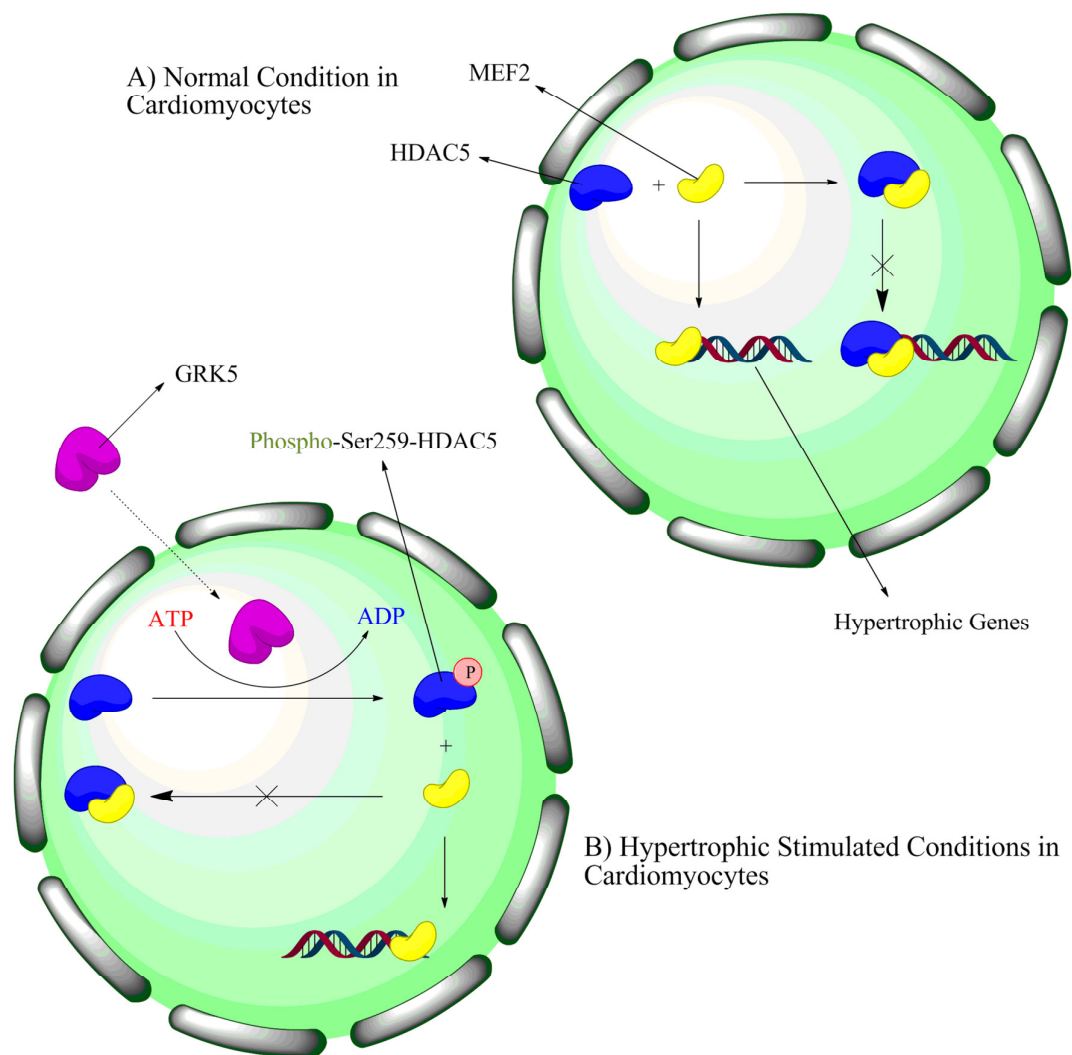


Figure 24: The function of HDAC5 in cardiomyocytes under normal conditions (A) and hypertrophic conditions (B) stimulated by activation of GPCRs triggering Gq signaling cascades.

Nucleophosmin (NPM1)

Nucleophosmin (NPM1), also termed B23 (Nucleolar Phosphoprotein B23), numatrin and NO38, is a plentiful nucleolar phosphoprotein, expressed ubiquitously and constantly travels between the nucleus and cytoplasm. It is involved in numerous cellular processes including regulation of chromatic structure (Naoe et al. 2006), centrosome duplication and cellular ploidy to sustain genomic stability (Sportoletti 2011), synthesis of ribosomal RNA (rRNA), assembly and transport of ribosome, cytoplasmic-nuclear trafficking (Naoe et al. 2006) and play important function in ARF-MDM2-p53 pathways to regulate tumor suppressors such as p53. It also has antiapoptotic effect and stimulates cell proliferation. In response to DNA damage, NPM1 shuffles from the nucleolus to the nucleoplasm to stabilize p53 and stimulate DNA repair. It also functions as histones' chaperone (Koike et al. 2010) and could interact with arrestins (So et al. 2012).

NPM1 is a member of the nucleoplasmin family. This family also includes two other proteins: nucleoplasmin (NPM2) and NPM3. There is an oligomerization domain shared in the N-terminal of all nucleoplasmin family. Two variants of NPM1 are identified: B23.1 and B23.2. The first variant has an additional 35 amino acids in its C-terminus. NPM1 polypeptide, with 294 amino acids, consists of an oligomerization domain (residues 1-119), histone binding region (residues 119-190) and DNA/RNA binding domain (residues 190-294) (Figure 25) (So et al. 2012). There are also a nuclear-export signal (NES) and a nucleolar localization signal (NuLS) in its C-terminus (Sportoletti 2011). The NPM1 gene is 25 kb and includes 12 exons (Naoe et al. 2006) and is frequently mutated in acute myeloid leukemia (AML). Its protein is over-

expressed in different types of solid tumors such as ovarian, colon, gastric and prostate carcinomas (Sportoletti 2011).

NPM1 is phosphorylated by polo-like kinase 1 (PLK1) during G2 to regulate the mitotic spindle assembly. This enzyme is located in centrosomes and regulates the entering of cells from G2 to M phase by phosphorylating cyclin B, WEE1, and cdc25c phosphatase (Michal et al. 2012). PLK1-mediated phosphorylation site of NPM1 is Ser4 (So et al. 2012). NPM1 is also phosphorylated at its Thr199 by CDK1 & CDK2 in response to DNA double-strand breaks (DSB) induced by ionizing radiation (IR) (Koike et al. 2010). The phosphorylation of NPM1 at both Ser4 & Thr199 is involved in regulating the centrosome duplication. NPM1 also could interact with N-terminal RH domain of GRK5 through its oligomerization domain and could be phosphorylated by this kinase both in vitro and in intact cells (HeLa cells (Henrietta Lacks-derived Cells)). The Ser4 has been identified as the major GRK5-mediated phosphorylation site of NPM1 in HeLa cells. This phosphorylation controls the sensitivity of cells to inhibition of PLK1, as cells with higher levels of GRK5 have lower sensitivity to PLK1 inhibition. In addition to Ser4, the Thr199 of NPM1 could also function as a minor GRK5-mediated phosphorylation site (Figure 25) (So et al. 2012).



Figure 25: The three domains of NPM1, and its two GRK5-mediated phosphorylation sites: Ser4 & Thr199.

N-terminal Oligomerization Domain: residues 1-119, containing Ser4, as the major GRK5-mediated phosphorylation site.

Histone Binding Domain: residues 119-190.

DNA/RNA Binding Region: residues 190-294, containing a nucleolar localization signal (NuLS), a nuclear-export signal (NES), and Thr199 as the minor GRK5-mediated phosphorylation site.

P53

P53 is a tumor suppressor gene that is activated by various stimuli, such as hypoxia, oncogene activation, DNA damage, and oxidative stress, to induce cell cycle arrest, apoptosis, senescence and DNA repair and inhibit cell growth and proliferation (Figure 26. A). It is mostly deleted or mutated in about half of all human cancers and tumors. P53 could also inhibit angiogenesis occurred in tumors and stimulate the transcription of genes related to DNA repair to resume DNA replication (Lenos and Jochemsen 2011; Tanikawa et al. 2012). The stability of p53 regulates its function, too. Its stability and activity could be mainly regulated by posttranslational modifications such as acetylation and phosphorylation. The Kinases such as ATM (ataxia

telangiectasia-mutated), ATM- and Rad3-related, DNA-dependent protein kinase, or CHK2 (checkpoint kinase 2) phosphorylate N-terminus of p53 to stabilize it by preventing its association with MDM2 (Chen et al. 2010a). MDM2 with MDMX (Murine Double Minute X) are two members of MDM family whose function is to regulate p53. They have a hydrophobic region in their N-termini that interacts with short alpha-helical domain in the N-terminus of p53. MDM2 is an E3 ubiquitin ligase that ubiquitinates p53 and causes its degradation (Lenos and Jochemsen 2011).

Recently a new kinase for p53 has been identified: GRK5. It has been shown that GRK5, but not GRK2 or GRK6, could phosphorylate p53 at Thr55 in vitro and in vivo (Chen et al. 2010a). It is interesting to know that GRK5-mediated phosphorylation of p53 enhances binding of p53 to MDM2 and results in degradation of p53. Thus, GRK5 serves as the negative regulator of p53 and inhibits p53-mediated apoptosis (Figure 26. B) (Chen et al. 2010a).

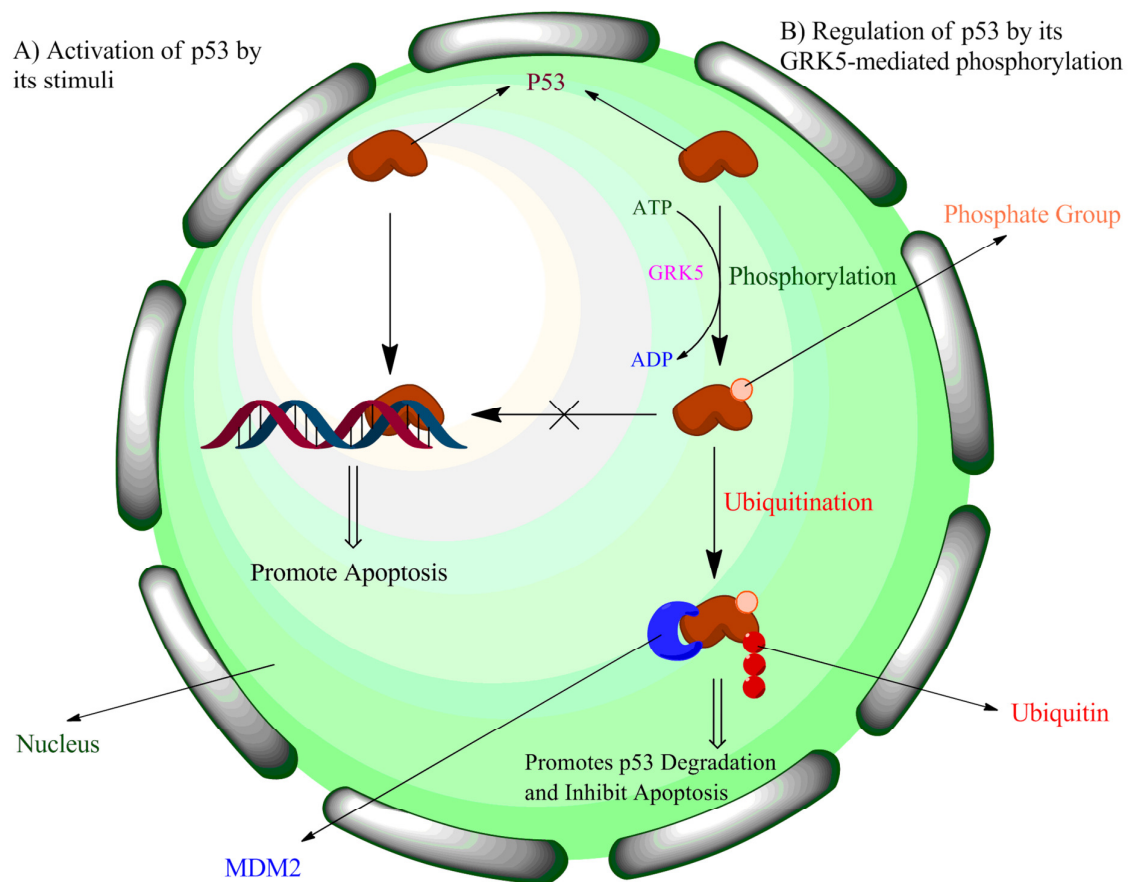


Figure 26: P53 in normal and phosphorylated conditions: A) P53 is activated by different stimuli such as DNA damage and hypoxia to activate transcription of genes involved in promotion of apoptosis. B) GRK5 positively regulates p53 affinity to MDM2 which ubiquitinates it, resulting in its degradation and prevention of apoptosis.

Two Substrates involved in Wnt signaling

Wnt molecules are extracellularly secreted glycoproteins functioning in regulation of tissue regeneration and organogenesis, adult neurogenesis, homeostasis, embryogenesis, cell proliferation, differentiation, and apoptosis, and are involved in

many diseases such as cancers and osteoporosis (Chen et al. 2009; Wang et al. 2009; Imura et al. 2010; Regimbald-Dumas and He 2011; Tanneberger et al. 2011). There are two types of cell surface co-receptors for Wnt ligands, activated by direct attachment of these factors: the heptahelical frizzled (Frz) receptors that are members of GPCR superfamily, and the single transmembrane receptors low-density lipoprotein receptor-related protein 5 and 6 (LRP5 and LRP6) functioning as the co-receptors for Frz receptors (Chen et al. 2009; Wang et al. 2009; Regimbald-Dumas and He 2011). In the absence of extracellular Wnt molecules, glycogen synthase kinase β (GSK3 β) and CK1 phosphorylate cytoplasmic β -catenin, involved in regulation of particular target genes transcription (Chandra et al. 2012). The phosphorylation of β -catenin occurs in a large multiprotein destruction complex which contains the scaffolding proteins Axin, its homologue Conductin (Axin2), and the tumor suppressor gene product adenomatous polyposis coli (APC) as well as GSK3 β and CK1 α (Wang et al. 2009; Regimbald-Dumas and He 2011; Tanneberger et al. 2011). Then, the phosphorylated β -catenin is degraded in proteasome by being ubiquitinated after its recognition by the β -transducin repeat-containing protein (β TrCP) 2 (Wang et al. 2009; Tanneberger et al. 2011). Upon binding of Wnt ligands to their receptors, Frz & LRP6 (or LRP5) receptors, multiple intracellular cascades are triggered. The canonical pathway of Wnt signaling results in stabilization of cytoplasmic β -catenin via suppressing the kinase activity of GSK3 β to increase the nuclear association of β -catenin with Lef-1/Tcf (lymphoid enhancer factor-1/T-cell factor) transcription factors after nuclear translocation of stabilized β -catenin to activate the transcription of Wnt target genes (Chen et al. 2009; Wang et al. 2009).

Two of components of canonical Wnt signaling are substrates of GRKs: APC (Wang et al. 2009) & LRP6 (Chen et al. 2009). The first one is not fully proved as the substrate phosphorylated by GRKs. Therefore, we will call it the putative substrate. We will explain about both of them in the next section.

1) *Tumor suppressor adenomatous polyposis coli (APC) (Putative Substrate)*

As mentioned above, the tumor suppressor adenomatous polyposis coli (APC) is a relative ubiquitously expressed protein with multiple roles in regulation of cell division, polarity and motility, microtubule stability, proper development of axon morphology, neural cell adhesion, glial migration and neurite outgrowth during neurogenesis based on in vitro studies (Imura et al. 2010; Chen et al. 2011). It is also involved in phosphorylation of β -catenin to prevent canonical Wnt signaling pathway. It is mostly mutated in colorectal cancers and has crucial function in preventing the cancers of colorectal epithelial cells (Zhang et al. 2000; Imura et al. 2010; Chandra et al. 2012).

APC is a shuttling protein found in both nucleus and cytoplasm, because it has two potential nuclear localization signals (NLS) containing amino acids 1767–1772 (NLS_{1APC}) and 2048–2053 (NLS_{2APC}), and two functional nuclear export signals (NES) (Zhang et al. 2000). There are other important motifs in the structure of APC: armadillo repeats that interact with and regulate the kinesin associated protein 3 (KAP3, a subunit of kinesin-2), IQ-motif-containing GTPase-activating protein (IQGAP) and the APC-stimulated guanine nucleotide exchange factors (ASEFs) which all play role in cytoskeleton regulation, and the

oligomerization region in N-terminus, the β -catenin binding motif, the mammalian homolog of Discs large (DLG1) binding domain involved in regulation of cell polarity and cell cycle, the microtubule plus end-binding protein 1 (EB1) and microtubules binding domain located in C-terminus (Figure 27 (Chen et al. 2011)) (Chen et al. 2011). There is also a serine-alanine-methionine-proline (SAMP) repeat motif in the midportion of APC that interacts with the RGS (regulator of G protein signaling) domain of Axin to promote the formation of multiprotein destruction complex responsible for β -catenin phosphorylation (Wang et al. 2009).

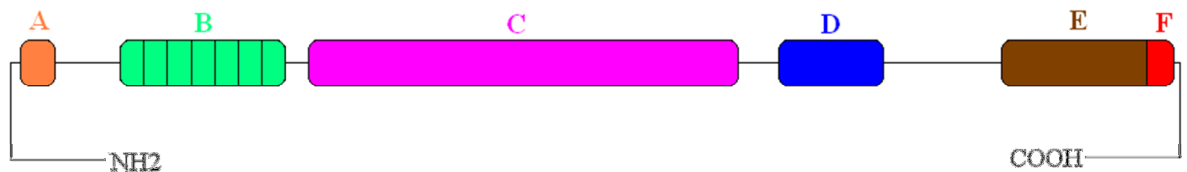


Figure 27: Important domains and regions of APC. A: Oligomerization Region, B: Armadillo repeats interacting and regulating KAP3, C: β -catenin binding motif, D: Microtubule binding region, E: EB1 (microtubule plus End-Binding protein 1) binding region, F: DLG binding region.

It has been shown that APC is phosphorylated at high levels in cell culture (Bhattacharya and Boman 1995). Moreover, it has been revealed that PKA could phosphorylate APC in vitro. Ser2054, the first serine located after NLS2_{APC}, is the residue phosphorylated by this kinase. The PKA-mediated phosphorylation of this serine residue is involved in regulation of nuclear import and nuclear activity of APC (Zhang et al. 2000). GSK3 β is another kinase

that could phosphorylate APC in vitro (Rubinfeld et al. 1996). This phosphorylation enhances the binding affinity of β -catenin for APC to assist the entry of β -catenin into the multiprotein destruction complex. Another kinase responsible for APC phosphorylation is Casein Kinase 1 ϵ (CK1 ϵ). CK1 ϵ regulates Wnt signaling positively and is involved in β -catenin regulation by directly phosphorylating APC at Ser1279 and 1392. It should be mentioned that both GSK3 β - and CK1 ϵ -mediated phosphorylation of APC are Axin-dependent (Rubinfeld et al. 2001). There is a novel kinase identified for axin-APC protein complex phosphorylation in HEK293 cells: GRK2. GRK2 can interact with SAMP repeat motif of APC via its N-terminal RGS domain. It is realized that the RGS domain of GRK2 is closely related to RGS domain of Axin, which is involved in protein-protein interaction between Axin and APC (SAMP repeat motif) to promote the assembly of multiprotein destruction complex. The interaction of GRK2 with APC is increased by the canonical Wnt signaling. But GRK2 phosphorylates a component of β -catenin destruction complex, containing axin-APC, to inhibit this signaling and regulates it in a negative feedback (Wang et al. 2009).

2) *Low-density Lipoprotein-Related protein 6 (LRP6)*

Low-density lipoprotein-related protein 6 (LRP6) is a single transmembrane receptor that functions as a critical co-receptor of Frz receptors for Wnt ligands (Chen et al. 2009) together with its structural analogous: LRP5. LRP6 belongs to LDLR (low-density lipoprotein receptor)

family and is also involved in regulation of glucose homeostasis, blood pressure, synthesis of triglycerides (TG) and fatty acids, TG and lipoprotein removal, and prevention of atherosclerosis. The LDLR family is a class of endocytotic receptors and includes LDLR, VLDLR (very low-density lipoprotein receptor), and LRP5 (LRP1, LRP2, LRP5 and LRP6) that bind and internalize extracellular molecules such as exotoxins and Apolipoprotein E (apoE) and Apolipoprotein B (apoB)-containing lipoprotein (chylomicron remnant, LDL & VLDL) to control their uptake. They are also involved in lipid metabolism and cholesterol homeostasis. LRP5/6 are the only members of this family that are involved in Wnt signaling pathway (Go and Mani 2012).

The structure of LRP6 is composed of three general domains: an extracellular N-terminal tail, a transmembrane anchor, and a cytoplasmic C-terminal tail. There are three types of domains in the extracellular N-terminal tail of LRP6: Epidermal growth factor (EGF)-like domain composed of several EGF repeats, YWTD-type B-propeller motif that participates in dissociation of ligand-receptor complex in a pH-dependent manner by the contribution of EGF repeats, and LDLR type A repeats that are in three clusters and involved in binding to ligands, such as apoB-100- and apoE-containing lipoprotein, Wnt and Dickkopf-1 (DKK1, an antagonist of LRP6 that suppresses its activity (Chen et al. 2009)) (Figure 28) (Go and Mani 2012). The cytoplasmic C-terminal tail of LRP6, which is phosphorylated by GSK3 β and casein kinase 1 γ (CK1 γ) to become activated upon exposure of Wnt ligands to Frz and LRP6 co-receptors, includes repeated PPPSPxS (Pro-Pro-Pro-Ser-Pro-X-Ser) domains. Phosphorylation of 5 of these repeated PPPSPxS domains, located near the Ser/Thr clusters, is an

important step in canonical Wnt signaling pathway, because it inhibits GSK3 β activity and makes docking sites for Axin. The Ser1490 at one of the PPPSPxS motifs is the GSK3 β -mediated phosphorylation site and the Thr1479 at Ser/Thr cluster is phosphorylated by CK1 γ (Tanneberger et al. 2011). GRK5 & 6 are the novel identified kinases that could positively regulate canonical Wnt signaling by phosphorylating LRP6 at repeated PPPSPxS domains and also other sites both in vitro and in cellulo. It has been discovered by in vitro studies that GRK5 could phosphorylate all five PPPSPxS domain in soluble LRP6 cytosolic tail, and GRK6 could phosphorylate three PPPSPxS domains. The Ser1490 residue resided in first PPPSPxS domains & Ser1509 residue located outside of the PPPSPxS domains are phosphorylated by both GRK5 and GRK6. The Ser1450 & Ser1448 are the other residues phosphorylated by GRK5 & 6, respectively. Thus, GRK5 & 6 are kinases that may stimulate the Wnt signaling by activating LRP6 (Chen et al. 2009).

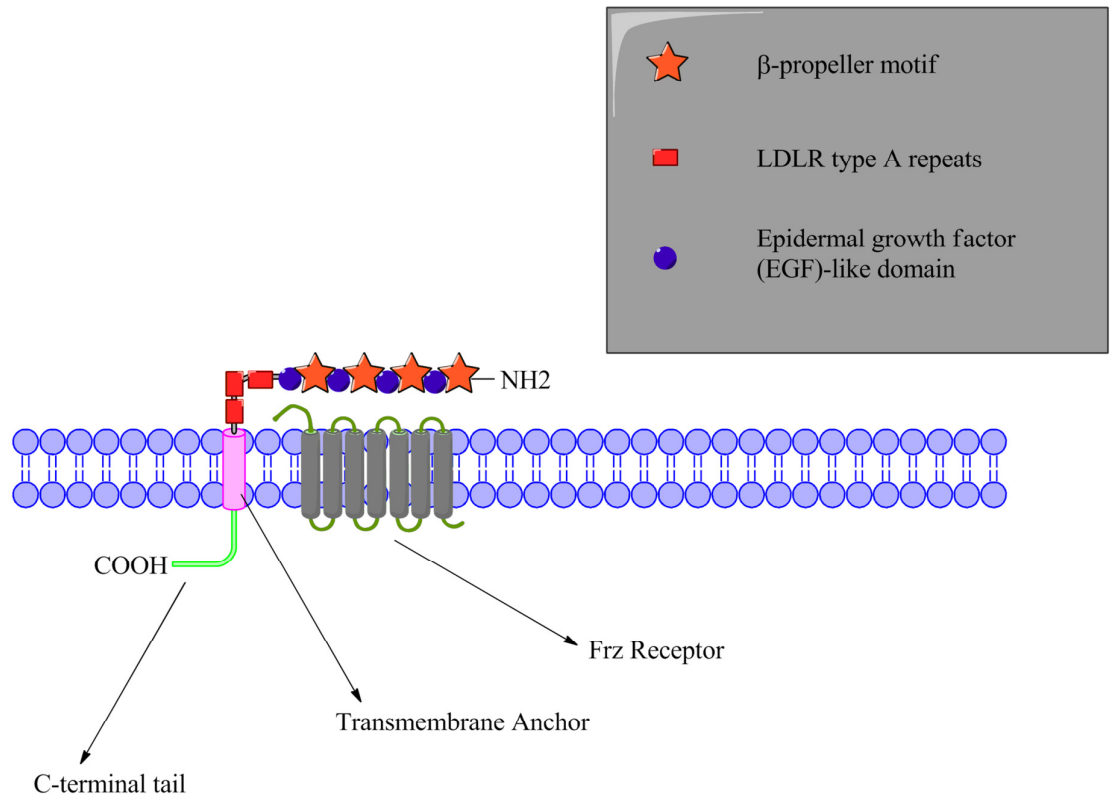


Figure 28: The schematic structure of LRP6 showing its N-terminal domains.

Inhibitor κ B ($I\kappa$ B) Family: nuclear factor κ B p150 (NF κ B p105) and inhibitor κ B α ($I\kappa$ B α)

Inhibitor κ B ($I\kappa$ B) family members are inhibitory subunits that regulate the activity of nuclear factor κ B (NF κ B), and contain $I\kappa$ B α , - β , - γ and - ϵ , Drosophila Cactus, NF κ B1 p105 (p105) (a precursor of p50), p100 (also called NF κ B2 and the precursor of p52), and Bcl-3 (B-cell leukemia 3) (Gilmore 2006; Parameswaran et al. 2006; Patial et al. 2009). However, p105 and p100 are sometimes categorized as members of NF κ B subfamily, because they have long C-terminal regions including multi copies of ankyrin repeats. These repeated domains have inhibitory effect on the action of their proteins, p105 and p100, and are proteolytically cleaved from the polypeptide to become active

DNA-binding factors, p50 and p52, respectively (Gilmore 2006). The I κ B proteins bind to NF κ B complex and sequester them in the cytosol (Cronstein et al. 1999). The NF κ B members are transcription factors that control the expression of genes participating in cell proliferation and apoptosis, innate and adaptive immunity, inflammation, cell adhesion and several pathologies of human (Basak et al. 2007; Patial et al. 2009). This family contains p65 (RelA), c-Rel (v-rel reticuloendotheliosis viral oncogene homolog (avian)), RelB, p52 (a product of p100) and p50 (a product of p105) (Parameswaran et al. 2006). By activation of NF κ B pathway, the I κ B proteins are phosphorylated by I κ B kinase (IKK) complex, composed of a scaffold, sensing protein called NF κ B essential modulator (NEMO) and subunits with kinase activity (IKK α and/or IKK β). The phosphorylation of I κ B proteins results in their ubiquitination and degradation through the ubiquitin-proteasome mechanism. Hence I κ B-bound NF κ B dimers are released and transported into the nucleus to influence the transcription of target genes via attaching to particular regions in their enhancer or promoter domains. These steps are the canonical model for NF κ B signaling activated by extracellular stimuli such as lipopolysaccharide (LPS) and Tumor necrosis factor α (TNF α) (Gilmore 2006; Parameswaran et al. 2006; Basak et al. 2007; Sorriento et al. 2008; Patial et al. 2009).

I κ B α is one of the most important members of I κ B family that is basically bound to NF κ B subunits p50 and p65 to sequester them in cytosol. After agonist-activation of NF κ B pathway, the bound I κ B α is phosphorylated (by IKK β), ubiquitinated and degraded. Therefore, the p50 and p65 subunits are released to enter the nucleus. It is discovered that IKK β phosphorylates I κ B α at Ser32/36. It has been shown that kinases such as ribosomal S6 Kinase, CK2 and IKK α phosphorylate I κ B α at these sites, too. By

Tumor necrosis factor α (TNF α)-treatment of Raw264.7 mouse macrophages two novel kinases of I κ B α have been identified: GRK2 & GRK5. Both of these kinases are highly expressed in immune cells such as macrophages. I κ B α could associate with GRK2 & GRK5 via its N-terminal region. By in vitro studies, it is observed that GRK2 & GRK5 could phosphorylate I κ B α at distinct residues. The residues Ser32/36 are phosphorylated by GRK5, the same as IKK β and other kinases named before. It is important to know that GRK2 & GRK5 have important roles in positively regulating TNF α -stimulated NF κ B pathway by phosphorylating I κ B α in Raw264.7 macrophages, whereas IKK β is not involved in this pathway (Figure 29). IKK β participates in regulating I κ B α -NF κ B pathway induced by lipopolysaccharide (LPS) (Patial et al. 2009). It is important to note that GRK5 binds to I κ B α by its RH domain, and their interaction results in accumulation of I κ B α in nucleus and sequestration of NF κ B transcriptional function (Sorriento et al. 2008).

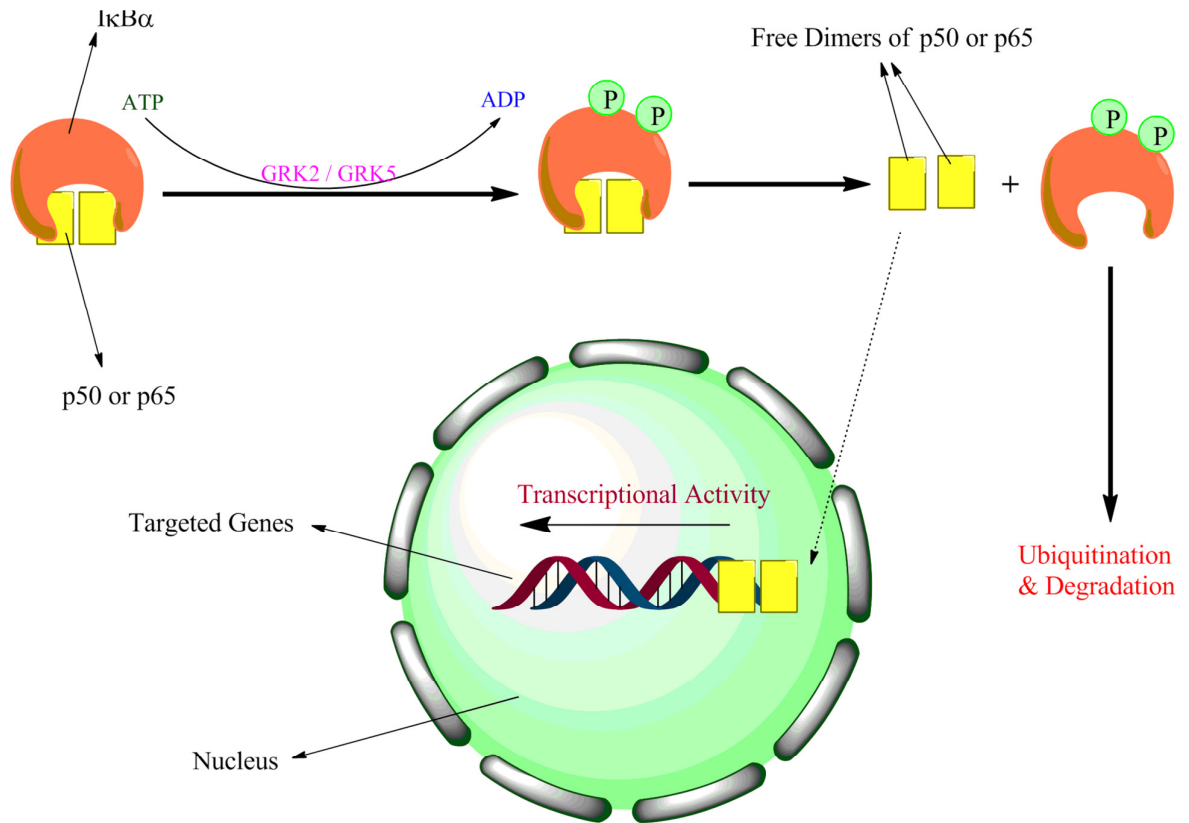


Figure 29: The proposed model for TNF α induced activation of NF κ B p50 or p65 by GRK2/5-mediated phosphorylation of I κ B α . The receptors and TNF α have not been shown in this figure.

Another member of I κ B family which some scientists categorize it as NF κ B subfamily member is p105. It is encoded by *Nfkb1* gene. p105 has some ankyrin repeats and a Rel homology region at its C-terminal and N-terminal tail, respectively (Figure 30 (Zhao et al. 2012)). As described previously, p105 is the precursor of p50. The C-terminal region of p105 is cleaved by proteasome to make the p50 monomers (Gilmore 2006; Zhao et al. 2012). p105 stoichiometrically binds to MAP3K TPL2 (Tumour Progression Locus-2) to keep it in inactive state under unstimulated conditions. Lipopolysaccharide (LPS) stimulation induces IKK-mediated

phosphorylation, ubiquitination and degradation of p105 in macrophages. Thus, the p105-bound TPL2 is released and could phosphorylate MEK1/2 (mitogen-activated protein-kinase/extracellular signal-regulated kinase kinase 1/2). MEK1/2 activates ERK1/2 by phosphorylating it (Parameswaran et al. 2006). It is realized that Ser927 & 932 in the PEST domain (polypeptide domains rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (Rechsteiner and Rogers 1996)) of p105 are the IKK-mediated phosphorylation sites following TNF α treatment (Lang et al. 2003). Another kinase that could effectively phosphorylate p105 is GRK5. GRK5 could interact with and phosphorylate p105 in vitro. The RH domain of GRK5 associates with C-terminal region of p105. β -arrestin 1 could also bind to this region of p105 in vitro (Parameswaran et al. 2006). As the C-terminal region of p105 is essential for activation of ERK induced by LPS, and both of GRK5 and β -arrestin 1 (Arrestin 2) bind directly to this region of p105, they could attenuate the LPS-induced activation of ERK1/2. Although RH domain of GRK2 can interact with C-terminus of p105, but it has been shown that GRK2 could phosphorylate p105 poorly (Parameswaran et al. 2006; Zhao et al. 2012).

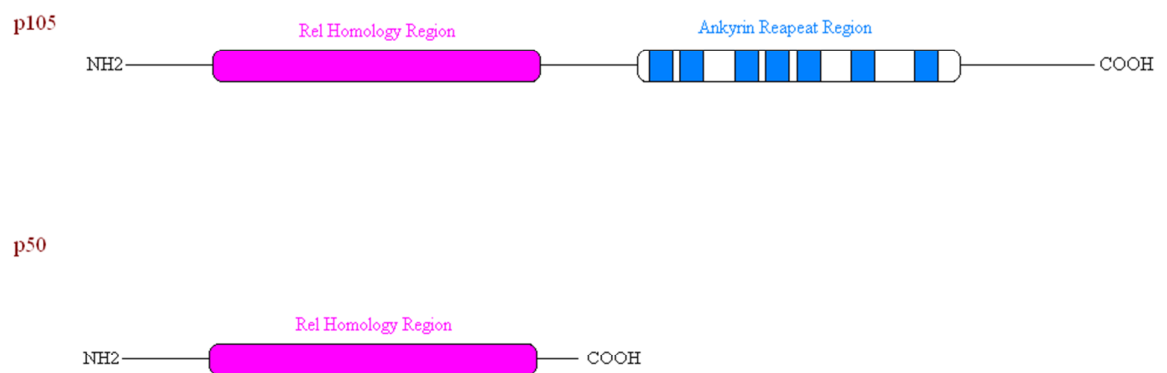


Figure 30: The structure of p105 & p50 with their main regions. The p105 is the precursor of p50. Its C-terminal ankyrin repeat region is cleaved proteolitically to generate p50.

Chapter 4: Discussion

Insufficiency of classical view about GRKs

According to classical model, three families of proteins are involved in GPCR desensitization: GRKs, arrestins and second messenger-dependent protein kinases (Ferguson 2007). GRKs and arrestins are critical components of homologous desensitization of GPCRs (Mushegian et al. 2012). Classically, scientists thought that GRKs phosphorylate only agonist-activated GPCRs (Pitcher et al. 1998b). But by discovering tubulin as the first non-GPCR substrate for GRKs in 1998 (Carman et al. 1998; Haga et al. 1998; Pitcher et al. 1998b) and other novel substrates later on, this canonical paradigm has become disabled to explain all the various roles of GRKs. Thus, in this thesis, we reviewed the novel substrates for GRKs which are not GPCRs. Our conclusion is that the classical view about GRKs and GPCR desensitization is not sufficient to explain all the functions of GRKs. The roles of GRKs are far more than that. We have tried to suggest novel functions for GRKs by introducing each of their non-GPCR substrates and the effect(s) of their phosphorylation by GRKs (Appendix).

Phosphorylation-dependent regulation by GRKs

We introduced all of non-GPCR substrates for GRKs discovered to date. They include a wide range of proteins found in cell membrane, cytoplasm and nucleus, such as non-GPCR receptors, nuclear proteins, tumor suppressor genes, and ion channels (Table 1) (Gurevich et al. 2012). We proposed new functions for GRKs in various cell processes.

Table 1: Categories of non-GPCR Targets for GRKs (Gurevich et al. 2012).	
Protein Groups	GRK Substrates
Non-GPCR Receptors	<i>EGFR, PDGRFβ, LRP6</i>
Membrane Proteins	<i>ENaCs, DREAM (KChIP3)</i>
Ribosomal Proteins	<i>P2</i>
Cytoskeletal Proteins	<i>Tubulin, Ezrin, Radixin</i>
Nuclear Proteins	<i>HDAC, NPM1</i>
Signaling Proteins	<i>β-Arrestin 1, Nedd4 & Nedd4-2, p38MAPK, IRS-1, NHERF, APC, PDEy, Phosducin & PhLP, Synucleins,</i>
Transcription Factors	<i>R-Smads, NFκB p105 & IκBα, P53</i>

For example, upon ischemic injury of cardiomyocytes GRK2 phosphorylates IRS-1 to reduce insulin sensitivity and glucose uptake (Evron et al. 2012). However, there are some debates going on regarding our work. Most of these targets we reviewed have been identified phosphorylated by GRKs via in vitro studies, or kinase and phosphorylation assays, but a few of them are studied in vivo (Appendix). As we will explain in next section, there are some problems and difficulties with cell-free approaches and methods to prove their phosphorylation by GRKs and their site(s) of phosphorylation. The results of in vitro studies are usually different with the results gained by in vivo experiments. Therefore, both of the results should approve each other to reach a consensus (Clark and Rich 2003). Thus, we propose more in vivo

studies to confirm that these proteins are real substrates for GRKs and to learn more about the consequences of their GRK-mediated phosphorylation.

Problems and difficulties in identification of GRKs' substrates and their phosphorylation sites

Clark & Rich discussed about difficulties in studies on the role of GRK2 in GPCR desensitization (Clark and Rich 2003). They believed that the number and location of GRK2-mediated phosphorylation sites of β -adrenergic receptor are different in cell-free experiments and in intact cell studies. The followings are the problems found within such studies (Clark and Rich 2003):

- 1) Results from intact cells and cell-free studies are not usually uniform with each other.
- 2) Regulation of most protein kinases by multiple pathways.
- 3) Hierarchical phosphorylation of targets.
- 4) The problem to identify GRK-mediated phosphorylation sites and their subsequent effects.
- 5) Regulations of endogenous and overexpressed substrates (receptors) are different.
- 6) Absence of specific inhibitors of kinases.
- 7) Absence of phospho-specific antibodies against GRK-mediated phosphorylation sites.

- 8) Overexpressing dominant negative protein kinases, or knocking out or down specific kinases rarely gives 100% inhibition, and specificity is difficult to achieve.
- 9) Protein kinase modulation of GPCRs is likely to vary between cell- and tissue-types.

Although the above problems are related to studies on "GPCR Desensitization", but we could generalize them to other phosphorylation studies. Therefore, more in vivo studies are required to reach to a consensus view on the roles of GRKs. The other debate we would like to elaborate on is the number 3 of the above problems, "Hierarchical phosphorylation of targets".

Hierarchical Phosphorylation

One of the most important ways to regulate cellular functions is phosphorylation of proteins (Kennelly and Krebs 1991). There could be 1 to 100 phosphorylation sites in phosphorylated target proteins. These sites are usually located at the regions that interact with or are accessible for related protein kinase(s). For some proteins with multiple phosphorylation sites, there is a mechanism that prior phosphorylation of a residue affects the phosphorylation of other residues subsequently. This process is called "hierarchical" or "synergistic" phosphorylation (Kennelly and Krebs 1991; Roach 1991). For example, in phosphorylation of glycogen synthase, both CK2 and glycogen synthase 3 are involved subsequently. The dephosphorylated glycogen synthase is first phosphorylated by CK2. It could not be

phosphorylated by glycogen synthase kinase 3, but after its phosphorylation by CK2, it could be phosphorylated by glycogen synthase kinase 3 at 4 Ser residues (Roach 1991). The other example is hierarchical phosphorylation of human tyrosine hydroxylase at Ser19, Ser31 and Ser40. The phosphorylation of Ser19 increases the phosphorylation of Ser40 in vitro and in situ in order to activate human tyrosine hydroxylase, whereas the Ser40 phosphorylation does not have any effects on Ser19 phosphorylation. The Ser31 phosphorylation also affects the phosphorylation of Ser40 in vitro and in intact cells (Lehmann et al. 2006). There are two possibilities to explain the function of phosphate group in hierarchical phosphorylation: First, the phosphorylated Ser residue could function as the recognition factor for secondary kinase. Second, the primary phosphorylation could alter the 3-dimensional structure of the target to enable it to be phosphorylated by secondary kinase without direct involvement of phosphate group (Roach 1991). We believe that these possibilities should be investigated about GRK targets, too. For example, multiple GRK2-mediated phosphorylation sites are predicted for Nedd4-2 (Arthur et al. 2006), or in the case of tubulin, there are two or three phosphorylation sites located near to each other (Yoshida et al. 2003). Thus, we suggest more research to prove the effects of every residue phosphorylation on phosphorylation of others, both in vitro and in vivo.

Phosphorylation-independent regulation by GRKs

In addition to kinase activity of GRKs, they also might be able to involve in GPCR signaling independent of their kinase activity (Ferguson 2007). Also, it has been shown that GRKs could interact directly also with non-receptor targets such as caveolin, RKIP

(Raf Kinase Inhibitor Protein), MEK, PI3K, Akt (Ribas et al. 2007; Sorriento et al. 2008), clathrin and G proteins (GRK2 & 3) to regulate their functions. For example, interaction of GRK2 with both PI3K and clathrin are important for internalization of agonist-activated β -adrenergic receptor (Evron et al. 2012). We suggest that this aspect of GRK activity would be a great topic for future research.

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Appendix

The coming table summarizes the following information: GRK's non-GPCR targets, their phosphorylation sites, methods and techniques that were used to identify them as a GRK substrate and the consequences of GRK-mediated phosphorylation. The blank space in the table means that we have not found any data, or it is still unknown or unidentified.

Substrate	β -Arrestin 1	Dream (KCHIP3)	ENaCs
GRK isoform(s)	GRK5 (more efficient) & GRK2 (Barthet et al. 2009)	GRK2 (more efficient) & GRK6 (Ruiz-Gomez et al. 2007)	GRK2 (Dinudom et al. 2004)
Phosphorylation site(s)	Ser412 (by GRK5) (Barthet et al. 2009)	Ser95 (by GRK2) (Ruiz-Gomez et al. 2007)	Ser633 (Dinudom et al. 2004)
Cultures/Cells	colliculi neurons and HEK293 cells (Barthet et al. 2009)	HEK293 Cells (Ruiz-Gomez et al. 2007)	Cultured salivary duct cell of male mice (Dinudom et al. 2004)
Consequences of GRK-mediated phosphorylation	Negative regulation of G protein-independent Src/ERK pathway triggered by 5-HT ₄ R activation (Barthet et al. 2009)	Reduction in the membrane expression of Kv4 potassium channels by inhibiting their trafficking to and localization on plasma membrane (Ruiz-Gomez et al. 2007)	Increase activity of phosphorylated-ENaC & Insensitive to ubiquitin protein ligase Nedd4 family, especially Nedd4-2 (Dinudom et al. 2004)
Other kinases	ERK1 & ERK2, Src Kinases (Barthet et al. 2009)	a kinase downstream from phosphoinositide 3-kinase/Akt (Ruiz-Gomez et al. 2007)	CK2 (Dinudom et al. 2004)
Methods used	In vitro kinase assay & immunoblotting (Barthet et al. 2009)	Rhodopsin Phosphorylation Assays, Two-dimensional Gel Electrophoresis and Mass Spectrometry Analysis (Ruiz-Gomez et al. 2007)	Phosphorylation Assays (Dinudom et al. 2004)

Substrate	Nedd4	Nedd4-2	Ezrin
GRK isoform(s)	GRK2 (Sanchez-Perez et al. 2007)	GRK2 (Sanchez-Perez et al. 2007)	GRK2 (Cant and Pitcher 2005)
Phosphorylation site(s)	Thr466 (Sanchez-Perez et al. 2007)	WW1 & WW2 Domains (Arthur et al. 2006; Sanchez-Perez et al. 2007) Multiple GRK2-mediated phosphorylation sites are predicted but not identified yet (Arthur et al. 2006)	Thr567 (Cant and Pitcher 2005)
Cultures/Cells	In vitro, cultured salivary duct and kidney cells of male mice (Sanchez-Perez et al. 2007)	In vitro, cultured salivary duct and kidney cells of male mice (Sanchez-Perez et al. 2007)	In vitro, cultured mammalian cells & HEK29 Cells (Cant and Pitcher 2005)
Consequences of GRK-mediated phosphorylation	Decrease the interaction of phosphorylated-Nedd4 with ENaC (Sanchez-Perez et al. 2007)		GRK2-mediated phosphorylation is required for ezrin activation and stabilization and also endocytosis of agonist-activated β 2AR (Cant and Pitcher 2005).
Other kinases	Akt & Sgk1 (Yang and Kumar 2010)	Akt & Sgk1 (Yang and Kumar 2010)	PKC α , PKC θ , Nck-interacting kinase, Rho kinase, myotonic dystrophy kinase-related Cdc42-binding kinase (Cant and Pitcher 2005; Kahsai et al. 2010)
Methods used	Immunoprecipitation of Nedd4 & Nedd4-2 with GRK2, phosphorylation reactions (Sanchez-Perez et al. 2007)	Phosphorylation reactions (Sanchez-Perez et al. 2007), sequence analysis using programs: PREDIKIN & NetPhos 2.0, homology modeling using the Mo1IDE framework, & Surface accessibility calculations of GRK2 to Nedd4-2 (Arthur et al. 2006)	In vitro phosphorylation assays, immunofluorescence, FACS analysis (Cant and Pitcher 2005)

Substrate	Radixin	Tubulin	PDGFR β
GRK isoform(s)	GRK2 (Kahsai et al. 2010)	GRK2 (Pitcher et al. 1998b) & GRK5 (Yoshida et al. 2003)	GRK2 (Freedman et al. 2002; Wu et al. 2005) GRK5 (Wu et al. 2006)
Phosphorylation site(s)	Thr564 (Kahsai et al. 2010)	Thr409 and Ser420 of β I-tubulin & Thr409, Ser420 and Ser444 of β III-tubulin (by GRK2) (Yoshida et al. 2003)	Ser1104 (for GRK2) (Hildreth et al. 2004) and Ser1005, Ser1006 (for GRK5) (Wu et al. 2006)
Cultures/Cells	In vitro, MDCK Cells (Kahsai et al. 2010)	HEK293 Cells & intact cells (for GRK2) (Pitcher et al. 1998b) in vitro (for both GRK2 & GRK5) (Carman et al. 1998)	For GRK2: rabbit aortic smooth muscle cells (Freedman et al. 2002), in vivo (Chen et al. 2008) HEK293 cells (Freedman et al. 2002; Wu et al. 2005), heterologous cellular systems, mouse embryonic fibroblasts (MEFs) & HEK293 Cells (Hildreth et al. 2004) For GRK5: in vitro, Mouse thoracic aortic Smooth muscle cells, HEK293 Cells & embryo fibroblasts (MEFs) (Wu et al. 2006)
Consequences of GRK-mediated phosphorylation	Stimulates cell migration & extension of membrane outgrowths, Increases the activity of Rac1 (Kahsai et al. 2010)	Enables phosphorylated-tubulin to interact with actin filaments and membranes (Carman et al. 1998)	GRK2-mediated phosphorylation of PDGFR β s results in its ubiquitination by c-Cbl or other potential ubiquitin E3 ligases (Freedman et al. 2002) and decreases its interaction with NHERF (Hildreth et al. 2004) GRK5-mediated Ser-phosphorylation of agonist-activated PDGFR β s is involved in its desensitization in Smooth muscle cell (Wu et al. 2006)
Other kinases	PKC α , PKC θ , Nck-interacting kinase, Rho kinase, myotonic dystrophy kinase-related Cdc42-binding kinase	tyrosine kinases, PKA, CamKII (Haga et al. 1998) and CK1 & 2 (Pitcher et al. 1998b)	agonist-activated PDGFR β (Wu et al. 2005), PDGFR β Tyr kinase (Wu et al. 2006)

	(Cant and Pitcher 2005; Kahsai et al. 2010)		
Methods used	Site-directed mutagenesis, kinase assay, immunofluorescence, western blot, overexpression & knockdown of GRK2 (Kahsai et al. 2010)	Phosphorylation assay, GST- β ARK fusion binding assay, binding and phosphorylation of Taxol-stable microtubules by GRKs & immunoprecipitation (Carman et al. 1998) preparation of GRK2C-GST-binding proteins, phosphorylation reaction, SDS-PAGE (Haga et al. 1998) immunoprecipitation of GRK2 and tubulin, phosphorylation reactions, phosphorylation of cellular tubulin & immunoelectron microscopy of HEK293 Cells for GRK2-Flag and tubulin (Pitcher et al. 1998b)	For GRK2: phosphoamino acid analysis, receptor immunoprecipitations, immunoblotting, immune complex kinase assays (Freedman et al. 2002) immunoprecipitations and immunoblotting, phosphoinositide hydrolysis (Hildreth et al. 2004) immunoprecipitation, immunoblotting (Wu et al. 2005) For GRK5: knock-out mice, immunoblotting and immunoprecipitations, in vitro phosphorylation with purified GRK5 (Wu et al. 2006)

Substrate	EGFR	NHERF	Hip
GRK isoform(s)	GRK2 (Freedman et al. 2002)	GRK6A (Hall et al. 1999b)	GRK5 (more efficient) & GRK2 (less efficient) (Barker and Benovic 2011)
Phosphorylation site(s)		Ser289 (Hall et al. 1999b)	Ser346 (Barker and Benovic) (Barker and Benovic 2011)
Cultures/Cells	In purified protein systems, intact cells, rabbit aortic smooth muscle cells (SMC)& HEK293 cells (Freedman et al. 2002)	In vitro, HEK293 cells (Hall et al. 1999b)	In vitro, HEK293 cells (Barker and Benovic 2011)
Consequences of GRK-mediated phosphorylation		In vitro analysis has shown that this serine phosphorylation, along with phosphorylation of Ser287 & 290, increase the inhibitory effect of NHERF on NEH3 (Hall et al. 1999b)	Stimulates the Hip-mediated endocytosis of CXCR4 after binding of CXCL12 (Barker and Benovic 2011)
Other kinases		PKA (Hall et al., 1999)	
Methods used	Receptor immunoprecipitations, immunoblotting, immune complex kinase assays, hydrolysis and phosphoamino acid analysis of the receptor (Freedman et al. 2002)	Immunoprecipitations & kinase assay (Hall et al. 1999b)	In vitro kinase assay, Co-immunoprecipitation (Barker and Benovic 2011)

Substrate	IRS-1	PDEy	p38 MAPKs
GRK isoform(s)	GRK2 (Usui et al. 2005)	GRK2 (Wan et al. 2001)	GRK2 (Peregrin et al. 2006)
Phosphorylation site(s)	Ser612/636 (Usui et al. 2005)	Thr62 in rod PDEy (Wan et al. 2001)	Thr123 (Peregrin et al. 2006)
Cultures/Cells	3T3-L1 Adipocytes (Usui et al. 2005)	HEK293 cells (Wan et al. 2001)	In vitro (Peregrin et al. 2006)
Consequences of GRK-mediated phosphorylation	Potentiates IRS-1 degradation ensuing to decrease of insulin signaling (Usui et al. 2005)	PDEy phosphorylated by GRK2 positively regulates the EGF- and thrombin-dependent activation of the p42/p44 MAPK pathway in HEK293 cells (Wan et al. 2001)	p38 MAPKs is phosphorylated by GRK2 to become inactivated (Peregrin et al. 2006)
Other kinases	Insulin receptor (Usui et al. 2005), TNF α (at Ser307) (Walker et al. 2012), Janus kinase, IKK, MAPK, GSK3, PKC, and mammalian target of rapamycin (Usui et al. 2005)	p42/p44 MAPK (Wan et al. 2003)	MKK3 & MKK6 (Peregrin et al. 2006)
Methods used	Overexpression of wild-type (WT)- & kinase-deficient (KD)-GRK2 in 3T3-L1 Adipocytes treated with ET-1, western blot using anti-insulin receptor (IR), PY20, or anti-GRK2 antibody, using phosphospecific IRS-1 antibodies against Ser307, Ser612, and Ser636 (Usui et al. 2005)	Immunoprecipitation, western blotting, phosphorylation incubations & site-directed mutagenesis (Wan et al. 2001)	Western blot, immunoprecipitation, site-directed mutagenesis (Peregrin et al. 2006)

Substrate	Phosducin	PhLP	Smad2
GRK isoform(s)	GRK2 (Ruiz-Gomez et al. 2000)	GRK2 (Ruiz-Gomez et al. 2000)	GRK2 (Ho et al. 2005)
Phosphorylation site(s)	Not identified exactly (serines & threonines between residues 204-245 of phosducin) (Ruiz-Gomez et al. 2000)	Not identified exactly (serines & threonines between residues 195-218 of phosducin-like protein) (Ruiz-Gomez et al. 2000)	Thr197 (Ho et al. 2005)
Cultures/Cells	In vitro (Ruiz-Gomez et al. 2000)	In vitro (Ruiz-Gomez et al. 2000)	In vitro, human hepatocarcinoma cells (Ho et al. 2005)
Consequences of GRK-mediated phosphorylation	GRK2-mediated serine/threonine phosphorylation of phosducin and PhLP decreases their affinity to G $\beta\gamma$, and inhibits their interaction. (Ruiz-Gomez et al. 2000)	GRK2-mediated serine/threonine phosphorylation of phosducin and PhLP decreases their affinity to G $\beta\gamma$, and inhibits their interaction. (Ruiz-Gomez et al. 2000)	R-Smads phosphorylated by GRK2 could not enter into the nucleus, so the signaling cascade of activin/TGF β is inhibited (Ho et al. 2005).
Other kinases	PKA (only for Phosducin) (Ruiz-Gomez et al. 2000)		TGF- β type I receptor, CamKII, MAPKs, PKC, and Erk-family MAP kinases (Singh et al. 2011)
Methods used	Phosphorylation assays, two-dimensional gel electrophoresis (Ruiz-Gomez et al. 2000)	Phosphorylation assays, two-dimensional gel electrophoresis (Ruiz-Gomez et al. 2000)	Northern blot, RT-PCR, Co-immunoprecipitation & in vitro kinase assay (Ho et al. 2005)

Substrate	Smad3	α -synuclein	β -synuclein
GRK isoform(s)	GRK2 (Ho et al. 2005)	GRK1, 2, 5, & 6 (Pronin et al. 2000)	GRK2 & 5 (Pronin et al. 2000)
Phosphorylation site(s)		Ser129 (Pronin et al. 2000)	Ser118 (Pronin et al. 2000)
Cultures/Cells	In vitro, human hepatocarcinoma cells (Ho et al. 2005)	COS-1 cells (Pronin et al. 2000), in vitro (Pronin et al. 2000; Fiske et al. 2011)	COS-1 cells (Pronin et al. 2000), in vitro (Pronin et al. 2000; Fiske et al. 2011)
Consequences of GRK-mediated phosphorylation	R-Smads phosphorylated by GRK2 could not enter into the nucleus, so the signaling cascade of activin/TGF β is inhibited (Ho et al. 2005).	Prevents the association of phosphorylated synucleins by PLD2 and phospholipids, results in disinhibition of PLD2 to arrange actin cytoskeleton and formation of vesicles for recycling and internalizing the receptor (Pronin et al. 2000) Phosphorylated α -synuclein at Ser129 will be dephosphorylated and degraded by the proteasome pathway and independent of ubiquitin (Machiya et al. 2010)	Prevents the association of phosphorylated synucleins by PLD2 and phospholipids, results in disinhibition of PLD2 to arrange actin cytoskeleton and formation of vesicles for recycling and internalizing the receptor (Pronin et al. 2000)
Other kinases	TGF- β type I receptor, CamKII, MAPKs, PKC, and Erk-family MAP kinases (Singh et al. 2011)	CK1 & 2, the pololike kinases (Fiske et al. 2011)	CamKII, CK1, CK2 (weak kinases) (Pronin et al. 2000)
Methods used	Northern blot, RT-PCR, Co-immunoprecipitation & in vitro kinase assay (Ho et al. 2005)	Co-transfection of COS-1 cells with cDNA for α -synuclein, immunoblotting, immunoprecipitation, phosphoamino acid analysis (Pronin et al. 2000)	Immunoblotting, immunoprecipitation, phosphoamino acid analysis (Pronin et al. 2000)

Substrate	γ -synuclein	Synoretin	P2
GRK isoform(s)	GRK2 & 5 (Pronin et al. 2000)	GRK2 & 5 (Pronin et al. 2000)	GRK2 (Freeman et al. 2002)
Phosphorylation site(s)	Ser124 (by GRK2) (Pronin et al. 2000)		Ser102 and Ser105 (Freeman et al. 2002)
Cultures/Cells	COS-1 cells (Pronin et al. 2000), in vitro (Pronin et al. 2000; Fiske et al. 2011)	COS-1 cells (Pronin et al. 2000), in vitro (Pronin et al. 2000; Fiske et al. 2011)	In vitro, HEK293 cells (Freeman et al. 2002)
Consequences of GRK-mediated phosphorylation	Prevents the association of phosphorylated synucleins by PLD2 and phospholipids, results in disinhibition of PLD2 to arrange actin cytoskeleton and formation of vesicles for recycling and internalizing the receptor (Pronin et al. 2000)	Prevents the association of phosphorylated synucleins by PLD2 and phospholipids, results in disinhibition of PLD2 to arrange actin cytoskeleton and formation of vesicles for recycling and internalizing the receptor (Pronin et al. 2000)	Has stimulatory effect on the translational function of P2 (Freeman et al. 2002)
Other kinases	CamKII and CK2 (Pronin et al. 2000)	CamKII and CK2 (weak kinases) (Pronin et al. 2000)	CK2 (Freeman et al. 2002)
Methods used	Immunoblotting, immunoprecipitation, phosphoamino acid analysis (Pronin et al. 2000)	Immunoblotting, immunoprecipitation, phosphoamino acid analysis (Pronin et al. 2000)	2D gel electrophoresis and mass spectrometry, applied biosystems API 165 quadrupole mass spectrometer, phosphorylation assays (Freeman et al. 2002)

Substrate	HDAC	NPM1	P53
GRK isoform(s)	GRK5 (Martini et al. 2008; Zhang et al. 2011)	GRK5 (So et al. 2012)	GRK5 (Chen et al. 2010a)
Phosphorylation site(s)	Ser259 (for HDAC5) (Martini et al. 2008)	Ser4 (in HeLa Cells) & Thr199 (So et al. 2012)	Thr55 (Chen et al. 2010a)
Cultures/Cells	In vitro (Martini et al. 2008) and in vivo, Cos7 Cells, transgenic mice (Zhang et al. 2011) cardiomyocytes, mouse heart (Martini et al. 2008) human neuroblastoma SHSY5Y cells (Liu et al. 2010)	In vitro, and intact cells (HeLa cells) (So et al. 2012)	In vitro and in vivo human osteosarcoma cells (U2OS & Saos-2 cells) GRK5-knock out mice (Chen et al. 2010a)
Consequences of GRK-mediated phosphorylation	Increases the transcriptional activity of MEF2 for hypertrophic genes by disinhibiting and derepressing it from GRK5-mediated phosphorylation HDAC leading to heart hypertrophy (Martini et al. 2008)	GRK5-mediated phosphorylation of NPM1 reduces the sensitivity of cells to PLK1 inhibition (So et al. 2012)	GRK5-mediated phosphorylation of P53 results in its degradation by binding to MDM2 in order to prevents its P53-mediated apoptosis (Chen et al. 2010a)
Other kinases	PKD, CamKII (Martini et al. 2008) and PKC (Zhang et al. 2011)	PLK1 (Michal et al. 2012), CDK1 & CDK2 (Koike et al. 2010)	ATM, ATM- and Rad3-related, DNA-dependent protein kinase, or CHK2 (Chen et al. 2010a)
Methods used	Immunoprecipitation , phosphorylation and HDAC activity assay (Martini et al. 2008) using HDAC assay kit (Liu et al. 2010) immunoblot analysis, RNA sequencing (Zhang et al. 2011)	Immunoblotting and immunoprecipitation , In vitro kinase assay, and flow cytometry (So et al. 2012)	Immunoprecipitation, gene knockdown, & in vitro kinase assay (Chen et al. 2010a)

Substrate	APC	LRP6	NFκB p105
GRK isoform(s)	GRK2 (Wang et al. 2009)	GRK5 & 6 (Chen et al. 2009)	GRK5 (Parameswaran et al. 2006)
Phosphorylation site(s)		All 5 repeated PPPSPxS domains (including Ser1490) of soluble LRP6 cytosolic tail + Ser1450 + Ser1509 (for GRK5) (Chen et al. 2009) 3 repeated PPPXPxS domains (including Ser1490) of soluble LRP6 cytosolic tail + Ser1448 + Ser1509 (for GRK6) (Chen et al. 2009)	
Cultures/Cells	HEK293 cells (Wang et al. 2009)	In vitro and in cellulo, mouse embryonic fibroblast (MEF) cell, HEK293 cells, zebrafish (Chen et al. 2009)	In vitro (Parameswaran et al. 2006)
Consequences of GRK-mediated phosphorylation	GRK2 phosphorylates APC to inhibit Wnt/β-catenin signaling (Wang et al. 2009).	GRK5 & 6-mediated phosphorylation of LRP6 may activate it to enhance the Wnt signaling cascade (Chen et al. 2009).	GRK5 interacts and phosphorylates with p105 to attenuate the activation of ERK1/2 induced by LPS (Parameswaran et al. 2006; Zhao et al. 2012)
Other kinases	PKA (Zhang et al. 2000), GSK3β, CK1ε (Rubinfeld et al. 2001)	GSK3β and CK1γ (Tanneberger et al. 2011)	IKK (Lang et al. 2003)
Methods used	Coimmunoprecipitation, immunoblotting, gene knockdown (Wang et al. 2009)	Gene knockdown & knockout, kinase phosphorylation assay and mass spectrometry, western blots and in situ hybridization for zebrafish embryos (Chen et al. 2009)	Western blotting, phosphorylation assays (Parameswaran et al. 2006)

Substrate	I κ B α
GRK isoform(s)	GRK2 & GRK5 (Patial et al. 2009)
Phosphorylation site(s)	Ser32/36 (for GRK5) (Patial et al. 2009)
Cultures/Cells	In vitro, Raw264.7 mouse macrophages, HEK293T cells (Patial et al. 2009)
Consequences of GRK-mediated phosphorylation	GRK2 & 5 phosphorylate I κ B α in order to increase the NF κ B activity after treatment of Raw264.7 macrophages by TNF α (Patial et al. 2009).
Other kinases	Ribosomal S6K, CK2 and IKK α (Patial et al. 2009)
Methods used	Overlay assay, in vitro phosphorylation assay, overexpression of GRK2 & 5, RNA interference (Patial et al. 2009)