

## Review

# The Role of G Protein-coupled Receptor Kinases in Cancer

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## Abstract

G protein-coupled receptors (GPCRs) are the largest family of plasma membrane receptors. Emerging evidence demonstrates that signaling through GPCRs affects numerous aspects of cancer biology such as vascular remodeling, invasion, and migration. Therefore, development of GPCR-targeted drugs could provide a new therapeutic strategy to treating a variety of cancers. G protein-coupled receptor kinases (GRKs) modulate GPCR signaling by interacting with the ligand-activated GPCR and phosphorylating its intracellular domain. This phosphorylation initiates receptor desensitization and internalization, which inhibits downstream signaling pathways related to cancer progression. GRKs can also regulate non-GPCR substrates, resulting in the modulation of a different set of pathophysiological pathways. In this review, we will discuss the role of GRKs in modulating cell signaling and cancer progression, as well as the therapeutic potential of targeting GRKs.

Key words: G protein-coupled receptor (GPCR), G protein-coupled receptor kinase (GRK), Cancer, Cell signaling

## Introduction

More than 700 genes have been identified as G protein-coupled receptors (GPCRs), which form the largest protein superfamily in the human genome [1]. GPCRs play key roles in mediating a wide variety of physiological events, from hormonal responses to sensory modulation (vision, olfaction, and taste) [2, 3]. GPCR-targeting drugs are used to treat many diseases. Over 40% of all FDA approved drugs are aimed at targeting GPCRs or their related pathways [4]. GPCRs are now being used as early diagnosis biomarkers for cancer, as they play integral roles in regulating and activating cancer-associated signaling pathways [5, 6]. Though GPCRs represent a growing share of all new anticancer therapies, “druggable” GPCRs represent only a small subset of receptors from this superfamily [5, 7]. This is mainly due to drug resistance, as studies that have used short- and long-term exposure to GPCR-targeting drugs have observed receptor desensitization [5, 8, 9]. Therefore, the pharmacological potential for GPCRs and their

downstream regulators requires further investigation in order to further develop therapies that can efficiently target cell signaling pathways in cancer [6].

GPCR signaling generally results in the transmission of amplified signals throughout the cell, and hyperactivation of these receptors may result in loss of normal cell physiological properties. There are several mechanisms including GTP hydrolysis, second messenger related protein kinases (e.g., PKA and PKC), G-protein-coupled receptor kinases (GRKs), and arrestins that prevent hyperactivation of GPCR signaling. However, the most important mechanism of terminating GPCR hyperactivation is GRK-mediated phosphorylation. Upon ligand-dependent receptor activation, GRK phosphorylates its target GPCR to prevent excessive cellular signaling. The details of this GPCR termination mechanism will be discussed in our review. Because GRKs act as negative regulators of GPCR activity, it has been suggested that they may play a role in cancer

progression by regulating cell proliferation, migration, apoptosis, invasion, and tumor vascularization in a cell type-dependent manner [10-14]. Therefore, a complete understanding of their role in GPCR signaling is of high clinical importance. In this article, we will review current research linking GRKs to various aspects of cancer biology and discuss the therapeutic potential of targeting GRKs.

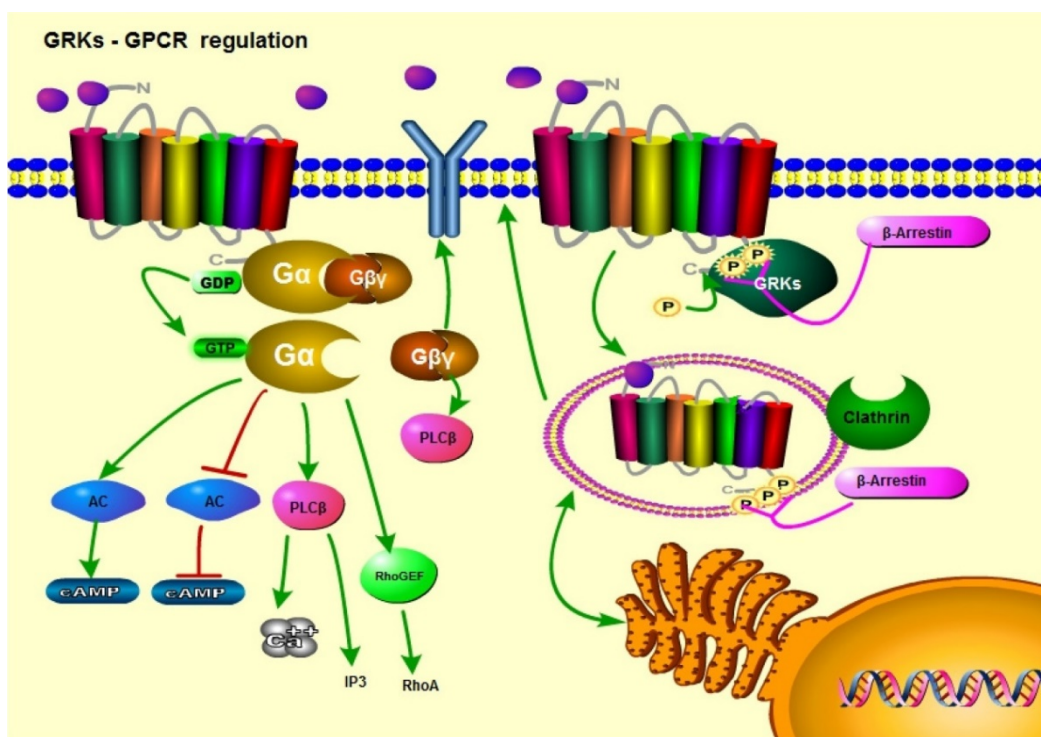
## Role of GRKs in GPCR signaling pathway

### GPCR signaling pathway

GPCRs serve as the interface between intracellular and extracellular environments. Receptors of this family are characterized by seven highly hydrophobic transmembrane helix structures. Stimuli, such as photons, small chemicals, ions, or protein ligands induce conformational changes in GPCR, which translates into cell signaling responses by activating coupled trimeric G protein complexes [15]. Based on sequence and structural similarity, GPCRs are classified into five subfamilies, namely rhodopsin, secretin, glutamate, adhesion, and frizzled [1]. The core structure of each GPCR can be divided into three domains: the extracellular region (ECR), which includes the N-terminus and three extracellular loops, the transmembrane region (TM), which includes seven  $\alpha$ -helices, and the intracellular region

(ICR) that contains three intracellular loops, an intracellular amphipathic helix, and the C-terminus [16]. Generally, the functional role of the ECR is to initiate binding of specific ligands. The TM region forms the receptor core structure, which is important for transducing extracellular stimulus through conformational changes. The TM region may also be involved in ligand binding in the majority of GPCRs. The ICR interacts with coupled G proteins, which are activated through conformational changes, and transduce downstream intracellular signaling [16].

Upon induction of conformational changes, GPCRs mediate signaling through G protein heterotrimers ( $G\alpha$ ,  $\beta$ , and  $\gamma$  subunits) [17]. These G proteins help transduce downstream signaling amplification based on the different functions of each  $G\alpha$  subunit isoform. Heterotrimeric G proteins transduce signals by exchanging GDP for GTP on the  $G\alpha$  subunit in response to ligand dependent activation of the GPCR. The  $G\alpha$ -GTP subunit can then separate from the  $G\alpha\beta\gamma$  heterocomplex and interact with its downstream signaling effectors. Because different GPCRs interact with a diverse array of intracellular coupled  $G\alpha$  protein isoforms, they can modulate a variety of downstream signaling pathways, and cause unique physiological responses when activated by different stimuli (Figure 1) [18].



**Figure 1. GPCR/GRK cell signaling; a general view.** Upon ligand binding, GPCRs activate downstream signaling pathways through their coupled  $G\alpha$  subunits. In response to GPCR conformational changes, G proteins activate downstream targets by converting GDP to GTP. GRKs phosphorylate the intracellular regions of GPCRs. This results in receptor desensitization and arrestin recruitment. Arrestins initiate receptor endocytosis, followed by receptor recycling or degradation.

## GRK family

The GRK family is comprised of seven serine/threonine kinases. The main function of these proteins is to inhibit activated GPCRs. Mammalian GRKs are classified into three subgroups based on sequence similarity (Figure 2): the rhodopsin kinases (GRK1 [rhodopsin kinase] and GRK7 [cone opsin kinase]), the  $\beta$ -ARK subgroup (GRK2 and GRK3), and the GRK4 subgroup (GRK4, GRK5, and GRK6). In humans, there are four GRK4 splice variants (GRK4- $\alpha$ , GRK4- $\beta$ , GRK4- $\gamma$ , and GRK4- $\delta$ ), and three GRK6 splice variants (GRK6A, GRK6B, and GRK6C) [19, 20].

All mammalian GRKs possess a similar protein structure, with a catalytic domain (~270 amino acids), an N-terminal domain (~185 amino acids), and a variable-length C-terminal domain (~105-230 amino acids). The N-terminal domain contains two conserved motifs for ligand binding and plasma membrane insertion [21, 22]. A single regulator of G-protein signaling (RGS) domain (~120 amino acids) is located within each of the N- and C-terminal domains, and both RGS domains contain a kinase catalytic domain (KD). The RGS domains regulate GPCR function via phosphorylation [23-25]. Phosphorylation occurs in response to ligand binding to GPCR. GRK-induced phosphorylation of cytoplasmic serine and threonine residues of GPCR triggers conformational changes in the receptor, revealing high affinity helical binding domains for  $\beta$ -arrestins. As a result,  $\beta$ -arrestin binds to the GPCR and inhibits downstream signaling [26]. Because GRKs also belong to protein kinase A, G, and C families (AGC), all GRKs share a conserved AGC sequence, which is important for kinase activity [27]. Unlike other GRKs, GRK2 and GRK3 have a pleckstrin homology domain (PH), which is critical for serine/threonine phosphorylation site recognition and termination of  $G\beta\gamma$ -induced signaling [28, 29]. In addition, the  $G\beta\gamma$  binding site of GRK2 is located in the N-terminal domain, which is involved in GRK2 cell surface localization and termination of  $G$ - $\beta\gamma$  downstream signaling [30]. Furthermore, GRK4 has a phosphatidylinositol-(4,5)-biphosphate (PIP<sub>2</sub>) motif in its N-terminal that can increase its catalytic kinase activity [31].

GRK4 is predominately expressed in the brain, kidney, testis, and human granulosa cells [32-35]. GRK1 and GRK7 are expressed mainly in retinal cells, where they mediate photo-transduction by phosphorylating rhodopsin receptors [36]. All other GRKs are expressed ubiquitously throughout the body and regulate the functions of a variety of GPCRs [37].

There are many more types of GPCRs than

GRKs, with each GRK interacting with and phosphorylating multiple GPCRs [13]. However, several groups have found that a subset of GRKs show substrate specificity with a select few GPCRs, despite the structural similarity of their domains. Therefore, the physiological roles of each GRK isoform may vary significantly [38]. In agreement, GRKs can find other regions of their targeted GPCR to phosphorylate even when the domain is mutated [39]. The N-terminus of each GRK is highly conserved and critical for GPCR identification. However, how GRKs recognize GPCR conformational changes is still unknown, making its role during GPCR activation unclear [40].

## Termination of GPCR signaling

The major function of GRKs is to mediate signaling termination through phosphorylation of activated GPCRs. The activated GPCR is directly regulated by two major mechanisms involving both GRKs and arrestins, where arrestins are recruited to the GPCR upon phosphorylation by GRKs. These two interacting regulatory proteins terminate GPCR intracellular signal transduction, and control G protein subunit coupling.

The mechanism of GPCR inhibition involves GRK phosphorylation of the intracellular regions of the target GPCR. Arrestins then bind to the phosphorylated GPCR domain to inhibit further G protein activation. This process leads to GPCR desensitization, internalization, and recycling or degradation, which require the recruitment of other proteins such as AP-2 and clathrin. To phosphorylate a ligand-bound GPCR, the GRK must be located at (or translocated to) the plasma membrane, where it forms a complex with the receptor. GRK1, GRK4, GRK5, GRK6, and GRK7 are normally found on the plasma membrane, where they bind and phosphorylate their target receptors. However, GRK2 and GRK3 require further steps in order to localize to the plasma membrane. These two kinases are mainly localized in the cytosol and endoplasmic reticulum. Only upon GPCR activation do they recognize detached  $G\beta\gamma$  subunits and undergo translocation to the plasma membrane to desensitize GPCRs [41]. Once a ligand binds to a GPCR, GRKs sense the intracellular conformational changes as it separates from coupled G protein subunits. They can then phosphorylate the C-terminus of the target GPCR [42]. Phosphorylation will then induce additional GPCR conformational changes and increase its affinity for arrestins. The binding of arrestins prevents further coupling of G protein subunits and inhibits second messenger signal transduction [43]. Furthermore, GRKs also attenuate GPCR signal transduction by controlling the



responsiveness of GPCRs to their ligands. This GPCR desensitization allows the cell to distinguish between different types of ligands, such as chemokines, and define the intensity of the cellular response [44]. Previous studies have shown that GRK specificity for a particular GPCR is determined largely based on the ligand bound to the GPCR [45].

### GPCR desensitization

GPCRs amplify signals in response to ligand binding in a dose-dependent manner. Stimulated receptors become less sensitive due to the amount of time they remain in their active form, a phenomenon known as receptor desensitization [24, 46, 47].

In 1983, Sitaramayya *et al.*, demonstrated that GRK1-mediated rhodopsin phosphorylation correlated with cGMP phosphodiesterase activity, and suggested that GRK1 was involved in GPCR desensitization [48]. Thereafter, the importance of pleckstrin homology/G $\beta\gamma$ -binding domain in the recruitment of GRK5 during GPCR desensitization was shown *in vitro* and *in vivo*. Furthermore, it was found that in the absence of phosphorylation by GRKs, GPCR receptor desensitization was greatly minimized [49]. Overexpression of GRK2 and  $\beta$ -arrestin in COS-7 cells resulted in GRK2-mediated phosphorylation of the  $\beta$ -adrenergic receptor, and subsequent recruitment of  $\beta$ -arrestins [50]. Several *in vivo* studies have identified co-localization of GRK3 and  $\beta$ -arrestin-2 in olfactory neuroepithelium in conjunction with dendritic knobs and cilia, and that GRK2 and  $\beta$ -arrestin-1 are not expressed in these tissues. In response to odorants such as citralva, GRK3-mediated GPCR desensitization was  $\beta$ -arrestin-2-dependent [51]. With regards to GRK4, no receptor substrate had been identified prior to 2000, when Sallese *et al.*, successfully demonstrated that metabotropic glutamate receptor 1 (mGlu1) signaling could be desensitized by GRK4 in an agonist-dependent manner in HEK293 cells [52]. GRK7 was the last GRK family member identified, and was co-expressed with GRK1 in cone photoreceptor cells [53]. In a zebrafish model, GRK7A deficiency affected cone bleaching adaptation and spontaneous decay, which highlighted the role of GRK7 in GPCR desensitization in scotopic vision [54].

### GPCR internalization and degradation

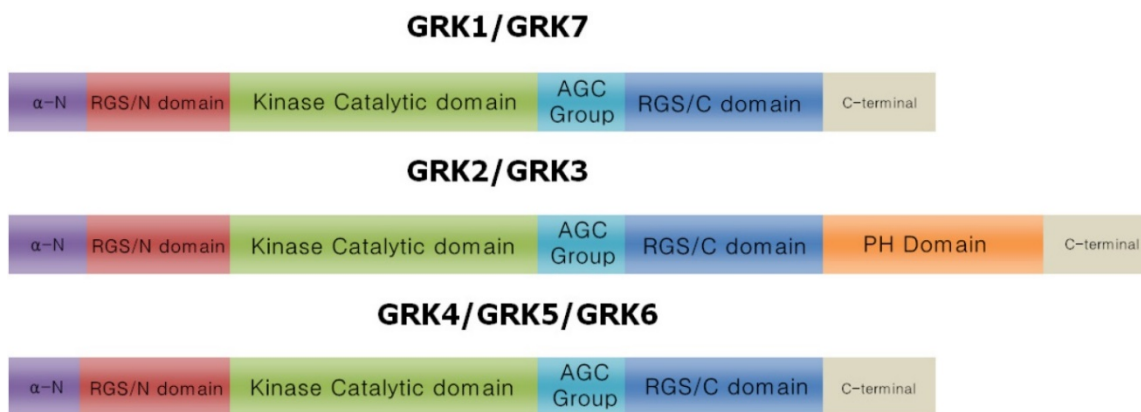
Internalization is an important mechanism used by cells to regulate GPCR activity and to allow recovery post desensitization. Internalization involves the endocytosis of activated receptors from the plasma membrane, a process that requires GRK activity. The presence of GRKs and their interaction with clathrin in endocytosed vesicles highlights the

role they play during receptor internalization [55]. GRKs interact with clathrin through a clathrin-box structure located within the C-terminus normally used to form clathrin-coated pits, and the removal of this domain led to loss of GPCR internalization [56]. For example, silencing of clathrin heavy chain protein inhibited  $\beta$ -adrenoceptor internalization and phosphorylation, suggesting clathrin may also regulate GRK activity [43, 57].

Goodman *et al.* previously demonstrated that  $\beta$ -arrestin subunits 1 and 2 were important in facilitating GPCR internalization [58]. The association of GRKs and arrestins with the C-terminus of various GPCRs led to shuttling of the internalized complex to lysosomes. Interestingly, the binding of  $\beta$ -arrestin to GPCRs was dependent on GRK phosphorylation of the target receptor. In cases of spontaneous  $\beta$ -arrestin-independent internalization, the receptor may also be recycled back to the plasma membrane [59].

In the case of  $\beta$ -adrenoceptor, upon ligand binding, receptor internalization occurred upon recruitment of phosphatidylinositol-4-5-bisphosphate 3-kinase catalytic subunit gamma (PI3K $\gamma$ ) to the cell surface [60]. Structural studies of the PI3K $\gamma$  protein suggested its PIK-domain interacted directly with GRK2 [61]. In addition, GRK2 mediated desensitization of G protein-coupled receptor 54 (GPR54) through inositol-(1,4,5)-triphosphate (IP3) formation in response to increasing concentrations of Kisspeptin-10 [62]. Co-transfection of melanocortin 1 receptor (MC1R) and GRK2 or GRK6 in heterologous cells has been shown to impair agonist-dependent cAMP signaling. This indicated that desensitization of MC1R occurred through GRK2 and GRK6 [63].

One of the key roles of GRKs is to regulate the number of GPCRs at the plasma membrane through receptor internalization, which can prevent hyperactivation of the target receptor. Internalized receptors are then degraded by endocytic trafficking to lysosomes. This process can occur either rapidly or over a longer period of time. The internalization and degradation of the  $\beta$ 2-adrenergic receptor occurs within seconds to minutes upon agonist binding, and can be reversed within minutes after removal of the agonist without new receptor synthesis, a process that is regulated by GRK2. Normally, a rapid reduction of cell surface receptors only temporarily desensitizes the receptor. However, slower regulation of receptor numbers requires receptor endocytosis in combination with synthesis of new receptors, which also involves ubiquitin-dependent protein degradation and GRK3 and GRK6 [64, 65].



**Figure 2. Molecular characteristics of the GRK protein family.** All GRKs (60-80kDa) possess a similar molecular structure with an  $\alpha$ -N-terminal domain followed by an N-terminal Regulator of G protein Signaling domain (RGS/N), which is important for receptor recognition and intracellular membrane anchoring [153, 154]. The protein kinase catalytic domain with a short AGC protein kinase domain is responsible for GRKs catalysis. The C-terminus has more motif variants than the RGS/C domain, which is critical for receptor recognition [14]. Unlike other isoforms, the GRK2 subfamily (GRK2 and GRK3) has a pleckstrin homology domain (PH), which is important for terminating G $\beta\gamma$  complex-related downstream signaling. The C-terminus length varies among all GRK subtypes (~105 to 230 AAs).

### GPCR biased signaling with GRK

The discovery of the concept of "biased signaling of GPCR" has revised the classical understanding of GPCR signaling. It is believed that activated GPCR conformation will promote either G-couple proteins or  $\beta$ -arrestin signaling or both, with a ligand known as "G-protein-biased agonist", " $\beta$ -arrestin-biased agonist", or "balanced agonist". The current molecular mechanism of this phenomenon has not been fully revealed yet. The prevailing hypothesis is that GPCR may have a special activated conformational state in response to each ligand leading to different exclusive downstream modulator(s).  $\beta$ -arrestin does not only serve as the terminator of GPCR signaling, but also acts as a signaling protein, in which the " $\beta$ -arrestin-biased signaling" always requires phosphorylated GPCR. Therefore, GRKs are suggested to be critical for therapeutic strategies of  $\beta$ -arrestin-biased signaling, because GRKs are able to promote high-affinity binding of  $\beta$ -arrestin to GPCRs. Interestingly,  $\beta$ -arrestin may have distinct responses to different GRK subtype phosphorylation of the same GPCR. Kelly *et al.* used  $\beta$ 2-adrenergic receptor ( $\beta$ 2AR) as an example to demonstrate that different GRK subtypes phosphorylate distinct intracellular sites [38]. After over-expression or silencing of GRK2 or GRK6 through transfection in HEK293 cells, and treatment with either unbiased full agonist (isoproterenol) or  $\beta$ -arrestin-biased agonist (carvedilol), the author used specific antibodies against 13 phosphorylation sites on the  $\beta$ 2AR to analyze phosphorylated sites by each GRK subtype. The result revealed six (Thr360, Ser364, Ser396, Ser401, Ser407, and Ser411) and two (Ser355 and Ser356) phosphorylation sites for GRK2 and GRK6, respectively. In addition, GRK2 suppressed

phosphorylation of the two sites phosphorylated by GRK6. Moreover, GRK6, but not GRK2, is essential for  $\beta$ -arrestin-dependent ERK1/2 activation. Additional evidence for  $\beta$ 2AR phosphorylation using PKA assay screening of Ser355 and Ser356 mutants was given by Xiaofang *et al.* [66]. Their data supported the hypothesis that GRK subtypes might have preferential phosphorylation and trigger unique conformational changes in GPCR. The different phosphorylation pattern therefore directly affects  $\beta$ -arrestin-dependent functions. Other receptors such as kappa opioid receptor, mu-opioid receptor, angiotensin II receptor type 1A, C-X-C chemokine receptor type 4, and vasopressin type 2 receptor were also reported to exhibit similar properties and showed a contradictory role between GRK2/GRK3 and GRK5/GRK6 in  $\beta$ -arrestin-mediated signaling [67-72]. However, there are limited studies on the physiological and pharmacological functions of GRK in GPCR-biased signaling. More importantly, because  $\beta$ -arrestin-biased signaling is involved in the cancer-related signaling pathway, the role of GRK in  $\beta$ -arrestin-biased signaling in cancer is worthy of attention.

### Role of GRKs in non-GPCR signaling pathway

GRKs play significant roles in non-GPCR-mediated signal transduction by phosphorylating proteins such as receptor tyrosine kinases (RTK). Previous studies have hypothesized a link between GRKs and RTK signaling that not only reveals the diverse role of GRKs in controlling intracellular signaling, but also highlights the potential benefit of targeting GRKs in cancer. In this section, we will discuss several examples of GRK regulation of non-GPCR signaling pathways.

Early evidence for GRKs as modulators of RTK signaling was based on the observation that ligand activation of epidermal growth factor receptor (EGFR) promoted GRK2 translocation to the plasma membrane to initiate EGFR internalization. EGFR activation of ERK/MAPK via phosphorylation was enhanced in HEK293 cells in response to overexpression of GRK2. These results indicate GRK2 regulated not only EGFR internalization but also its ability to transduce signals [73]. Furthermore, EGF stimulation induced the formation of a GRK2/EGFR complex in a G $\beta$  $\gamma$ -dependent manner [74], and pretreatment with the EGFR tyrosine kinase inhibitor, AG 1478, reversed this effect [74]. Other targets of GRK2 include the retinal photo-receptor type 6 cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE $\gamma$ ), which is found in the internal membranes of retinal photoreceptors, where it reduces cGMP in rod and cone outer segments in response to light [75]. Wan *et al.* demonstrated that GRK2 targeted PDE $\gamma$  in a complex with c-Src whereby threonine 62 of the PDE $\gamma$  was phosphorylated [76]. This is consistent with earlier reports that Src mediates GRK2 phosphorylation of other plasma membrane proteins [77]. In addition, GRKs mediate the cross talk between RTK and GPCR signaling pathways, such as that which exists between the  $\delta$ -opioid receptor (DOR), the  $\mu$ -opioid receptor (MOR), and EGFR [78].

Reduced platelet-derived growth factor receptor (PDGFR) signaling is also found to be associated with GRK2 expression. By modulating the levels of GRK2 expression, Freedman *et al.* showed that phosphorylation of PDGFR could be altered by GRK2 [79]. It was subsequently found that GRK2 enhanced the ubiquitination of PDGFR leading to its desensitization. In smooth muscle cells, overexpression of full-length GRK2 significantly decreased PDGFR activity in a G $\beta$  $\gamma$ -independent manner, but this effect was abrogated in cells expressing a truncated version of GRK2 consisting of only its pleckstrin homology/G $\beta$  $\gamma$ -binding domains [80]. However, in the case of GRK5 that lacks pleckstrin homology domain, PDGFR could still be phosphorylated [81]. Moreover, knocking out GRK2 in HEK293 cells strongly enhanced downstream PDGFR signaling, resulting in increased AKT activation [82]. Therefore, the catalytic activity of PDGFR is dependent on the inhibitory activities of GRK2 and GRK5.

Interestingly, an opposite effect was found with regards to the modulation of insulin-like growth factor 1 receptor (IGF-1R) signaling by GRK2, where reduced GRK2 levels inhibited IGF1-R activation. Silencing of GRK2 also enhanced the expression of

cyclins and IGF1-R in human hepatocellular carcinoma (HepG2) cells [83], and silencing GRK5 and GRK6 also impaired IGF1-R signaling. However, silencing GRK3 had no effect on the status of IGF1-R [84]. In this study, GRK2 and GRK6 were found to counteract each other with regards to IGF-1R activation and ERK phosphorylation. It was then suggested that IGF1-R interacts with both GRK2 and GRK6 to promote  $\beta$ -arrestin recruitment [84].

Previous studies have illustrated an interaction between vascular endothelial cell growth factor receptor (VEGFR) and GRK in the cardiovascular system and during tumor angiogenesis. Here, GRK5 was shown to regulate VEGF signaling and activation of its downstream effectors (AKT, ERK1/2, and GSK-3) in human coronary artery endothelial cells (HCAECs). However, Sandeep *et al.* demonstrated that GRK6 depletion enhanced tumorigenesis and metastasis using a GRK6 knock-out mouse model of Lewis lung cancer [85].

There have also been reports of interactions of GRKs with a wide range of other membrane or intracellular proteins, such as nucleophosmin, cyclin-dependent kinase 2 (CDK2), toll-like receptor 4 (TLR4), HSP90, HSP70 interacting protein (Hip), similar mothers against decapentaplegic homolog (SMAD), Rho, synucleins, phosphoinositide 3-kinase (PI3K), histone deacetylase 5 (HDAC5), activin receptor-like kinase 1/5 (ALK1/5), glucose transporter type 4 (GLUT4), mammalian STE20-like kinase 2 (MST2), never in mitosis gene related kinase 2A (NEK2A), tubulin, ezrin, and many other functional proteins [86-102] (summarized in Figure 3). As an example, GRK2 does not directly phosphorylate sphingosine-1 phosphate receptor (S1PR), but via G-protein-coupled receptor kinase interacting protein-1 (GIT1) to activate the MEK/MAPK/ERK cascade to induce epithelial cell migration [103]. In summary, GRKs play diverse roles in regulating the activity of GPCRs, RTKs, and other receptors to control cell behaviors and maintain homeostasis.

### The role of GRK in GPCR transactivation

In the past decade, GPCR transactivation has further broadened this signaling network, with the two known patterns being from GPCRs to receptor tyrosine kinases (RTK) and receptor serine/threonine kinases (RS/TK). Two mechanisms, ligand-dependent and ligand-independent, are generally accepted for GPCR-RTK transactivation. Membrane-bound matrix metalloproteases (MMPs) play predominant roles in ligand-dependent receptor transactivation. Following GPCR activation, MMP levels increase to enhance the release of membrane-anchored heparin-binding EGF-like growth factor (HB-EGF)[104]. HB-EGF



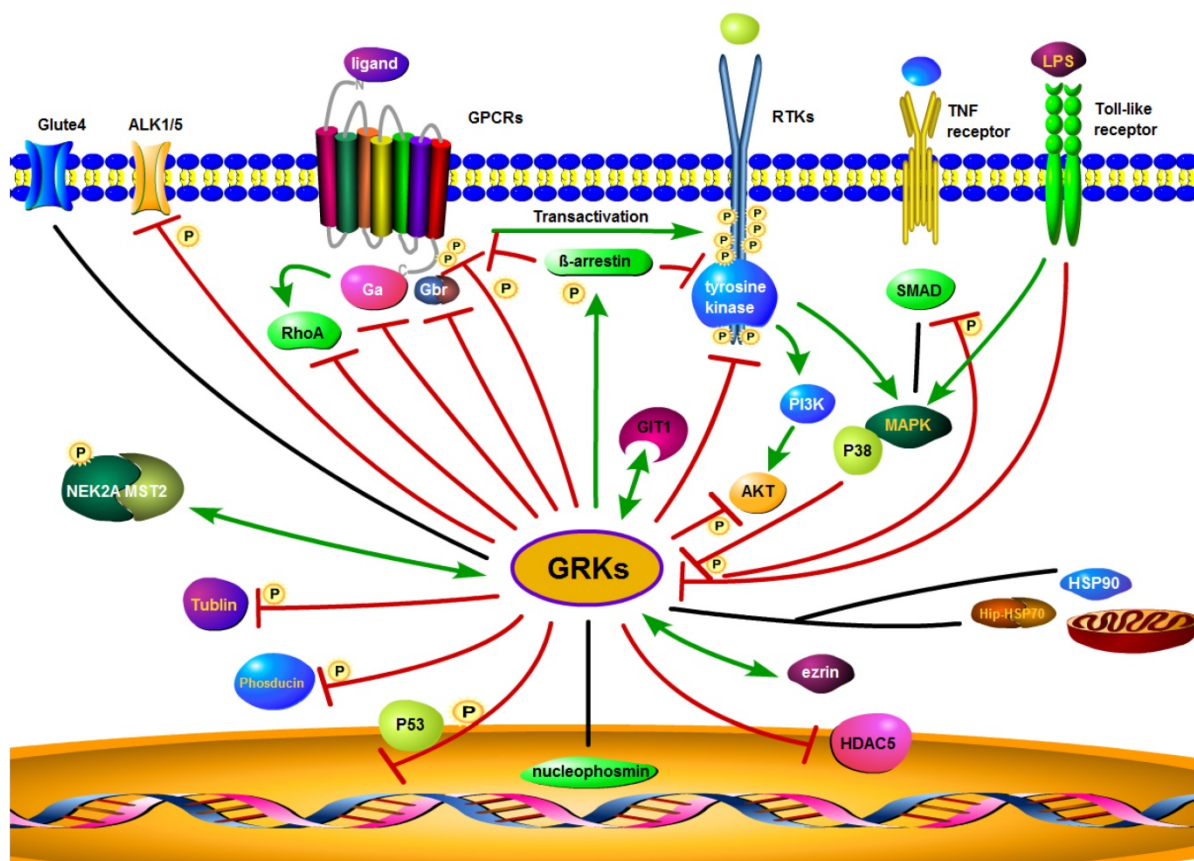
further activates EGFR and hence triggers the metastatic and invasive behaviors of cancers. The ligand-independent pathway is based on GPCR activation of Src family proteins, which phosphorylate the intracellular tyrosine residues of RTK to activate the downstream signaling pathway. Examples of GPCR-RTK transactivation in cancers are well summarized in articles written by Almendro *et al.* [105].

Regarding RS/TK transactivation by GPCR, recent accumulating evidence establishes a GPCR-ROCK-Integrin-TGFBR (ALK5) pathway. Burch *et al.* properly demonstrated that thrombin activated PAR-1 to transactivate TGF-beta receptor by integrin binding to LLC with conformational alternation that led to TGF-ligand initiation of ALK5 downstream pathway in vascular smooth muscle cells [106]. Also this report supported an early research model on integrin-LLC-ALK [107].

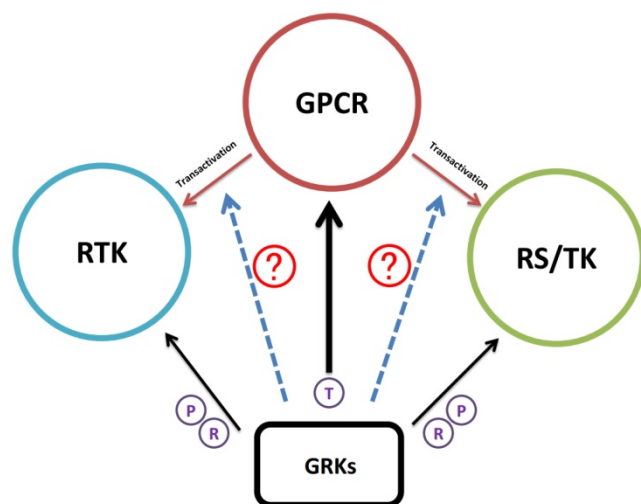
As discussed above, current studies of GRKs involve GPCR-dependent and RTK-dependent mechanisms. As recently evidenced by Kamato *et al.* using RNA-sequencing in vascular smooth muscle cells, over 50% GPCR-activated gene expression was accounted for by RTKs and RS/TKs transactivation [108]. Thus, the role of GRK in GPCR-RTK or GPCR-RS/TK transactivation regulation is becoming more apparent and important in cancer research (Figure 4).

### The role of GRKs in cancer pathology

Because GPCR signaling is an important contributor to tumor growth and metastasis, discerning how GRKs regulate GPCR activity in cancer cells may greatly improve our understanding of tumorigenesis and oncogenesis, and help develop novel anticancer therapies. In the following section, we discuss the role of GRKs in cancer progression (an overview is summarized in Table 1).



**Figure 3. Proposed GRK interaction networks.** GRK plays a diverse role in intracellular signaling. GRKs promote  $\beta$ -arrestin binding to both GPCRs and RTKs. Through their interaction with clathrin coated plasma membrane pits, GRKs mediate receptor internalization, followed by receptor degradation or recycling. GRKs also interact with other intracellular or membrane proteins and modulate interrelated signaling through phosphorylation. Red lines indicate an inhibitory effect of GRKs, while green lines indicate a stimulatory effect. The black lines represent no inhibitory or stimulatory activity.



**Figure 4. Schematic illustration of GRK regulation research progress.** Accumulating evidence suggested that GRKs have the regulatory function in GPCR, RTK, and RS/TK signaling pathways. As GPCR induced transactivation of RTK or RS/TK that further broadened the GPCR network map, the next research area will be certainly turning into the potential functions of GRKs during the transactivation process. Black arrow; studies have confirmed interaction between GRKs and the target substrate. Dotted blue arrow; potential function of GRK in the transactivation of RTK or RS/TK. T in circle; signaling termination. P in circle; receptor phosphorylation. R in circle; regulation of receptor activities after interaction.

### GRK1 and GRK7

Because GRK1 and GRK7 are almost exclusively expressed in retinal tissue (cone and rod cells) and phosphorylate opsin GPCRs during visual acquisition, only a handful of cancers have been implicated in dysfunctional GRK1/7 activity. Several groups have shown that GRK1 and GRK7 may be involved in embryogenesis, but also indirectly interact with proteins such as Rho GDP-dissociation inhibitor (RohGDI) and PDE $\gamma$ , both of which have been found to be aberrantly regulated in cancer [109, 110]. Furthermore, reductions in GRK1 activity due to a mutation or deletion causing nonfunctional protein, have been shown to contribute to the onset of Oguchi disease (stationary night blindness Oguchi type-2) [111]. A direct link between reduced GRK1/7 activity and cancer progression has not yet been confirmed. However, GRK1/7 may play a role in cancer-associated retinopathy through its interaction with recoverin (a calcium binding protein) in patients with lung cancer [112].

### GRK2

The role of GRK2 in cancer has been well-characterized. High levels of GRK2 expression are found in differentiated thyroid carcinoma, whereas GRK5 levels are significantly decreased when compared to normal thyroid tissue. The levels of GRK2 protein are correlated with its activity, as it rapidly desensitizes thyroid-stimulating hormone

receptor (TSHR), whereas GRK5 inhibits desensitization of this receptor. In thyroid carcinoma, activation of TSHR increased cancer cell proliferation [113]. In human hepatocellular carcinoma HepG2 cells, GRK2 negatively regulates Insulin-like growth factor-1 receptor (IGF1-R) signaling. In GRK2 knockdown cells, reduced GRK2 levels enhanced cyclin and IGF1-R expression. These results highlight the role of GRK2 in suppressing cell cycle progression [83]. Another study using hepatocellular carcinoma cells (HCCs) demonstrated that GRK2 inhibits IGF1 signaling, thereby regulating proliferation and migration [12]. It is generally believed that IGF-1 induces the expression of early growth response protein 1 (EGR1) through reactive oxygen species (ROS)-dependent activation of ERK1/2/JNK and PI3-K/PKB [114]. Modulating the levels of GRK2 expression in HCCs demonstrated that GRK2 regulated EGR1 expression after IGF1 treatment. Altogether, these studies reveal the therapeutic potential of GRK2 by inhibiting IGF1-mediated responses [12, 83, 84]. Moreover, in clinical studies, high GRK2 expression correlated with a high tumor (T) stage and poor survival rates of patients with pancreatic cancer. However, these levels could not be considered as an independent prognostic marker [115].

Yao *et al.* used a rat osteosarcoma model to evaluate the association between GRK2 and cancer-related pain levels in dorsal root ganglion neurons. Implanted breast carcinosarcoma cells in the tibia resulted in increased expression of GRK2. Nerve growth factor (NGF), together with GRK2, promoted the phosphorylation of opioid receptors, and an increase in pain. In addition, anti-NGF therapies mediated the effects of GRK2 and arrestins to significantly relieve cancer bone pain [116].

GRK2 is also involved in miRNA mediated pathways. MiR-K3, a Kaposi's sarcoma-associated herpesvirus (KSHV) encoding miRNA, facilitates endothelial cell migration and invasion. Interestingly, overexpression of GRK2 in KSHV infected tumor cells reversed this miR-K3-dependent induction. In addition, miR-K3 directly up-regulated GRK2 expression. Moreover, downregulation of miR-K3 by GRK2 inhibited the migration and invasion of KSHV infected HUVEC cells. These results suggest that GRK2 plays a role in suppressing KSHV-associated tumor progression [117].

GRK2 has also been implicated in breast cancer progression. GRK2 levels are elevated in precursor lesions of mammary glands in mouse mammary tumor virus with Her-2 amplification (MMTV-HER2) mice. The expression of GRK2 is also dependent on estrogen receptor alpha (ER $\alpha$ ) signaling in human



breast cancer cell lines. Moreover, increased GRK2 levels may contribute to cellular transformation by promoting mitogenic and anti-apoptotic activities during tumor development. Data obtained from breast cancer patients showed that GRK2 levels were significantly increased in infiltrating ductal carcinomas. This strongly suggests that a relationship exists between GRK2 activity and breast cancer progression [118]. Interestingly, a recent study demonstrated that up-regulation of GRK2 was associated with increased histone deacetylase 6 (HDAC6) expression. GRK2 increased the growth of both luminal and basal breast cancer cells in an HDAC6- and peptidylprolyl-cis/trans-isomerase (Pin1)-dependent manner, and inhibition of GRK2 increased the sensitivity of these cells to commonly used chemotherapeutic compounds. Therefore, the GRK2-HDAC6-Pin1 axis may be a potential therapeutic target for combination therapy [119]. Finally, GRK2 was found to be a negative regulator of CXCR4 (C-X-C chemokine receptor type 4), a chemokine receptor that mediates metastasis and is

typically used as an indicator of patient prognosis [120].

GRK2 also plays a role in gastric cancer progression, as studies using human gastric carcinoma MKN-45 cells have shown that GRK2 mediated the homologous desensitization of H2 receptors in poorly differentiated cancers [121]. Desensitization of H2 receptors by histamine was inhibited in response to treatment with a GRK2, but not GRK6, antisense phosphorothioateoligo-DNA (PON). This indicates that GRK2 and GRK6 play significantly different roles in modulating H2 receptor pathways in MKN-45 cells.

With regards to the role of GRK2 in skin cancer, low expression of GRK2 in melanoma cells could enhance cAMP production in response to MC1R agonists. MC1R has been shown to regulate pigmentation and differentiation of epidermal melanocytes and contributed to melanoma progression. This effect was reversed by overexpressing GRK2 and GRK6 [63]. Thus, GRK2 and GRK6 may regulate the development of skin cancer by modulating MC1R signaling.

**Table 1.** Summary of previous studies investigating GRKs in cancer.

GRK subtype	Type of cancer	Interacting protein	Function	Ref
GRK2	thyroid carcinoma	TSHR	↓ proliferation through rapid desensitization	[113]
	hepatocellular carcinoma cell	IGF1-R	↓ proliferation and migration	[12]
	human hepatocellular carcinoma (HepG2)	IGF1-R	↓ cell cycle progression	[83]
	pancreatic cancer	N/A	--T-stage and poor survival rate	[115]
	breast carcinosarcoma	NGFR	↓ bone cancer pain	[116]
	Kaposi's sarcoma-associated herpesvirus infected tumor cell	CXCR2	↓ migration and invasion	[117]
	basal breast cancer with Her-2 amplification /infiltrating ductal carcinoma	Her-2/ER-α	↑ promoting mitogenic, anti-apoptotic activities -survival and progression	[155]
	luminal and basal breast cancer	HDAC6/Pin1	↑ sensitiveness of breast cancer cells to traditional chemotherapeutic treatment	[119]
	breast cancer	CXCR4	↓ metastasis	[120]
	human gastric carcinoma cell line (MKN-45)	H2 receptor	--poor differentiation	[121]
GRK2/5/6	human breast cancer	N/A	↑ tumor growth ↓ angiogenesis	[98, 122]
	gastric cancer (SSTW-2)	recoverin	--tumor progression, metastasis	[11]
GRK2/6	melanoma	melanocortin 1 receptor	--determinant for skin cancer	[63]
GRK3	breast cancers (MDA-MB-231,MDA-MB-468)	CXCR4	↓ metastasis ↓ migration	[123]
	prostate cancer(PC3)	N/A	↑ metastasis, tumor progression, angiogenesis	[124]
	retinoblastoma(Y-79)	CRF1 receptor	↑ stress adaptation	[125]
	oral squamous carcinoma	β2-adrenergic receptor	--tumor malignancy and invasion	[126]
GRK4	ovarian malignant granulosa cell tumor	FSHR	--benign and malignant transformation in tumor development	[35]
GRK5	glioblastoma	N/A	--proliferation rate and WHO Grade	[130]
	thyroid carcinoma	TSHR	↓ proliferation through slow desensitization	[113]
	prostate cancer(PC3)		↓ proliferation, cell cycle	[131]
	prostate cancer (PC3, DU145, LNCaP )	Moesin	↓ migration, invasion ↑ cell adhesion	[132]
	prostate cancer	N/A	↑ tumor growth, invasion and metastasis	[133]
GRK6	osteosarcoma (U2OS,Saos-2)	P53	↓ cell apoptosis	[90]
	hepatocellular carcinoma	N/A	--proliferation maker in early diagnosis	[134]
	hypopharyngeal squamous cell carcinoma(FaDu)	Methylation of GRK6	--cancer progression ↓ invasion	[135]
	medulloblastoma	CXCR4/ EGFR/PDGFR-Src	↑ migration	[136]
	lung cancer	CXCR2	↓ cancer development	[85]
GRK1/7		recoverin	--cancer-associated retinopathy	[112]

N/A= not provided by research ↑ =increase or promoting related mechanism ↓ =decrease or diminish related mechanism --=has association with.

A systematic study by Penela *et al.* described the regulatory role of GRK2 in tumor vessel formation, as it modulated the proliferation and migration of endothelial cells, and promoted hypoxia and macrophage infiltration during tumor angiogenesis [122]. Rivas *et al.* further suggested that GRK2 is involved in tumor vessel formation and acts as a regulator of angiogenesis through neovascularization in breast cancer. Using hemizygous GRK2 (GRK2+/-) and endothelium-specific GRK2 knockout mice, it was also shown that reduced GRK2 levels enhanced tumor growth and promoted new blood vessel formation [98].

### GRK3

Fitzhugh *et al.* firstly showed a role for GRK3 in breast cancer progression as knockdown of this gene in MDA-MB-231 and MDA-MB-468 cells inhibited CXCL12-mediated chemotaxis, suggesting that GRK3 regulates CXCR4 activation of CXCL12 [123]. *In vivo*, stable knockdown of GRK3 resulted in a high metastatic rate of xenograft breast cancer. Interestingly, according to the study by Billard *et al.*, GRK3 regulated CXCR4 signaling in triple negative and other molecular subtypes of breast cancer [13]. In these studies, GRK3 protein and mRNA expression levels were sensitive to chemokine-mediated migration. This observation was supported by other studies that used extracted data from The Cancer Genome Atlas (TCGA) database. Here, the authors demonstrated that the ratio between GRK3 and CXCR4 may be a key factor in controlling tumor migration and metastasis. Although these authors did not perform *in vivo* experiments to confirm their findings, they clearly showed that breast cancer metastasis was inhibited by GRK3 through regulation of CXCR4 signaling.

Prostate cancer has also been used as a model to discern the role of GRK3 in metastasis, tumorigenesis, and angiogenesis. Li *et al.* found significantly increased migration of endothelial cells in response to elevated expression of GRK3. One group implanted GRK3 knockdown cells into the prostates of SCID mice, and observed enhanced proliferation and metastasis of primary tumors. Microvessel density was modulated by overexpression of GRK3 in primary tumor cells, indicating GRK3 mediated angiogenesis. In agreement, the expression levels of GRK3 were found to be significantly higher in patients with metastatic disease than those with early stage disease [124].

The first report involving GRK3 in retinoblastoma progression dates to 2001. By measuring corticotropin-releasing factor (CRF)-

stimulated intracellular cAMP production and PKA signaling pathway activation in Y-79 retinoblastoma cell line, Dautzenberg *et al.* found that GRK3 controlled desensitization of the CRF1 receptor. Because the CRF1 receptor is known to increase stress adaptation in cancer cells [125], decreased levels of GRK3 may be beneficial to cancer cell survival.

Finally, recent studies have shown that GRK3 is aberrantly expressed in oral squamous carcinoma cells. The mRNA expression levels of GRK3 in tumor samples from patients with oral squamous cell carcinoma were significantly higher than in normal tissues. This study suggests that high expression of GRK3 is associated with oral squamous cell carcinoma tumorigenesis, possibly through the activation of  $\beta$ 2-adrenergic receptor [126].

### GRK4

GRK4 is expressed primarily in the testis, ovaries, brain, kidney, and myometrium [127]. Due to the low-level expression of GRK4, there are few reports regarding its role in cancer. Several studies have shown that GRK4 modulates arterial angiotensin type 1 (AT1) and dopamine D receptor signaling [128, 129]. In malignant ovarian granulosa cells, the expression of GRK4 $\alpha/\beta$  was also found to be significantly lower than in benign granulosa cells [35]. Silencing of GRK4 $\alpha/\beta$  resulted in decreased kinase activity and impaired follicle-stimulating hormone receptor (FSHR) desensitization. This desensitization may play a crucial role during ovarian granulosa cell transformation, although further investigation is required to fully discern the role of GRK4 in this process.

### GRK5

GRK5 plays various roles during tumorigenesis. The expression of GRK5 is associated with worse prognosis in patients with stage II to IV glioblastoma. Kaur *et al.* showed that the levels of expression of GRK5 were significantly higher in high-grade primary and recurrent glioblastoma multiforme (GBM) than low-grade glioblastomas. The samples obtained from patients with recurrent diseases had higher GRK5 expression than tumor samples taken prior to recurrence. Knockdown of GRK5 was further shown to decrease the rate of proliferation and expression of stem cell markers in glioblastoma cells derived from a patient with GBM [130]. Note, as previously discussed, GRK2 and GRK5 have opposite roles in thyroid cancer, as GRK2 inhibited the activity of the TSH receptor, while GRK5 promoted its activation [113].

In prostate cancer, it appears that increased expression of GRK5 correlates with tumorigenesis

and oncogenesis. Silencing of GRK5 inhibited the proliferation of prostate cancer PC3 cells by decreasing the number of cells in G1 and increasing those in the G2/M phase of the cell cycle. These results suggest that GRK5 regulates cell cycle progression in prostate cancer cells [131]. Other studies found that GRK5 was involved in migration, invasion, and cell adhesion of prostate cancer cells through a possible interaction with moesin, a protein known to regulate cell spreading. Overexpression of phosphomimetic moesin-T66D in PC3, DU145, and LNCaP prostate cancer cells significantly reduced cell migration through phosphorylation of moesin by GRK5. Moreover, expression of a phosphorylation-deficient moesin-T66A protein enhanced its activity [132]. *In vivo* studies have also demonstrated that knockdown of GRK5 in mice with xenografted prostate cancer cells suppressed tumor growth, invasion, and metastasis [132, 133]. Finally, it has been shown that GRK5 could directly phosphorylate P53 tumor suppressor in U2OS and Saos-2 osteosarcoma cells, and promote its degradation, thereby inhibiting tumor cell apoptosis [90].

### GRK6

Studies investigating the function of GRK6 in patients with hepatocellular carcinoma using immunohistochemistry have demonstrated a positive correlation between GRK6 and Ki-67 expression, pathological disease stage, metastasis, and survival rate. These authors hypothesized that GRK6 could be used as a biomarker for the early diagnosis of hepatocellular carcinoma [134]. Furthermore, recoverin, which is functionally associated with GRK6 (as well as GRK2 and GRK5), was aberrantly expressed in SSTW-2 gastric cancer cells [11].

GRK6 mRNA and protein expression was also found to be lower in hypopharyngeal squamous cell carcinomas than normal tissues [135]. Cancer progression appears to be linked to aberrant methylation of *GRK6*, whose expression correlates with cancer cell invasion and disease stage. *In vitro*, GRK6 expression levels are elevated and invasion is inhibited in FaDu cells in response to 5-aza-2'-deoxycytidine [135].

In medulloblastoma cells, PDGFR/Src signaling may mediate GRK6 activity to promote CXCR4 activation of CXCL12 with increased rate of cell migration [136].

GRK6 depletion also enhanced tumorigenesis and metastasis in a Lewis lung cancer mouse model, as MMP-2 and MMP-9 expression was significantly increased in *GRK6*<sup>-/-</sup> animals. Pharmacological inhibition of CXCR2 activation abrogated this effect in an NF- $\kappa$ B-dependent manner [85].

### GRK inhibitors

Increased GRK expression levels are correlated with chronic or acute use of GPCR-targeted drugs. In a study investigating GPCR-targeted drug tolerance in the brain, it was shown that increased GRK expression might be responsible for drug resistance [137, 138]. In addition, various pathological conditions such as heart failure, depression, Alzheimer's disease, and Parkinson's disease are associated with the modulation of endogenous GRK expression [139-143]. Because scientists are beginning to understand more about the biology of GPCR/GRK signaling in cancer, targeting GRKs are emerging as a new anticancer strategy. The most likely methods of developing highly selective GRK inhibitors will be to target their unique kinase domains or decrease GRK expression using selective RNA aptamers [144]. To date, no effective GRK inhibitors have been approved for clinical practice [145]. Because GRKs are a subfamily of AGC kinases, and their kinase domains are relatively similar in structure, nonselective GRK inhibitors may likely cross-react with other AGC kinases [146]. However, one highly selective GRK2 inhibitor known as Takeda Compound 103A that inhibits GRK2 activity 50-fold more than other AGC kinases has been developed by Takeda Pharmaceuticals [147]. Other highly selective drugs such as paroxetine (GRK2), GSK180736A (GRK3), balanol (GRK5), Takeda Compound 101 (GRK2), and sanigivamycin (GRK6) have been widely studied [148]. Although they are reasonably selective for GRKs, they all show cross-reactivity with other kinases. The RNA aptamer, C13, first described by Mayer *et al.*, [149] competes with the alkaloid, staurosporine, for the kinase domain binding on GRK2 without binding to the N- or C-terminus [144, 149]. To our knowledge, the effective inhibitor targeted regions are frequently mutated in the population, limiting the drug's therapeutic potential [150].

### Conclusion and perspectives

In conclusion, GRKs cooperate with arrestins and clathrin to control GPCR signaling. Because GPCRs and RTKs are the primary signal transducers of extracellular stimuli, GRKs act as important regulators to protect cells from overstimulation. Consequently, GRK activity could directly modulate the ability of endocrine hormones to influence the function of cells and tissues. In addition, GRKs help maintain homeostasis by inhibiting signal transduction and facilitating cellular communication in response to extracellular stress.

Recent studies have shown that GRK expression



levels are implicated in almost every facet of cancer biology, from proliferation, invasion, and migration to metastasis and oncogenic transformation [151]. Significant levels of GRK expression alternation in different cancers indicate that GRKs could be promising molecular targets to be considered to modulate GPCR responsiveness. Due to the heterogeneity of GRK structures, each one appears to play a different role in cancer progression. Investigation of the interesting contradictory roles of each subtype of GRKs may enable development of potent drugs that inhibit some isoforms but activate others for better disease control. GRKs regulate hundreds of unique GPCRs simultaneously, making it complex to fully discern how they modulate cellular signaling. As more GPCR-targeted anticancer therapies are developed, it is important to identify these mechanisms. Our current hypothesis suggests that GRKs primarily act as feedback loops that alter GPCR activity, especially for arrestin-biased signaling. Furthermore, targeting GRKs may result in unintended consequences with regard to the effects of GPCR-targeting drugs, and may induce drug resistance. Because GPCR signaling is becoming a more attractive topic in cancer pharmacology research [152], it is necessary for us to gather a full understanding of these GPCR-GRK interactions to inform more practical and efficient drug design and development. Collectively, pharmacological intervention of GRKs provides us with a novel concept in cancer therapy.

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## Competing Interests

The authors have declared that no competing interest exists.

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