Chapter 4

Biological Consequences of Priming Phosphorylation in Cancer Development

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Abstract

Multisite phosphorylations on a single polypeptide mediated by protein kinase(s) are commonly observed. In some cases, hierarchical phosphorylations occur when first priming event triggers second processive phosphorylation. Hierarchal multisite phosphorylation that is mediated by a priming kinase and a processive kinase is a fail-safe system that accurately regulates physiological processes, including cell cycle progression, survival, migration, metabolism, differentiation and stem cell renewal. Here, we summarize the findings of cancer-associated priming kinases (CK1 and DYRK family) and processive kinase (GSK3). GSK3 has an unusual ability to accurately regulate the wide variety of cellular processes via the priming phosphorylation of its substrates. Therefore, dysregulation of priming phosphorylation gives rise to pathological disorders such as cancer.

Keywords: priming phosphorylation, multisite phosphorylation, hierarchical phosphorylation, priming kinase, CK1, DYRK1A, DYRK2, processive kinase, GSK3, NFAT signaling, Wnt signaling, β-catenin, SCF, β-TRCP, FBW7, LRP signalosome, protein stability, cancer

1. Introduction

Protein phosphorylation is the most frequent post-translational modification that regulates the function, interaction and stability of various proteins. Multisite phosphorylations on a single polypeptide, which are mediated by protein kinase(s), are commonly observed. In some cases, hierarchical phosphorylations occur when first phosphorylation event triggers second subsequent phosphorylation. Here, such a first phosphorylation is called as "priming phosphorylation." Priming phosphorylation is mediated by "priming kinase" and serial phosphorylation is mediated by "processive kinase" (**Figure 1**).

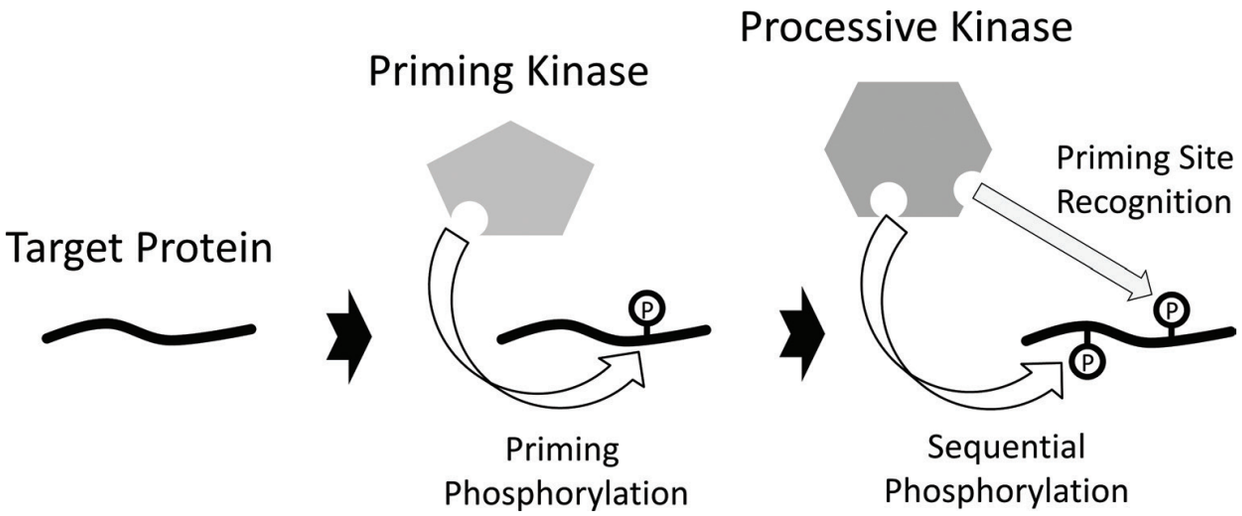

Figure 1. Schematic illustration of multisite phosphorylation mediated by a priming kinase and a processive kinase.

Processive kinases, such as glycogen synthase kinase 3 (GSK3), are ubiquitously expressed in mammalian tissues and involved in numerous cellular processes. In this context, priming kinases provide a basis for selective action of the individual cellular process regulated by processive kinases.

Hierarchical multisite phosphorylations by the priming kinase and the processive kinase are the fail-safe mechanism that accurately regulates physiological processes, including cell cycle progression, survival, migration, metabolism, differentiation and stem cell renewal. Therefore, loss of priming phosphorylation caused by impairment of priming kinases gives rise to pathological disorders, such as cancer.

2. Priming kinases

2.1. Casein kinase 1 (CK1)

The casein kinase 1 (CK1) family is evolutionary conserved serine/threonine protein kinases that are ubiquitously expressed in eukaryotic organisms from yeast to human [1]. In human, six CK1 isoforms (α, γ1, γ2, γ3, δ and ε) are encoded by distinct genes. These isoforms differ in length and sequence of N-terminal and C-terminal domain [2, 3].

The name casein kinase arose from the protein kinase activity using casein as an in vitro substrate [4]. Because casein is a highly phosphorylated protein, the casein kinase was initially characterized by a phosphate-directed protein kinase [5, 6]. However, it became evident that CK1 does not only phosphorylate phospho-primed substrates but also displays a prominent phosphorylation activity targeting the site that contains cluster of acidic amino acids, immediately N-terminal (−1 to −5) of the phospho-acceptor site [7–9]. The canonical consensus sequence for CK1 is shown in **Figure 2**.

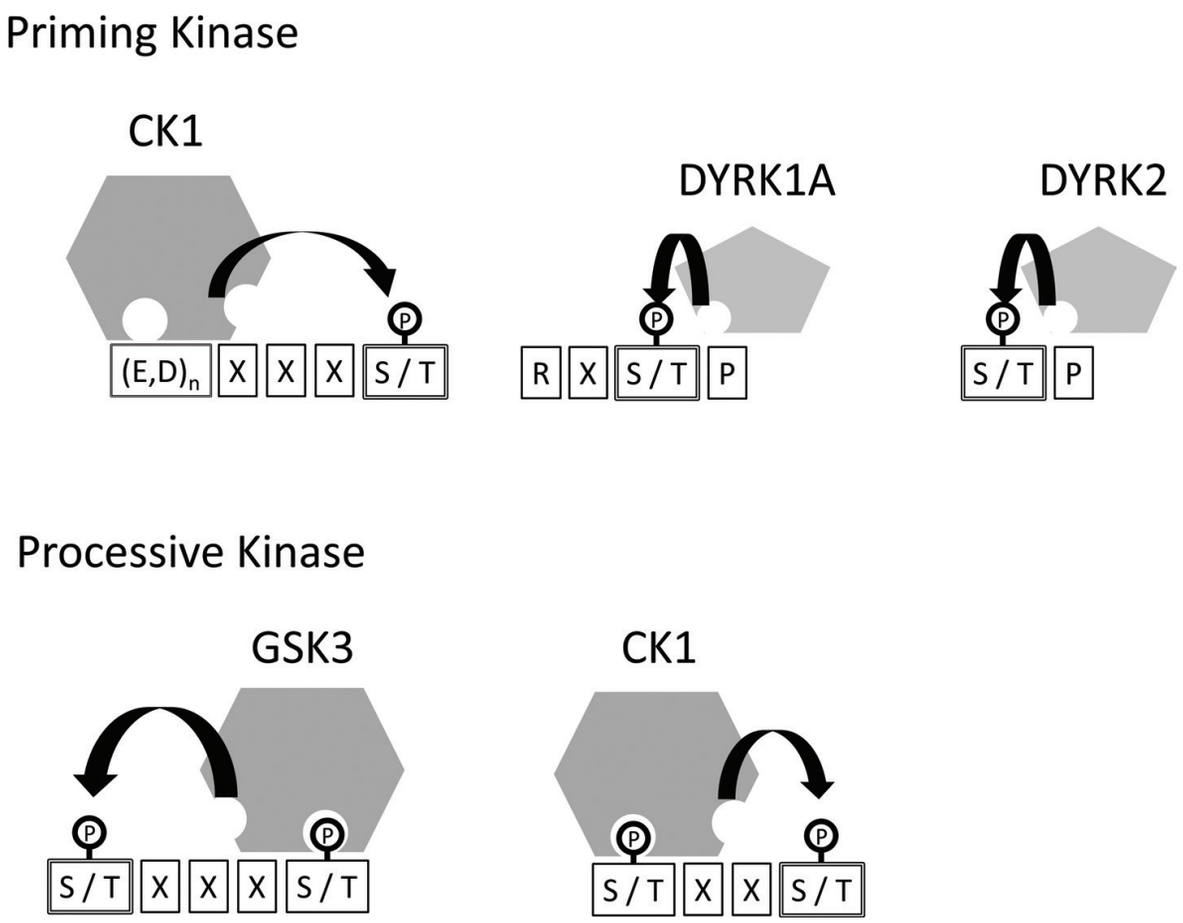

Figure 2. Consensus sequence of priming kinases and processive kinases. $(E,D)_n$ denotes acidic amino acids cluster; X denotes any amino acid and S/T denotes Ser or Thr. CK1 recognizes the negatively charged amino acid cluster. DYRK1A is a proline- and arginine-directed kinase. DYRK2 is a proline-directed kinase. GSK3 typically phosphorylates "primed" substrate that is pre-phosphorylated by a priming kinase. CK1 also behaves as a phosphate-directed processive kinase.

Members of the CK1 family are ubiquitously expressed but their expression levels differ in tissue and cell type [10–12]. Recently, an increasing number of substrate proteins have been identified, which are phosphorylated by CK1 family *in vitro* and *in vivo* [2, 13, 14]. According to a global weblogo analysis to a database of 35,000 non-redundant phosphosites, CK1 targets are responsible for the generation of 9.5% of the human phosphoproteome [15].

The wide range of substrates suggests that the members of CK1 family regulate diverse and important cellular functions. For instance, they are involved in Wnt signaling, Hedgehog signaling, Hippo signaling, neurodegenerative disease, circadian rhythms, vesicular trafficking, cytoskeleton dynamics, nuclear localization, DNA processing and repair, apoptosis, cell division, proliferation and differentiation [2, 13, 14, 16]. Consequently, deregulation or dysfunction of CK1 in these pathways responsible for growth, proliferation, and apoptosis may result in pathological condition, such as tumorigenesis [3, 17, 18]. CK1δ and CK1ε isoforms are overexpressed and activated in many tumor types, such as colon and pancreatic cancers [19, 20]. By contrast, the downregulation of CK1α leads to increased proliferation and invasive growth of melanoma cells [21, 22].

2.2. Dual specificity tyrosine phosphorylation-regulated kinases (DYRKs)

Dual specificity tyrosine phosphorylation-regulated kinase (DYRK) family is an evolutionally conserved eukaryotic protein kinases belong to CMGC protein kinase group [23, 24]. In human, five DYRK members (DYRK1A, DYRK1B, DYRK2, DYRK3 and DYRK4) are encoded by distinct genes [25].

Their kinase activity depends upon the autophosphorylation of a tyrosine residue in activation loop of catalytic domain [26, 27]. The autophosphorylation of tyrosine is an intramolecular event that is mediated by a short-lived translational intermediate of itself [28–30]. Once phosphorylated on the tyrosine residue, DYRKs lose tyrosine kinase ability and retain only serine/threonine kinase ability. Although DYRKs are potentially proline-directed kinase, they differ in their target recognition sequence and its preference for an arginine residue is a feature of DYRK1A but not of DYRK2 and DYRK4 [31–34]. The canonical consensus sequence for DYRK1A and DYRK2 is shown in **Figure 2**.

With some exceptions [35–37], DYRK1A and DYRK2 have been characterized as a potential tumor suppressor [38–49]. In contrast, DYRK1B (also known MIRK), closely related to DYRK1A, have been characterized as a positive regulator of cancer cell survival [50–61]. It is still not known to the details of the biological functions for DYRK3 and DYRK4.

Human DYRK1A is the most well-characterized member in the DYRK family [62, 63] because the gene is localized in the down syndrome (DS) critical region in chromosome 21 [64–67]. In mouse, DYRK1A is essential for embryonic development, especially in the nervous systems, and unbalanced gene dosage causes developmental delay and abnormal brain morphology [68–75]. In neuronal progenitor cells, overexpression of DYRK1A bring to the attenuation of cell proliferation that promotes the switch to a quiescent state or differentiation. DYRK1A mediates direct phosphorylation of p53 at Ser-15 that leads to the induction of p53 target genes, such as $p21^{CIP1}$, and impaired G1/G0-S phase transition [76].

It is known that individuals with DS have a significantly reduced incidence of solid tumors [77–79]. DS model mouse exhibits that considerable growth protection against transplantation of allogeneic tumors is caused by a deficit in tumor angiogenesis arising from suppression of nuclear factor of activated T cells (NFAT) transcriptional regulator pathway [38]. DYRK1A phosphorylates NFAT proteins in nucleus, thereby priming the subsequent phosphorylation by additional kinases (GSK3 and CK1), then fully phosphorylated NFAT proteins are exported from the nucleus to the cytoplasm (**Figure 3**). Cytoplasmic accumulation of NFAT proteins represses the NFAT pathway associated with tumor progression [80]. Paradoxically, children with DS have a remarkably increased risk of developing leukemias, including most types of acute megakaryoblastic leukemia (AMKL) and acute lymphoblastic leukemia (ALL). It has been suggested that DYRK1A is also a potent AMKL-promoting gene that modulates megakaryoblastic expansion through the inhibition of the NFAT pathway [35].

Although precise regulation of NFAT pathway is essential for vertebrate development and function, NFAT isoforms are overexpressed and activated in human solid tumors and leukemias.

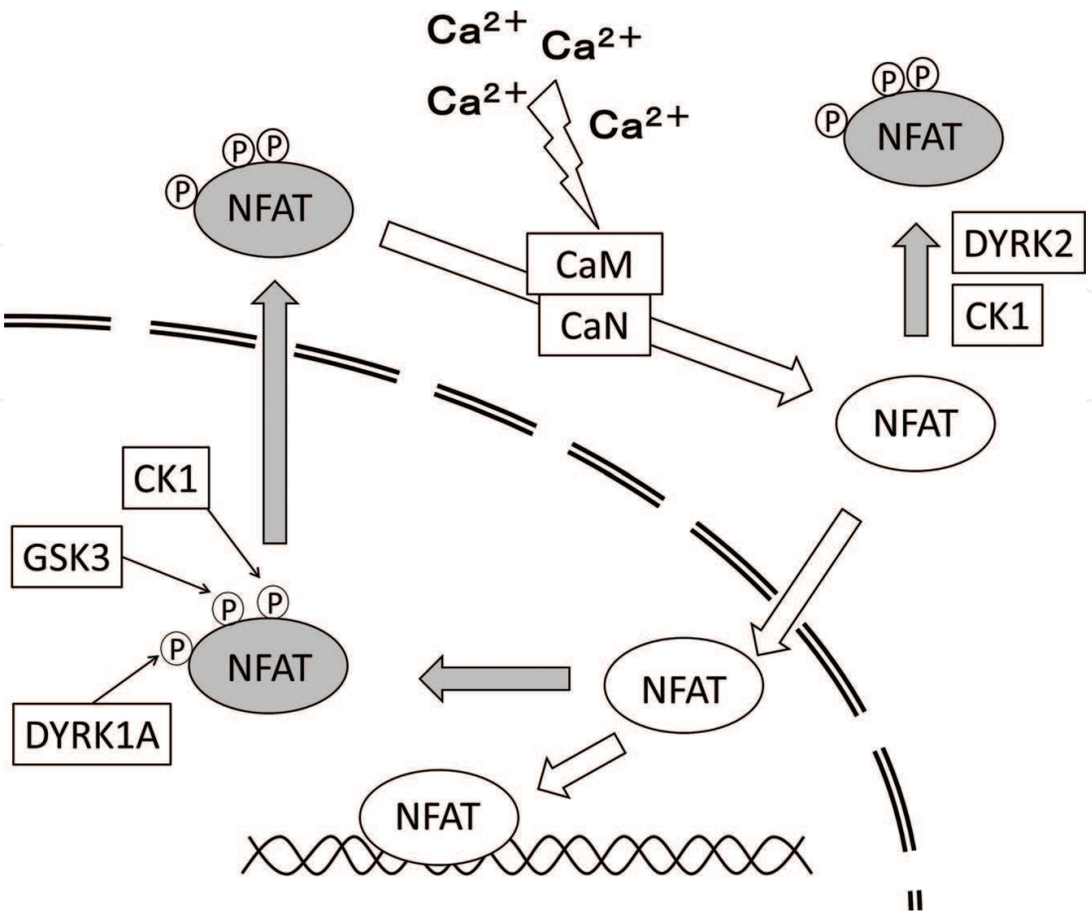

Figure 3. Schematic diagram of NFAT translocation. DYRK1A acts as an export kinase in the nucleus. DYRK2 acts as a maintain kinase in the cytoplasm. These kinases mediate the priming phosphorylation of NFAT proteins. Fully phosphorylated NFAT proteins are sequestered in cytoplasm. Increased intracellular Ca^{2+} levels activate the calmodulin (CaM)/calcineurin (CN) phosphatase complex. Dephosphorylated NFAT proteins relocate into nucleus and promote gene transcription.

This aberrant expression of NFAT proteins leads to the induction of the target genes that promote malignant phenotype that is associated with tumor progression, such as proliferation, survival, migration and invasion [80–82]. In the basal state, NFAT proteins are sequestered and inactivated as a phosphorylated form in the cytoplasm. DYRK1A acts as an export kinase that phosphorylates NFAT proteins inside the nucleus and induces its relocation to the cytoplasm. As a counterpart of export kinase, DYRK2 acts as a maintenance kinase that phosphorylates in the cytoplasm, where they keep NFAT proteins in a phosphorylated state, and prevents their translocation to the nucleus (**Figure 3**). DYRK1A and DYRK2 can directly phosphorylate the conserved SP3 motif of the NFAT regulatory domain and thereby can prime for the subsequent phosphorylation by GSK3 and/or CK1 [83].

Under normal conditions, DYRK2 is predominantly expressed in the cytoplasm and constitutively degraded by MDM2 ubiquitin ligase in the nucleus. Upon exposure to genotoxic stress, ATM protein kinase phosphorylates DYRK2 at Thr-33 and Ser-369. As a result, DYRK2 enable to escape from MDM2 and to induce the kinase activity toward p53 at Ser-46 in the

nucleus. Phosphorylation of Ser-46 following severe DNA damage increases the transcriptional activity of pro-apoptotic genes [84, 85]. The other functional role of DYRK2 in DNA damage response may be link to DNA double-strand break repair pathway [86].

DYRK2 was found to be mutated in breast and central nervous system tumors, in nonsense and missense mutation, respectively [87, 88]. Loss of function of DYRK2 in cancer cells accelerated cell proliferation due to stabilization of oncogenic transcription factors, c-Jun and c-Myc [89]. This stabilization is caused by the loss of priming phosphorylation that is necessary to generate a phosphodegron that leads to subsequent SCF (Skp1-Cullin1-F-box protein) ubiquitin ligase-mediated degradation. Snail, a zinc finger protein to promote epithelial-mesenchymal transitions (EMT), is stabilized by the DYRK2 knockdown, probably in the same fashion as c-Jun/c-Myc, and allows cancer cells to represent loss of epithelial features and gain of invasiveness [90, 91] (**Figure 4**). Moreover, it is recently demonstrated that impairment of DYRK2 augments cancer stem-like traits of breast cancer cells [92].

2.3. Other priming kinases

It is also known that cAMP-dependent kinase (PKA), AMP-activated protein kinase (AMPK), cyclin-dependent kinase 5 (CDK5), DNA-dependent protein kinase (DNA-PK), calcium and calmodulin-dependent protein kinase II (CAMKII) and mitogen-activated protein kinases

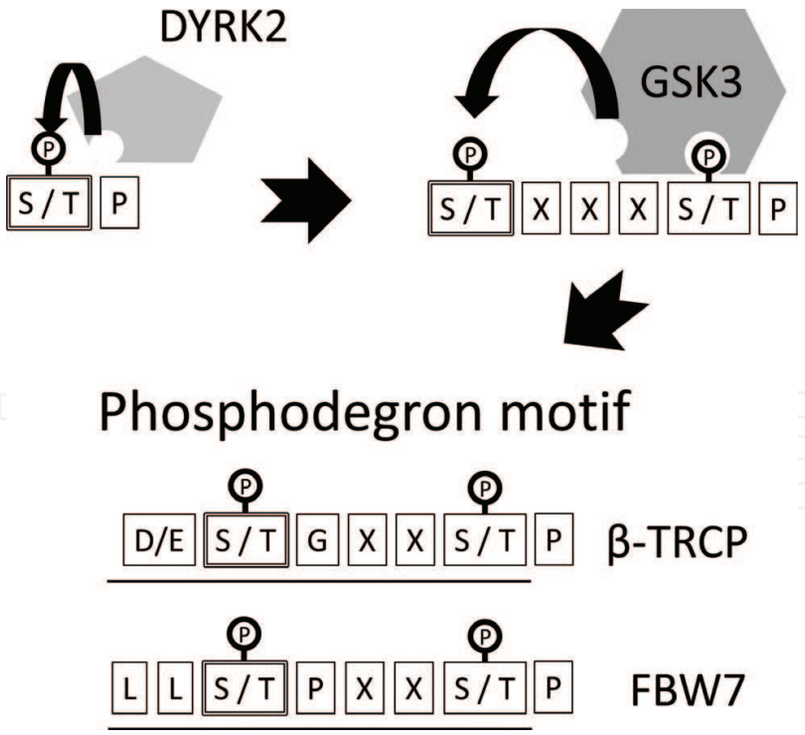

Figure 4. Consensus sequence of phosphodegron motif. These phosphodegron motifs are created by several priming kinases such as DYRK2 and processive kinase GSK3. Underlined sequence indicates the canonical sequence of β-TRCP and FBW7. Loss of priming phosphorylation leads to dysgenesis of phosphodegron and results in stabilization of their target proteins.

(MAPKs) can act as priming kinases for GSK3 [93]. CDK1 functions as a priming kinase for polo-like kinase 1 (PLK1) that is a key regulator of cell cycle progression [94, 95].

3. Processive kinases

Phosphate-directed protein kinases, such as CK1, CK2 [96, 97] and GSK3, function as the processive kinases. CK1 not only act as a priming kinase but also proposed as a processive kinase (**Figure 2**). Here, we focus on GSK3.

GSK3 was originally identified as a protein kinase that negatively regulates glycogen synthesis by phosphorylating and inactivating glycogen synthase [98]. In mammals, two GSK3 isoforms (GSK3α and GSK3β) are encoded by distinct genes [99]. These two GSK3 isoforms, which are expressed ubiquitously in tissues, have many overlapping functions, but they do not always compensate for each other.

Substrate recognition by GSK3 is an unusual preference for target proteins that are priorly phosphorylated at an approximately 4 residues C-terminal to GSK3 target site. The canonical consensus sequence for GSK3 is shown in **Figure 2**. Although priming phosphorylation is not stringently required for the recognition of GSK3, the efficiency of substrate phosphorylation is greatly increased by priming phosphorylation [100]. This substrate recognition mechanism means that GSK3 reduces crosstalk among different signaling pathways. In other words, GSK3 must be colocalized with the priming kinase that is involved in the specific signaling pathway. For example, in NFAT pathway, processive kinases GSK3 and CK1 are distributed throughout the entire cell. However, priming kinases DYRK1A and DYRK2 are localized to specific location, which is nuclear and cytoplasm. GSK3 thus has an unusual ability to accurately regulate the wide variety of cellular processes. We now know that the enzyme is a key regulator of various cellular processes, such as Wnt signaling pathway, hedgehog signaling pathway, NFAT pathway, mTOR pathway, EMT, cell cycle and proliferation regulation. Large number of proteins involved in a wide spectrum of cellular processes have been proposed as putative substrates of GSK3 [93]. It is noteworthy that the consensus sequence of GSK3 is included in the "phosphodegron motif" that is recognized by SCF ubiquitin-ligase complex (**Figure 4**). Therefore, most GSK3-targets receive the proteasomal degradation that relies on a phosphodegron created by dual phosphorylation and mediated by priming kinase and GSK3 [101, 102].

Unlike most protein kinases, GSK3 is constitutively active in resting cells, and its activity can be inhibited by a variety of extracellular signals that typically induce cell survival and growth, such as insulin, growth factors and nutrients. Numerous stimuli lead to activate the GSK3 inactivating kinase pathways, such as PI3K-Akt and mTOR pathway. These kinases lead to inactivation of GSK3 through the phosphorylation of N-terminal serine residue. Phosphorylated N-terminal segment creates a primed pseudosubstrate that intramolecularly binds to substrate-binding pocket (**Figure 5**). Inactivation mechanism of GSK3 by the Wnt signaling pathway is mentioned below.

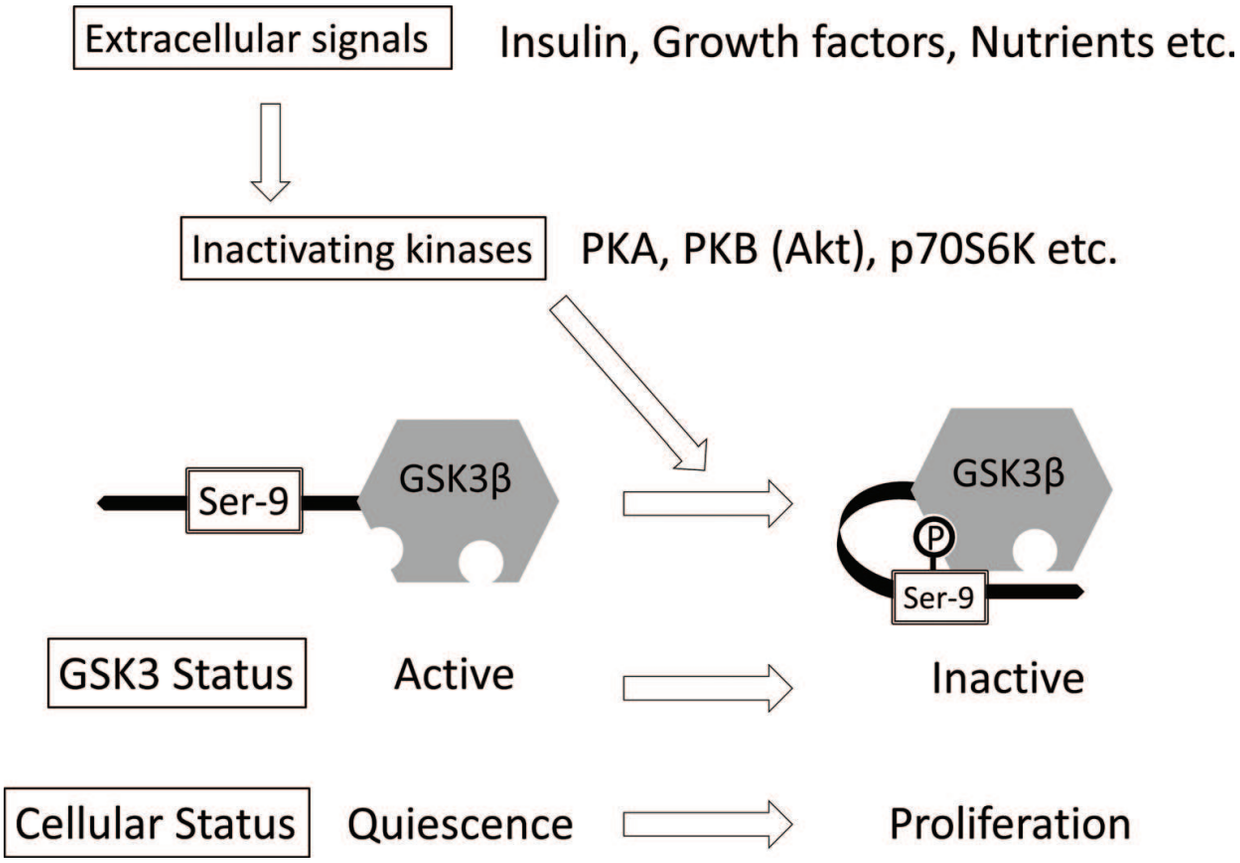

Figure 5. Regulation of GSK3 activity and cellular status. GSK3 is constitutively active in quiescent cells. Stimulation of cells with insulin, growth factors or nutrients causes inactivation of GSK3 through several kinases belonging to each signaling cascade. These GSK3 inactivating kinases phosphorylate the N-terminal serine residue of GSK3 and create a primed pseudosubstrate that binds to catalytic site and inhibits the kinase activity.

4. Priming phosphorylation regulates cellular processes

4.1. Wnt signaling pathway

Wnt signaling pathway plays crucial roles in proliferation and differentiation of stem and progenitor cells during embryogenesis and adult tissue homeostasis [103–105]. Aberration of this signaling is implicated in a variety of human cancers [106–109].

The β-catenin-dependent Wnt pathway is commonly referred to as the canonical pathway, which is characterized by the stabilization and the nuclear translocation of transcriptional co-activator β-catenin.

In the absence of Wnt ligand, β-catenin is sequestered in the cytoplasm and constantly degraded by the action of a "destruction complex," which is composed of adenomatous polyposis coli (Apc), Axin, CK1α and GSK3 [110–114]. The degradation of β-catenin is based on two steps regulated by priming phosphorylation. (1) CK1α mediates the priming phosphorylation of Apc that leads to sequential phosphorylation by GSK3, and this phosphorylation enhances the binding affinity to β-catenin [115–118]. (2) CK1α leads to phosphorylation of

β-catenin on Ser-45, which creates a priming site for GSK3 [119]. Then, GSK3 phosphorylates Thr-41, Ser-37, and Ser-33 in a sequential manner [120, 121]. Priming-dependent phosphorylation by GSK3 generates the consensus motif of β-transducin repeat-containing protein (β-TRCP) recognition site at the N-terminal domain of β-catenin. After being released from the destruction complex, phosphorylated β-catenin is recognized by $SCF^{\beta\text{-TRCP}}$ E3 ubiquitin ligase and degraded by the ubiquitin-proteasome pathway [122–125] (**Figure 6**).

From a conditional knockout mouse model, the deficient of CK1α in gut epithelium shows a lot of the features of human colorectal tumors in addition to β-catenin stabilization and strong Wnt signal activation [126]. Additionally, a genome-wide, reporter-based, screening in human haploid cells reveal that CK1α is a critical negative regulator of canonical Wnt signaling pathway [127].

In the presence of Wnt ligand, β-catenin is stabilized by escaping from phosphorylation-mediated degradation and is translocated into the nucleus. After that, it binds to the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factor and activates Wnt target gene expression. At this time, the function of destruction complex is suppressed by the priming phosphorylation-dependent manner (**Figure 6**).

Signaling of Wnt family requires the cell-surface receptors, frizzled (Fzd) that is related to the GPCR superfamily and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) that is a single-span transmembrane receptor [128–131]. Wnt-Fzd-LRP5/6 triple complex recruits a Fzd-associated scaffold protein, Dishevelled (Dvl) and triggers the membrane-associated clustering into ribosome-sized LRP signalosomes [132]. In turn, Dvl promotes phosphorylation of the cytoplasmic tail of LRP5/6 mediated by membrane-bound CK1γ and the phosphorylated LRP5/6 is followed by the recruitment of Axin away from the degradation complex [133–136]. The phosphorylation sites of LRP5/6 contain five PPPSPxS motifs. Membrane-associated GSK3 phosphorylates the first Ser (or Thr) within these motifs and serves a priming site for the CK1-mediated phosphorylation [137, 138]. In this case, membrane-associated GSK3 acts as a priming kinase and CK1 acts as a processive kinase. The phosphorylated PPPSPxS repeats provide an optical-binding site for Axin and recruit cytoplasmic Axin-GSK3 complex to LRP signalosome [135, 137, 139]. Importantly, phosphorylated LRP5/6 cytoplasmic tail that creates a primed pseudosubstrate can directly inhibit GSK3 activity [138, 140–142], suggesting that LRP signalosome formation may be an aggressive mechanism for sequestration of GSK3 activity from cytosol. Endocytosed signalosomes that colocalize with the late endosomal markers Rab7 and Vps4 mature into multivesicular bodies [143]. As a result of the LPR signalosomes formation, β-catenin protects from phosphorylation and escapes from ubiquitylation and proteasome degradation, which enables it to accumulate in the cytosol and nucleus. As it turns out, stabilized β-catenin binds TCF/LEF to initiate the cellular transcriptional program that is usually directs to proliferation, survival and inhibition of differentiation (**Figure 7**).

4.2. Priming phosphorylation regulates protein stability

The ubiquitin-proteasome system controls degradation of the majority of regulatory proteins, including transcription factors and protein kinases, that play key roles in tumorigenesis.

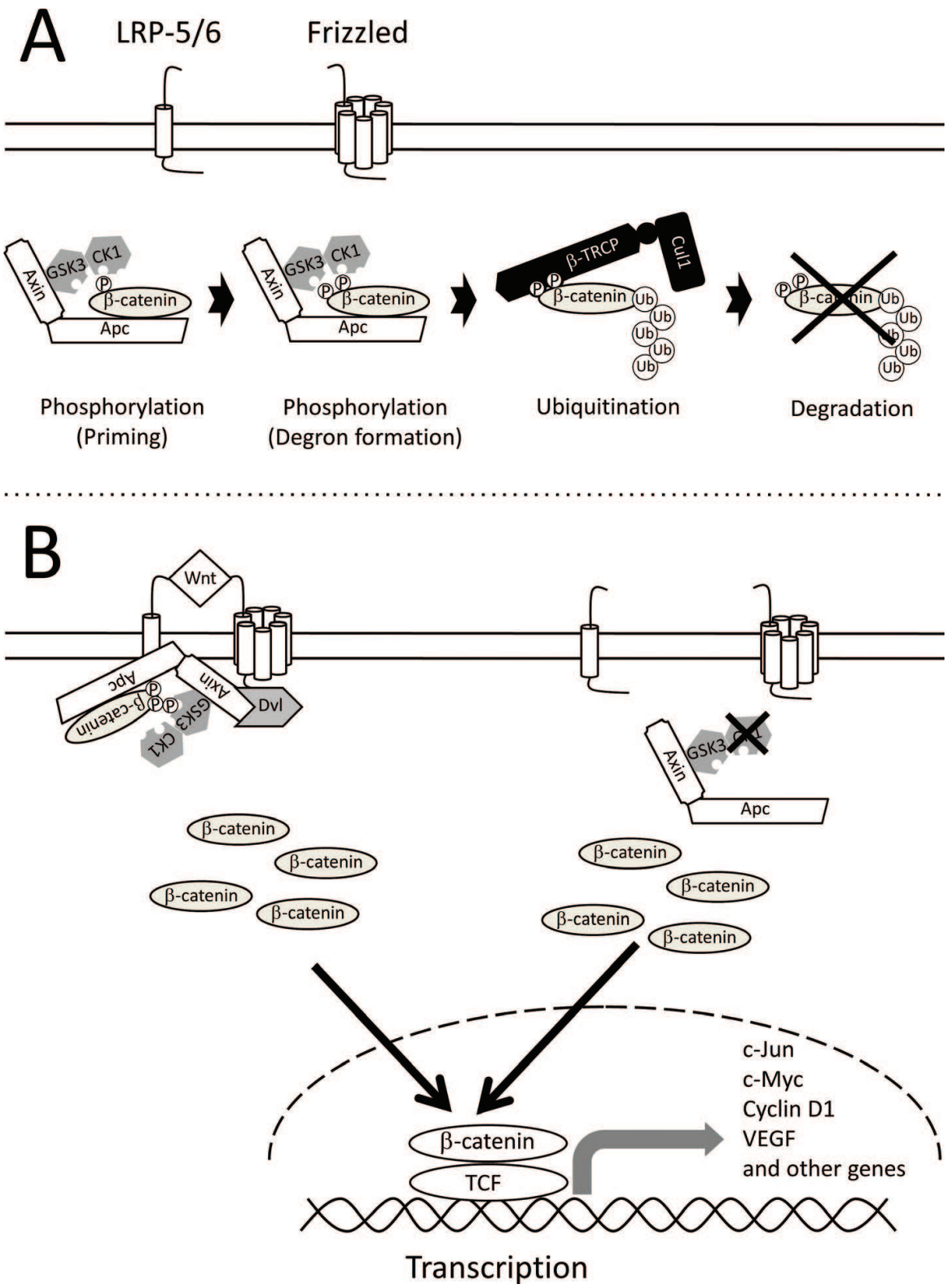

Figure 6. Canonical Wnt signaling pathway. (A) Schematic representation of constitutive degradation of β-catenin mediated by the destruction complex and $SCF^{\beta\text{-TRCP}}$ complex in resting cell. (B) Upon Wnt signaling, β-catenin is stabilized by sequestration and inactivation of destruction complex at cell membrane. It is known that the deficient of CK1α in gut epithelium shows a lot of the features of human colorectal tumors in addition to β-catenin stabilization and strong Wnt signal activation.

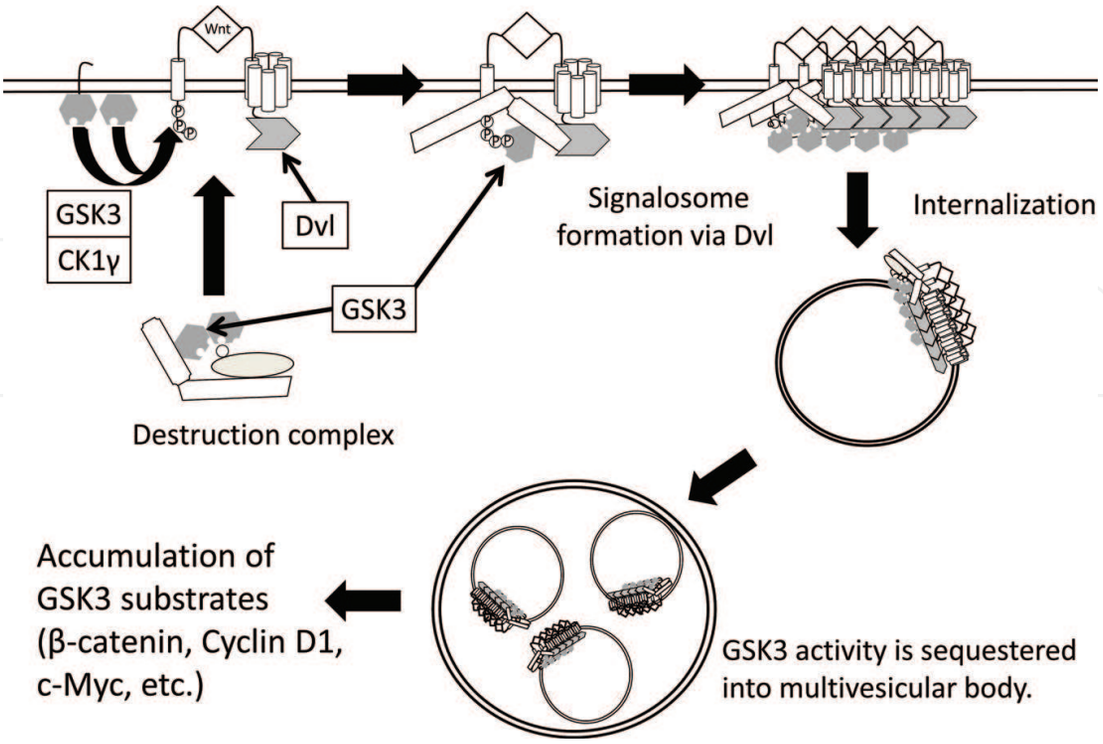

Figure 7. Wnt signaling stabilizes the GSK3 target proteins. Wnt signaling promotes the signalosome formation via priming phosphorylation of C-terminal tail of LRP5/6 and oligomerization of Dvl. The signalosome sequesters a fraction of cytosolic GSK3 into multivesicular bodies. Consequently, accumulation of GSK3 substrates including oncogenic proteins is caused by a loss of phosphodegron formation.

E3 ubiquitin ligases determine the substrate specificity for given substrates. SCF E3 ubiquitin ligase is important for the recognition of specifically phosphorylated substrates. F-box protein, which is a subunit of SCF, mediates the recognition and binding of the phosphorylated substrate. In most cases, phosphorylated substrates have a short motif that is a recognition signal for F-box proteins, namely phosphodegron. Since the consensus sequence of GSK3 is corresponding to special phosphodegron motifs that are recognized by two subfamily of F-box protein FBW7 and β-TRCP, a lot of GSK3 targets phosphorylated by priming kinases receive the proteasomal degradation [93, 102]. It is known that FBW7 and β-TRCP are involved in cell cycle regulation and tumorigenesis by targeting proteins in these processes. Thus, priming phosphorylation to create the phosphodegron processed by GSK3 is presumed to be significant consequence for cancer development.

FBW7 is generally considered as a tumor suppressor because of its loss of function phenotype found in multiple type of human cancer. FBW7 recognizes a lot of oncogenic substrates, including cyclin E, c-Myc, c-Jun, Mcl-I, mTOR and Notch-1 [144]. Among these substrates, c-Myc, c-Jun and potentially mTOR are phosphorylated by DYRK2, which creates a priming site for GSK3 [89, 145]. Therefore, the loss of priming phosphorylation may denote the direction of cancer progression in the GSK3-FBW7 axis. On the other hand, β-TRCP contributes to the degradation of β-catenin and snail, which is implicated in human cancer progression. Of note, both are phosphorylated by priming kinases [146]. In contrast to FBW7, β-TRCP is regarded as an oncogene on account of the fact that higher expression of β-TRCP is validated in various type of human

cancer. Furthermore, overexpression of β-TRCP exerts its tumorigenic activity in mouse model [147] and mutations in β-TRCP are uncommon in human cancers [144]. However, due to the fact that β-TRCP substrates include both oncogene products such as β-catenin and tumor suppressors such as IκB, an inhibitor of NF-κB, it is difficult to characterize the function of β-TRCP as an oncogene or a tumor suppressor. In this context, the contribution of β-TRCP to tumor progression may become altered in the tissue specific- or cellular context-dependent manner.

Bioinformatic analysis reveals that about 20% of the human proteome contains three or more putative GSK3 phosphorylation sites. As mentioned above, LRP6 signalosome induced by the canonical Wnt signal sequesters GSK3 into multivesicular bodies and the sequestration results in the cytosolic GSK3 activity level decreased to below 40%. Accordingly, the half-life of numerous cellular proteins including GSK3 substrates is extended [143]. In proliferating cells, Wnt signaling peaks in the G2/M phase of cell cycle, and in this phase, G1 activators such as cyclin D1 and c-Myc are accumulated to progress the cell cycle [148, 149]. Moreover, depletion of GSK3 activity with a chemical inhibitor treatments or siRNA knockdown experiments stabilizes cellular proteins as similar to Wnt treatment [143]. This means that GSK3-dependent protein catabolism is more universal, beyond the cell cycle and Wnt signaling. Therefore, it is predicted that the dysregulation of priming phosphorylation influences the cellular protein homeostasis through the processive phosphorylation by GSK3. Priming kinase-GSK3-SCF axis emerges as a principal regulator of cancer development.

5. Conclusion

Hierarchical multisite phosphorylation by a priming kinase and a processive kinase is the fail-safe mechanism that accurately regulates the physiological processes, including cell cycle progression, survival, migration, metabolism, differentiation and stem cell renewal. Loss of priming phosphorylation caused by impairment of priming kinases, such as CK1 family and DYRK family, gives rise to pathological disorders as a result of the abnormal localization and/or half-life of cellular proteins. These priming kinases create the recognition site for further phosphorylation by the processive kinase, GSK3. The consensus sequence of GSK3 is corresponding to phosphodegron motif that is recognized by SCF ubiquitin-ligase complex. Therefore, a lot of GSK3 targets including oncogenes or tumor suppressors receive the proteasomal degradation that depends upon a phosphodegron. GSK3-dependent protein dissimilation is more universal, beyond the cell cycle and Wnt signaling. Consequently, priming kinase-GSK3-SCF axis manifests as a key regulator for cancer development.

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