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Invited Mini Review

Phosphorylation-dependent regulation of Notch1 signaling: the fulcrum of Notch1 signaling

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Notch signaling plays a pivotal role in cell fate determination, cellular development, cellular self-renewal, tumor progression, and has been linked to developmental disorders and carcinogenesis. Notch1 is activated through interactions with the ligands of neighboring cells, and acts as a transcriptional activator in the nucleus. The Notch1 intracellular domain (Notch1-IC) regulates the expression of target genes related to tumor development and progression. The Notch1 protein undergoes modification after translation by posttranslational modification enzymes. Phosphorylation modification is critical for enzymatic activation, complex formation, degradation, and subcellular localization. According to the nuclear cycle, Notch1-IC is degraded by E3 ligase, FBW7 in the nucleus via phosphorylation-dependent degradation. Here, we summarize the Notch signaling pathway, and resolve to understand the role of phosphorylation in the regulation of Notch signaling as well as to understand its relation to cancer. [BMB Reports 2015; 48(8): 431-437]

INTRODUCTION

Notch signaling is a highly conserved process and plays an important role in the regulation of cellular growth, cell cycle arrest, and cellular development (1, 2). In mammals, there are four Notch receptors (Notch1-4) and the abnormal regulation of Notch1 signaling can promote cancer and other disorders (3, 4). Notch1 regulates the expression of target genes such as Hes1, Hes5, and Hey1, for the acceleration of cell growth (5). Notch1 is a single transmembrane receptor, which perceives signal transduction mediated by ligands (6). After Notch1 is activated by docking ligands, sequential cleavage occurs to produce Notch1 intracellular domain (Notch1-IC). Notch1-IC enters the nucleus and promotes the displacement of repressive complex which promotes the activation.

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Proteins are regulated by post translational modifications (PTMs), which alter the stability and activities of the proteins. PTMs are not template-based since they differ between single and multiple combinations to promote the functions of proteins in a time- and signal-dependent manner. Proteins are subjected to various PTMs, including phosphorylation, acetylation, methylation, hydroxylation and ubiquitination. Phosphorylation is the addition of a phosphate group, taken from ATP, to serine, threonine, or tyrosine, in order to control enzymatic activity and protein-protein interactions. Acetylation is the transfer of an acetyl group from acetyl coenzyme A to a lysine residue, which regulates the protein stability and function. Methylation is the transfer of a methyl group to the lysine or arginine residue of a protein, previously shown to regulate the epigenetic control of gene silencing and activation; however, protein methylation has also been shown to regulate non-histone protein expression to control gene regulation and protein stability. Hydroxylation is the modification of a hydroxyl group to a proline residue under hypoxic conditions in order to adapt to the microenvironment. Ubiquitination is the addition of a ubiquitin conjugate to a lysine residue to regulate cellular location and protein stability. PTMs are reversibly and competitively regulated to control the signaling cascades responsible for the maintenance of cellular homeostasis.

Particularly, protein phosphorylation controls the activation of signaling cascades in response to various stimuli, regulating cell growth and survival. The phosphorylation of Notch1-IC interrupts the formation of the Notch1-IC-induced transactivation complex and promotes ubiquitin-dependent modification targeted by the phosphorylation signal (7, 8). Various kinases are known to phosphorylate Notch1, and the expression of these kinases is different in cancers. In addition, Notch1 is mutated to mimic the phosphorylation-deficient form in order to avoid degradation in cancer. Here, we provide an overview of the Notch signaling pathway, and resolve to understand the role of phosphorylation in the regulation of Notch1 signaling.

NOTCH: RECEPTORS AND LIGANDS

Notch is a single transmembrane receptor involved in signal transduction as a transcription regulator (9). Notch was first discovered over 100 years ago by Morgan and colleagues, who found that the mutation of Notch genes resulted in a de-

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fect of wings in the fruit fly, *Drosophila melanogaster*. These flies have two ligands, Delta and Serrate, which induce the Notch signal of neighboring cells. *Caenorhabditis-elegans* has two receptors (Lin-12 and Glp-1) and four ligands (APX-1, LAG-2, ARG-1 and DSL-1). In mammals, there are four Notch receptors (Notch1-4) and five ligand genes (Delta-like 1, 3, 4 and Jagged1, 2). Notch ligands have a Delta/Serrate/Lag-2 (DSL) domain which allows the Notch receptor to identify the ligand and to receive signaling (Fig. 1) (4). Notch consists of various domains that precisely regulate the function. The Notch receptor has 36-epidermal growth factor (EGF)-like repeats essential for the binding of ligands. There are three juxtamembrane repeats subjected to proteolysis in the processing of Notch. Also, Notch has ankyrin repeats, a transactivation domain (TAD) involved in the transactivation of Notch, and a proline, glutamic acid, serine, and threonine (PEST)-degradation domain critical for the short half-life of Notch1 (10).

THE CANONICAL NOTCH1 SIGNALING PATHWAY

Notch1 is initially produced as a 300 kDa monomer, which exists as a heterodimer at the cell surface prior to signaling. Before Notch1 is transported from the ER to the Golgi apparatus, proper glycosylation modifies the EGF repeats that have a consensus motif for glycosylation (11). Glycosylation-deficient Notch1 is non-functional and cannot be transported to the Golgi apparatus. During the maturation of Notch1 in the trans-Golgi, cleavage occurs by a furin-like convertase (S1 cleavage) for the transportation to the cell membrane (12). This cleavage separates Notch1 into two fragments, making a heterodimer. They move to the membrane and form the transmembrane protein complex. Then, cell-to-cell communication and proteolytic processing occurs when the extracellular domain of Notch1 is docked onto the DSL domain of a ligand. Secondary hydrolysis is promoted by ligand binding, which cleaves the extracellular domain of the Notch1 receptor (13, 14). The extracellular domain is subjected to secondary hydrolysis by ADAM (A Desintegrin And Metalloproteinase) protease and gamma secretase then cleaves the inner fragment, resulting in the release of the signaling fragment, Notch1 intracellular domain (Notch1-IC) (15). Then, Notch1-IC trans-locates into the nucleus and interacts with CSL (CBF/recombining binding protein suppressor of Hairless [RBP-Jκ] in mammals, SuH [Suppressor of Hairless] in *Drosophila melanogaster* and Lag-1 in *C. elegans*) and MAML (mastermind-like) and functions as a transcriptional activator to regulate its target gene (16, 17).

NUCLEAR CYCLE WITH THE NOTCH1 INTRACELLULAR DOMAIN

Without Notch1 activation, RBP-Jκ suppresses the transcription of target genes by forming a complex with co-repressors (18, 19). When Notch1 is activated by ligands, nuclear-translocated Notch1-IC interacts with RBP-Jκ through the RBP-J associated molecule (RAM) domain and dissociates the corepressor complex. In addition, Notch1-IC recruits the general co-activator, p300/CBP/Mastermind (MAML-1), and another histone-modifying enzyme (17, 20, 21). This complex leads to the transcriptional activation of target genes, including members of the Hairy/enhancer-of-split (HES) family, the Hairy/enhancer-of-split related with YRPW motif (Hey) family, nuclear

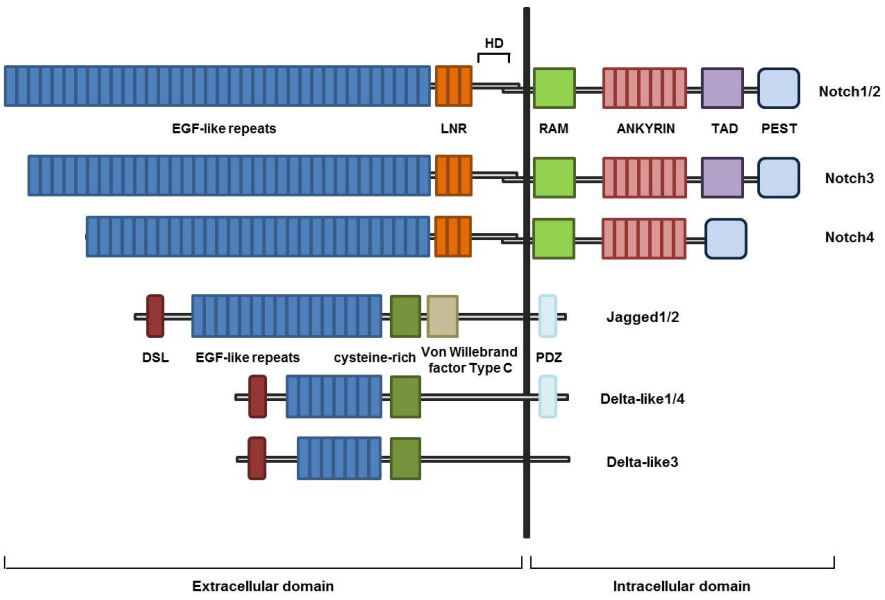


Fig. 1. The molecular structure of the Notch receptor and ligand in mammals. The Notch receptor consists of Notch1-4, and is a heterodimer complex spanning the plasma membrane. Notch has a functional domain, which regulates cellular process. Members of the Delta-like (1, 3 and 4) and Jagged (1, 2) families serve as ligands for signal transduction using the DSL residue. The ligands have a cysteine domain and an EGF-like repeat to specifically bind to the Notch receptor and the Von Willebrand factor type C domain for ligand dimerization.

factor-kappa B (NF-κB), the vascular endothelial growth factor receptor (VEGF), cyclin D1, c-Myc, p21, p27, Akt, etc. (22). In mammals especially, the best-described Notch1 target genes are the transcription factors Hes1, Hes5, and Hey1, the roles of all of which have been well demonstrated in tumor development and progression (23). However, Notch1-induced transactivation is terminated by the phosphorylation of Notch1-IC. The phosphorylation begins with mastermind and the ski-interacting protein (SKIP), which recruits kinases to the TAD or PEST domain (24). Then, the FBW7/ SEL-10 E3 ligases identify the phosphorylation of the PEST domain to promote ubiquitin-mediated degradation. After Notch1-IC is degraded by the Notch1-targeted turn over, the repressors form a complex and inhibit the transcriptional activity of Notch1 target genes (Fig. 2) (25).

PHOSPHORYLATION-DEPENDENT REGULATION OF THE NOTCH1 INTRACELLULAR DOMAIN

To balance the threshold of Notch1 activity, it is effective and economic to down-regulate protein stability. Various reports have suggested that Notch1 is regulated by posttranslational modification such as phosphorylation and ubiquitination, during the multiple steps of signal transduction (6). The ubiquitination of Notch ligands is necessary to activate the Notch1 signaling pathway. Mind bomb ubiquitinates Delta and induces endocytosis and signal transduction in the signal-receiving cell (26). A Delta mutant that does not have the residue for ubiquitination will fail to induce signal transduction of the Notch1 receptor, while Neuralized (Neur) promotes the endocytosis and degradation of Delta (27). FBW7 recruits the components of an SCF ubiquitin ligase complex through the F-box protein, and recognizes a phosphor-epitope, CPD (Cdc4 phosphodegron; a short linear motif activated by the addition of one or more phosphate groups). Within these substrates via the WD40 domain, ubiquitin-mediated degradation is induced by the proteasome (28, 29). Three isoforms, FBW7 α, β, and γ, are distributed in the nucleoplasm, cytoplasm, and nucleolus, respectively, after alternative splicing (30). In particular, phosphorylation of the PEST domain is a substrate for recognition by FBW7 in the Notch1 pathway. Mastermind promotes the recruitment of the Cyclin C-CDK8 complex and the hyperphosphorylation of the PEST domain (31). Recently, Cyclin C has been shown to enhance the activity of CDK3 and CDK19,

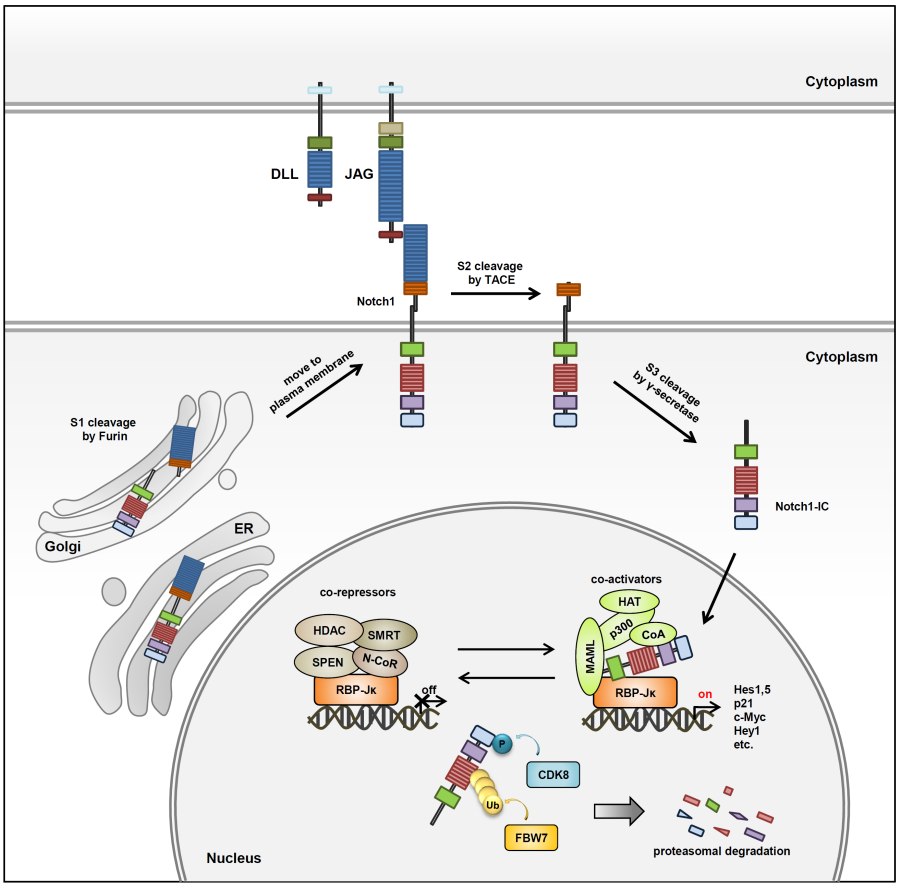


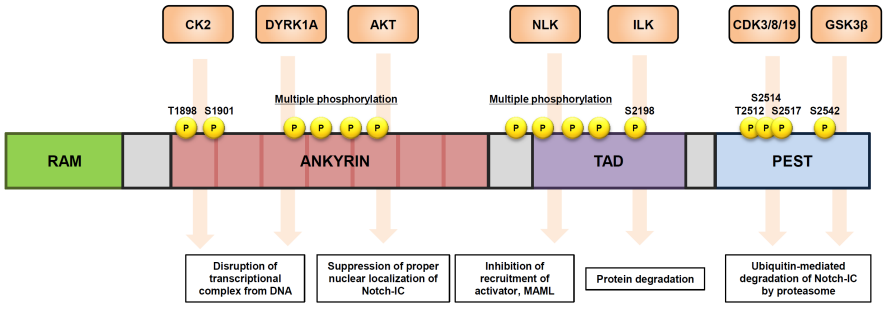
Fig. 2. The processing of the Notch signaling pathway and the nuclear cycle. Notch1-IC is produced by sequential cleavage via ligand binding. Notch1-IC enters into nucleus and promotes the displacement of a repressive complex and the transactivation of Notch target genes. Notch1-IC activation is regulated by phosphorylation and ubiquitination with CDK8 and FBW7, respectively. HDAC: histone deacetylase, SPEN: split-ends, N-CoR: nuclear receptor corepressor, SMRT: silencing mediator for retinoid and thyroid hormone receptors, MAML: Mastermind, HAT: histone acetyltransferase, CoA: co-activator.

and to phosphorylate the PEST domain of the Notch1-IC of Thr2512, Ser2514 and Ser2517 (32). The GSK3β-mediated phosphorylation of Notch1-IC protects from proteolysis by Itch (33-35). An integrin-linked kinase (ILK) is a component of integrin signaling and phosphorylates Ser2173 of mouse Notch1 (Ser2198 in human Notch1) and down-regulates the protein stability of Notch1-IC, thus decreasing transcriptional activity (36). In addition, the phosphorylation of other domains influences transcriptional regulation. The phosphorylation of the ankyrin domain of Notch1 determines the transcriptional activity of the Notch1 target gene (37). The DYRK1A gene can suppress Notch1-induced transactivation, in a kinase-dependent manner, without affecting protein stability (38). Akt promotes hyperphosphorylation and disrupts the translocation of Notch1-IC, resulting in the inhibition of the transcriptional regulation (39). Nemo-like kinase (NLK) phosphorylates the membrane-tethered Notch1 protein, as well as the Notch intracellular domain. NLK-mediated phosphorylation does not interfere with the nuclear localization of Notch1-IC, but decreases the interaction of Mastermind and the association of the Notch active transcription complex (Fig. 3) (40).

EFFECT OF NOTCH SIGNALING ON CANCER

Several tumors exploit the potential of Notch1 as an oncogene and tumor suppressor. According to COSMIC data, hematopoietic and lymphoid cancers show a high mutation rate of Notch1 at 72%, and solid tumors in the upper aerodigestive tract, large intestine, lung, skin, stomach, and breast also show aberrant expression of Notch1. Previous studies reported the Notch1 protein stabilized in T-cell acute lymphoblastic leukemia (T-ALL) and solid tumors such as breast cancer, murine mammary cancer and lung cancer (41, 42). Patients of T-ALL have shown epigenetic mutations, including the translocation of the 3′-region of Notch1 in T-cell receptor beta locus. The Notch1 intracellular domain (Notch1-IC) is over-expressed, resulting in the activation of target genes. The aberrant expression of Notch1-IC induces the accumulation and cell-cycle arrest of bone marrow progenitor cells, resulting in the tumorigenesis of lymphoid cancer (41). The abnormal expression of Notch1 was also shown in breast cancer with high levels of Notch1-IC being expressed in 20 breast cancer tissues and the negative regulator of Notch1 signaling, Numb, being down-regulated in these tissues (43). According to research in Chinese breast cancer patients using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry, the aberrant expression of Notch1 was shown, especially in those with stage 2 lobular carcinoma (44). In another study, the levels of Notch1-IC and p21 in 109 cases of gastric cancer, a major disease in developing countries, was examined using immunohistochemistry (45). The expression of Notch1-IC increased in the more advanced stage cancers, while the expression of p21 was down-regulated in these cases. This negative correlation consequently promotes the invasion and phenotypic characteristics of the tumor.

In addition to a mutation on the hetero-dimerization domain, the main Notch1 active mutation is a frame shift to codon 2515 in the PEST domain, producing insensitivity to FBW7 (46). The numerous cancer-associated mutations within the CPDs of FBW7 substrates disrupt sensitivity to FBW7 degradation (47). Peptidyl-prolyl cis-trans isomerase 1 (Pin1) regulates the isomerization and inactivation of FBW7 and stabilizes



Observed Phospho-peptide	Residue	Kinase	Site implicated in	Reference
RKK**S**QDG	S2198	ILK	Protein degradation Ubiquitination	Mo et al., 2007
LTP**S**PES	S2514 S2517	CDK8	Protein degradation	Fryer et al., 2004
SPP**T**SMQ	T2542	GSK3β	Protein degradation	Foltz et al., 2002
PFL**T**PSP	T2512	unknown	Protein degradation Ubiquitination Molecular association Phospho-Degron motif	O'Neil et al., 2007
DQW**SSSS**PHS	S2522-5	unknown	unknown	Chiang et al., 2006

Fig. 3. The domain architecture of mammalian Notch1 receptors and the regulation of Notch-IC by kinases. Notch1 is a highly conserved transmembrane protein including an ectodomain (EGF-like repeats and LNR domain), a heterodimerization domain (HD), and an intracellular domain (RAM domain, ankyrin repeats, TAD, and PEST domain). Notch1-IC is regulated by the phosphorylation of several kinases, mainly on the ankyrin repeats, to regulate the formation of the transcriptional complex. Furthermore, Notch1-IC phosphorylation of the PEST domain controls the protein stability of Notch1-IC (59, 60).

Notch1-IC, by inhibiting the FBW7-mediated degradation of Notch1-IC (48). Notch1 signaling is engaged in epithelial-mesenchymal transition by regulating the transcription regulators such as Snail, Slug, TGF-β, FGF, and PDGF and supports the phenotypic and functional changes during tumor progression (49, 50). Notch1 activation promotes the migration and angiogenesis of cancer and increases the self-renewal activity of cancer stem cells (51). Notch1 induces the expression of Sox2, NANOG and Oct4 to support the activity of tumor-initiating cells, cancer stem cells, which are highly related to tumor migration and recurrence (52).

CROSS-TALK BETWEEN THE NOTCH1 SIGNALING PATHWAY AND OTHER SIGNALING PATHWAYS

Notch1 signaling regulates various factors that are the main regulators of other signaling pathways, including the PI-3K/Akt, NF-κB, mTOR, and TGF-β signaling pathways, and interacts with oncogenic proteins (22). A phosphatase, PTEN, is a negative regulator of Akt signaling by dephosphorylation of the active phosphor residue, and is typically mutated in cancer (53). HES1, one of the Notch1 target genes, binds to the promoter region of PTEN and down-regulates the expression, resulting in the stabilization of Akt activity. Furthermore, Akt promotes the protection of cancer cells from apoptosis via the NF-κB signaling pathway, and angiogenesis via the mTOR signaling pathway (54). NF-κB, which plays a role as a transcription factor of the immune system and cell proliferation, activates the expression of Notch1 target genes. Notch1-IC activates NF-κB signaling through direct interaction, and through the RBP-Jκ-mediated transcriptional regulation of p100 and p52, which are subunits of NF-κB (55). In mTOR signaling, mTOR is phosphorylated by the PI-3K/Akt pathway and vice versa to regulate cell growth, differentiation, cell survival and autophagy. Notch1-IC inhibits the expression of the tumor suppressor, p53, which is a key regulator of cell apoptosis, by decreasing functionally active phosphor-residues (Ser15, 20, and 392) via the mTOR and PI-3K/Akt pathways (56). Notch1 signaling is related to growth factors, such as the platelet-derived growth factor (PDGF), HER/ErbB interactions with epithelial growth factors and transforming growth factor-α, vascular endothelial growth factor (VEGF), and transforming growth factor (TGF)-β (22). TGF-β induces Hey1, a Notch target gene, and Jagged1, a Notch ligand, to promote epithelial-mesenchymal transition. Notch1-IC forms a transcriptional complex with Smad3, a component of canonical TGF-β signaling and regulates the expression of Hes1 by binding to the promoter (57). These signal cross-talks between oncogenic signaling pathways are very sophisticated regulation processes during cell fate determination and cancer development (22).

CONCLUDING REMARKS

We briefly provided an overview of the Notch1 signaling pathway, and elucidated the role of phosphorylation in the regulation of cancer progression and development. Turnover of Notch1-IC expression was shown to be a key determinant in the regulation of this activity. Several kinases were shown to promote the phosphorylation of Notch1 in the ankyrin domain and PEST domains, which affects the transactivation and protein stability of Notch1. In particular, the phosphodegron, recognized by the E3 ubiquitin ligase FBW7 for degradation, exists in the PEST domain. Additionally, cancer cells were shown to avoid the degradation of Notch1-IC by promoting C-terminal truncation. Cyclin C and its partners CDK8, CDK3, and CDK19 have been suggested to phosphorylate Notch1-IC on the phosphodegron along with its nearby residues. The Cyclin C-CDK complex promotes the degradation of Notch1-IC and is regarded as a tumor suppressor. However, Cyclin and Cyclin dependent kinases (CDKs) are controlled temporally, according to the cell cycle mechanism, and Notch1 functions in cell fate determination and differentiation in steady state (58). Thus, further studies focused on the discovery of other kinases targeting the phosphodegron of Notch1-IC may have great potential in suggesting a target protein for cancer therapy. While cancer therapies such as surgery, irradiation therapy, and chemotherapy are primarily used, inducible kinases may be effective in managing cancer growth and survival by down-regulating Notch signaling. Therefore, further studies focused on discovering a new regulator of Notch signaling should be carried out to improve the treatment and control of cancer in humans.

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