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POST-TRANSLATIONAL MODIFICATIONS OF NR5A1

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Abstract- Steroidogenic factor 1 (SF1/NR5A1/AD4BP), plays essential roles in the development and functions in endocrine tissues, including adrenal gland, gonads, and pituitary. Post-translational modifications are known to influence for controlling functions of proteins and subsequently for regulating cell fate as well as transcriptional activity of numerous nuclear receptors. In this mini-review, we focus on the post-translational modifications of NR5A1 and discuss their functions on regulating NR5A1 activity.
Key words – NR5A1, phosphorylation, acetylation, SUMOylation, ubiquitination

INTRODUCTION

Nuclear receptors (NRs) mediate the transcriptional responses to a wide variety of physiological stimuli and thus function as important regulators of development, homeostatsis, metabolism, and reproduction. By binding to specific DNA sequences (hormone response elements), NRs serve as platforms for the recruitment of various coregulatory factors (coactivators and/or corepressors) that influence gene regulation. In early 1990s, steroidogenic factor 1 (SF1/NR5A1/AD4BP), a 53-kilodalton protein, was isolated and characterized by two independent research laboratories, Dr. Keith Parker in the United States and Dr. K. Morohashi in Japan, when searching promoter-binding factors for steroidogenic P-450 genes [1, 2]. Throughout this min-review we will refer to this protein as NR5A1, and to the corresponding genes in mouse and human as *Nr5a1* and *NR5A1*, respectively. Subsequent experiments by numerous research teams revealed that NR5A1 plays a crucial role in the regulation of steroid hormone biosynthesis, endocrine development of the gonads and adrenal glands, as well as sex development and differentiation [3-5].

Several genes, including *AMH/Amh* [6], *CYP11A1/Cyp11a1* [7], *CYP17A1/Cyp17a1* [8], *CYP19A1/Cyp19a1* [9], *CYP21/Cyp21* [10], *FSHB/Fshb* [11], *GnRHR/Gnrhr* [12], *HSD3B2/Hsd3b2* [13], *INHA/Inha* [14], *LHB/Lhb* [15], *MC2R/Mc2r* [16], *NR0B1/Nr0b1* [17], and *STAR/Star* [18] have been identified as targets of NR5A1. The conserved binding consensus sequence for NR5A1 in the promoter regions of target genes is AGGTCA. Regulation of these genes involves the concerted action of NR5A1 with multiple transcription factors with which it can synergize, such as EGR1 [19], GATA4 [20], SOX9 [6], PITX1 [21], and WT1 [22]. Numerous transcriptional co-activators, such as nuclear receptor coactivator 1 (NCOA1) [23], cyclic AMP response element-binding protein (CREB)-binding protein/EP300 [24], transcriptional intermediary factor 2 (TIF2) [25], and CTNNB1 (also known as β-catenin) [26], have been reported to interact with NR5A1 and likely participate in NR5A1-mediated transcriptional activation. On the other hand, several factors such as nuclear receptor corepressor 1 (NCOR1) [27], DDX20 [28], and NR0B1 [29] appear to play an inhibitory role by limiting NR5A1 function. Recent extensive studies have found that NR5A1 is associated with developmental disorders and birth defects, such as adrenal agenesis and aplasia [30], androgen insensitivity syndrome [31], ovarian insufficiency [32], and impaired sex development [33]. Therefore, NR5A1 is not only important for steroidogenesis but also essential for organ development and functions in the adrenal gland, gonads, and pituitary.

The structural features of general NRs comprise of a N-terminal regulatory domain (contains domain of activation function 1, AF1), a DNA binding domain (DBD) in the amino terminus, a hinge region (regulatory domain), and a ligand-binding domain (LBD) localized in the carboxyl terminus which usually contains domain of activation function 2 (AF2). In contrast to most NRs, the AF1 domain of NR5A1 is localized in hinge region [34]. Unlike most NRs, NR5A1 binds DNA as a monomer. NR5A1 is well conserved among species, and the overall amino acid identity between human and mouse is 93.5%. The major difference between human and mouse NR5A1 is localized in the hinge region (Figure 1).

This mini-review focuses on the roles and functions of post-translational modifications of NR5A1. The endocrine functions of NR5A1 and transcriptional regulation of *NR5A1/Nr5a1* gene has been reviewed elsewhere.

POST-TRANSLATIONAL MODIFICATION

Post-translational modifications are essential mechanisms for controlling functions of proteins and subsequently for regulating cell fate.

Phosphorylation of NR5A1

Phosphorylation is one of the most important and common post-translational modifications and plays an essential role in a wide range of cellular processes. It is estimated that at least 1/3 of total proteins are phosphorylated in some cellular state. The reversibility of phosphorylation thus provides an important regulatory mechanism in organisms. Hormone stimulation (such as GnRH, FSH, LH, ACTH) often enhances cAMP levels in target cells, in turn leading to PKA cascade activation, which phosphorylates target proteins. Most of the cAMP target genes are also the target genes of NR5A1 [35-38]. Therefore, short after the discovery of NR5A1, the search for the possibility of NR5A1 phosphorylation was on the way.

The first evidence was that cAMP-dependent PKA elicits phosphorylation of NR5A1 *in vitro* and serine 430 was suggested to be the potential consensus phosphorylation site [9, 37, 39-41]. However, cAMP does not directly alter NR5A1 phosphorylation status *in vivo* and no physiologically functional phosphorylation site by PKA was reported [42]. Interestingly, PKA also activates phosphatases (such as MKP1), leading to decreased phosphorylation of NR5A1 and subsequently activates human *CYP17* transcriptional activity [43, 44]. Therefore, the balanced and integrated phosphorylation and de-phosphorylation of NR5A1 is required for human CYP17 transcriptional activity.

Further studies suggest that PKA-mediated NR5A1 transcriptional activation is mainly involved DNA binding activity, cofactor recruitment, and stabilization of NR5A1 protein [16, 24, 42-43]. In 1999, Hammer et al. reported that NR5A1-medaited transcription is dependent on phosphorylation at serine 203 (S203) located in a major activation domain (AF1) of the protein *in vivo* [34] (also see Figure 1).

Further studies confirmed that Ser203 is the only physiological phosphorylation site found so far in NR5A1 [45, 46]. An NR5A1 S203A mutant attenuates NR5A1 activation, while S203D (mimics phosphorylation of NR5A1) results in a dose-dependent increase in NR5A1-mediated transcription [45]. Interestingly, phosphorylation at S203 on NR5A1 also mimics stabilization of ligand binding effect [46]. Moreover, phosphorylation-dependent NR5A1 activation is likely mediated by MAPK/ERK signaling pathway [34, 45, 47-48]. S203 phosphorylation is required for transcriptional activation of numerous NR5A1 target genes, including *SRY/Sry* and *STAR/Star* [49, 50]. Recent studies have demonstrated that NR5A1 interacts with CDK7 and S203 is phosphorylated by CDK7-mediated process [51, 52].

Inactive form of CDK7 represses NR5A1-mediated transcriptional activity due to blockade of phosphorylation at S203 [51]. These results suggest that S203 can be phosphorylated by both ERK and CDK7 pathways. More recently, peptidyl-prolyl cis/trans isomerase PIN1 interacts with phosphorylated NR5A1 and facilitates NR5A1-mediated transcriptional activity [53], suggesting PIN1 functions as a novel regulator in NR5A1-mediated reproductive development and functions.

Acetylation of NR5A1

Acetylation is an essential chemical reaction for various biological processes including gene regulation. Acetylation of histones and tumor protein p53 are solid examples. Since numerous NRs (such as estrogen receptors, androgen receptors, and thyroid receptors) are acetylated [54-56], many groups predict NR5A1 would be acetylated at a conserved acetylated site. In addition, coactivators with histone acetyltransferase activity interact and enhance NR5A1-mediated transcriptional activity. Acetylation of NR5A1 by histone acetyltransferase GCN5 was first reported by Jacob et al. in 2001 [57]. In that study, the major acetylation site on NR5A1 is K72 and the minor sties K34 and K38 (see Figure 1). Both acetylation of NR5A1 by GCN5 and inhibition of deacetylation by trichostatin stimulate NR5A1's transcription activity and stability. A tertiary GCN5/SRC1/NR5A1 complex on *Cyp17* promoter proposed by Sewer group [58] further supports the importance of NR5A1 acetylation. Since the acetylation motif on p53 is KXXKK [59], Chung group from Taiwan searched other possible acetylation site(s) on NR5A1 and reported that KQQKK motif, adjacent to DNA-binding domain, on NR5A1 is acetylated by p300 [24] (also see Figure 1). Mutation of KQQKK motif significantly reduces p300-mediated activation and DNA-binding activity of NR5A1. Interestingly, cAMP pathway stimulation increases NR5A1 colocalization with p300 in nuclear loci, suggesting NR5A1 activity is activated by cAMP via p300-mediated acetylation, recruitment to active loci, and enhanced DNA binding activity. To date, there is no evidence suggests how NR5A1 is deacetylated. The potential candidate would be SIRT1, HDAC1, and HDAC2. A previous report has demonstrated that NR4A1 induces zinc finger protein *ZNF461* gene expression, and ZNF461 further acts as a novel corepressor of NR5A1 via recruitment of HDAC2 [60]. Therefore, future studies are necessary to determine how NR5A1 is deacetylated.

SUMOylation of NR5A1

Post-translational modification of proteins involving conjugation members of the small ubiquitin-related modifier (SUMO) family, which was discovered in 1997 and is highly conserved from yeast to humans, has been shown to regulate and influence diverse cellular processes and pathways, including cancer development and metastasis [61-62], cell cycle regulation [63-64], chromosome segregation [65-66], DNA repair [67-68], formation of sub-nuclear structures [69-70], nuclear transport [71-72], protein stability [73-74], and regulation of transcription [75-76]. Three laboratories simultaneously investigated SUMO modifications that control NR5A1 function and found that NR5A1 could be conjugated by SUMO both *in vivo* and *in vitro* in late 2004 and early 2005 [77-79]. NR5A1 physically interacts with UBE2I (SUMOylation E2 enzyme), PIAS1, and PIAS3 (SUMOylation E3 enzymes) [77, 80]. NR5A1 could be SUMOylated at lysines 119 and 194 (with K194 is the major SUMO site) based on *in vitro* and cell culture system [77-79] (also see Figure 1). The experimental

results suggest that SUMOylation of NR5A1 is necessary for repression of transcriptional synergy through synergy control (SC) motif which is localized adjacently to K194. Moreover, loss of SUMOylation of NR5A1 and SOX9 further enhances synergistic transcription of NR5A1 target genes, suggesting SUMO modification regulate transcription through affecting selective and cooperative interaction among factors constituting transcriptional complexes [77]. Though SUMOylation of NR5A1 alters its nuclear and subcellular localization [52, 78], the overall DNA binding activity of NR5A1 is not affected by its SUMOylation status. Furthermore, though K119 resides adjacent to DBD and K194 resides adjacent to LBD, the structure and coregulator recruitment of NR5A1 LBD are not altered by its SUMOylation [79]. Overexpression SENP1 (deSUMOylation enzyme) increases NR5A1-mediated transcriptional activity [79]. On the other hand, overexpression of PIASy and PIASxα promote NR5A1 SUMOylation [79]. Further studies show that SUMO modification of NR5A1 is HDAC-independent and NR0B1-independent [79]. Interestingly, DDX20 (DEAD-box RNA helicase) strongly interacts with SUMOylated NR5A1 and increases transcriptional repression, suggesting DDX20 is directly coupled to transcriptional repression by SUMOylation [79]. Furthermore, preventing NR5A1 SUMOylation increases the mRNA and protein levels of multiple sateroidogenic enzyme genes, suggesting the essential role of NR5A1 SUMOylation on steroidogenesis [52, 79]. Since ACTH-mediated signaling cascades coordinate a cyclic pattern of NR5A1-dependent transcriptional activation [16], we analyzed the role of NR5A1 SUMOylation on *Star* promoter. The analysis indicated that blockade of NR5A1 SUMOylation leads to an increase in overall promoter occupancy but does not alter the oscillatory recruitment dynamics in response to ACTH [52]. Notably, though ERK and CDK7 both are able to phosphorylate NR5A1 [51-52], CDK7 binds preferentially to the SUMO-less NR5A1 and CDK7 inhibition reduces NR5A1 phosphorylation [52]. Therefore, CDK7 plays an essential role in NR5A1-coordinated modification model. Since the extent of SUMOylation appears extremely low for most substrates (5-10% total substrates) [52, 77-79], the importance and contribution of SUMOylation cycle remains unclear *in vivo*. More recently, using a knockin mouse model, Lee et al. have shown that SUMO-less NR5A1 inappropriately activates hedgehog signaling and targets, leading to marked endocrine abnormalities in mutant mice [81]. This important *in vivo* mouse model suggests that SUMOylation cycle of NR5A1 is essential for endocrine development as well as functional capacity of transcriptional activity.

Ubiquitination of NR5A1

Ubiquitination is an important and essential post-translational modification in regulating protein degradation. Impaired ubiquitination process accumulates unwanted proteins and leads to certain diseases and genetic disorders. It has been shown that increasing the ubiquitination and degradation of NR5A1 by HDAC inhibitors leads to reduced *CYP11A1* transcriptional activity [82]. Moreover, recent study has demonstrated that ubiquitination of NR5A1 is phosphorylation-dependent and PIN1-mediated in regulating gonadotropin gene transcription [53]. However, more studies are needed to determine the impact of ubiquitination on NR5A1 activity as well as the crosstalk between ubiquitination and other post-translational modifications.

Interaction of post-translational modifications

Extensive studies have demonstrated the interaction and regulation between phosphorylation and SUMOylation, such as phosphorylation-dependent SUMO modification (PDSM) [83] and SUMOylation-regulated protein phosphorylation [84]. Mounting evidence indicates that SUMOylation inhibits and phosphorylation activates NR5A1 [34, 52, 77-79, 85], while recent structural analyses have revealed that phospholipids can serve as activating NR5A1 ligands [86-88]. Therefore, the interplay between SUMOylation and phosphorylation might play an important in regulating NR5A1's function. We and others have demonstrated that whereas NR5A1 SUMOylation is independent of S203 phosphorylation and is unaffected by ACTH treatment, loss of SUMOylation at K194 leads to enhanced NR5A1 phosphorylation at S203 [52, 85]. This highlights the interaction between post-translational modifications in regulating NR5A1. As stated earlier, one report has found that ubiquitination of NR5A1 is phosphorylation-dependent [53]. Therefore, more extensive studies are necessary to determine the physiological roles of crosstalk among post-translational modifications such as phosphorylation, SUMOylation, acetylation, and ubiquitination in regulating NR5A1 activity.

CONCLUSION

NR5A1 is a nuclear receptor selectively expressed in the adrenal gland, gonads, and pituitary, where it mediates the hormonal stimulation of multiple genes involved in steroid hormone biosynthesis. Through the extensive studies by numerous groups, NR5A1 activity is regulated by various post-translational modifications. Future studies will indeed be required to focus on the crosstalk among those post-translational modifications in regulating stability, transcriptional activity, ligand-binding of NR5A1.

DISCLOSURE SUMMARY

The authors have nothing to disclose.

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ABBREVIATIONS

ACTH: Adrenocorticotropic hormone
AF1: Activation function 1
AF2: Activation function 2
AMH: Muellerian-inhibiting factor
CDK7: Cyclin-dependent kinase 7
CTNNB1: Catenin beta-1
CYP11A1: Cholesterol side-chain cleavage enzyme
CYP17A1: Steroid 17-alpha-hydroxylase/17,20-lyase
CYP19A1: Cytochrome P450 19A1 (aromatase)
CYP21: Steroid 21-hydroxylase
DBD: DNA binding domain
DDX20: ATP-dependent RNA helicase (DP103)
EGR1: Early growth response protein 1
FSH: Follicle-stimulating hormone
FSHB: Follitropin subunit beta
GATA4: Transcription factor GATA-4
GnRH: Gonadotropin-releasing hormone
GnRHR: Gonadotropin-releasing hormone receptor
HDAC1: Histone deacetylase 1
HDAC2: Histone deacetylase 2
HSD3B2: 3 beta-hydroxysteroid dehydrogenase type 2
INHA: Inhibin alpha chain
LBD: Ligand binding domain
LH: Luteinizing hormone
LHB: Lutropin subunit beta
MC2R: Adrenocorticotropic hormone receptor
NCOA1: Nuclear receptor coactivator 1
NCOR1: Nuclear receptor corepressor 1
NR: Nuclear receptor
NR0B1: Dax-1
NR4A1: Nuclear hormone receptor Nur77
NR5A1: Steroidogenic factor 1 (SF1/AD4BP)
PDSM: Phosphorylation-dependent SUMO modification
PIAS1: E3 SUMO-protein ligase PIAS1
PIAS3: E3 SUMO-protein ligase PIAS3
PITX1: Pituitary homeobox 1
PKA: Protein kinase A
SIRT1: NAD-dependent deacetylase sirtuin-1
SOX9: Transcription factor SOX-9
STAR: Steroidogenic acute regulatory protein (STARD1)
SUMO: Small ubiquitin-related modifier
UBE2I: SUMO-conjugating enzyme UBC9
WT1: Wilms tumor protein
ZNF461: Zinc finger protein 461 (GIOT1)

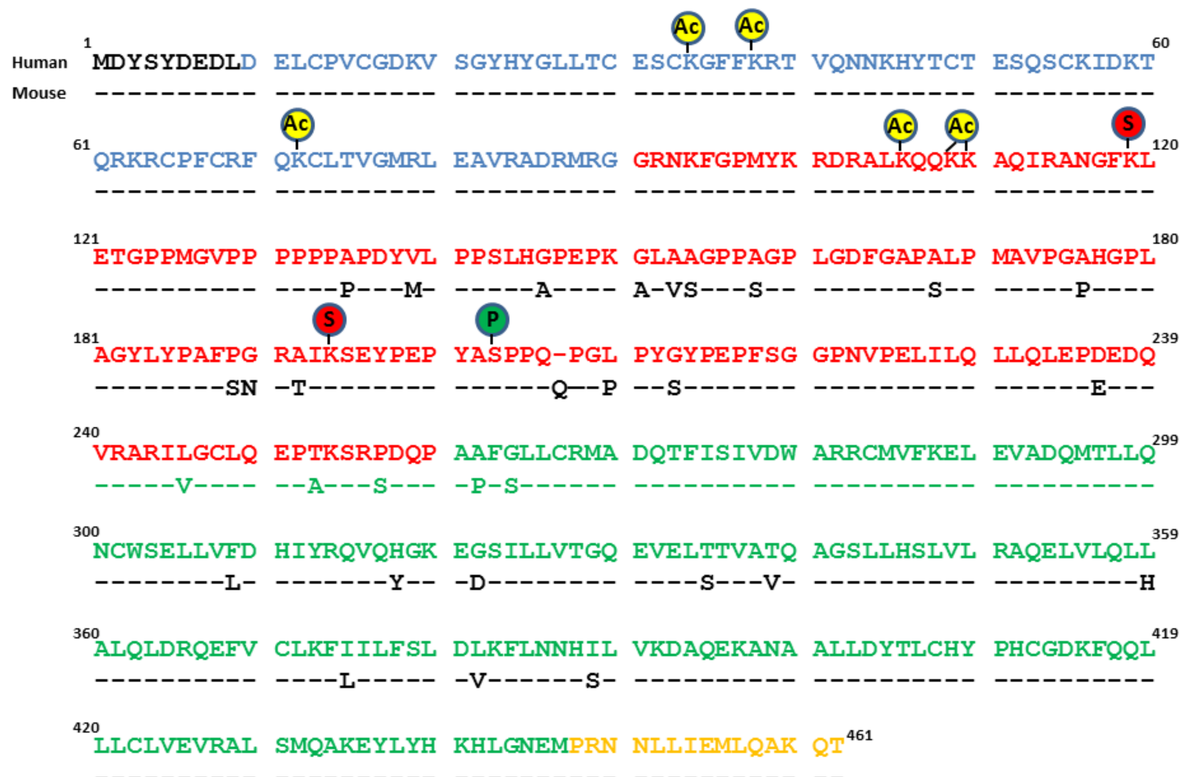

Fig. 1- Sequence alignment of human and mouse NR5A1 with known post-translational modification sites is shown. The sequence numbering is indicated at the beginning of every new line. The DNA-binding domain (DBD) is blue colored, the hinge region is red-colored, the ligand-binding domain (LBD) is green-colored, and the AF2 region is orange-colored. Ac, acetylated. P, phosphorylated. S, SUMOylated.