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DNA 손상 반응 유전자의 전사종결에서 RPRD1A 와 히스톤 H3 45 번 Threonine 잔기 인산화와의 관계

The Relation between RPRD1A and H3-T45 Phosphorylation in Transcriptional Termination of DNA Damage Response Gene

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이 상 호

A thesis of the Degree of Master of Philosophy

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by

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ABSTRACT

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Core histones undergo diverse post-translational modifications to regulate the transcription process. On our previous report, we showed that AKT1 phosphorylates 45th threonine of histone H3 (H3T45) under DNA damage conditions. It enhances transcription by facilitating transcriptional termination. However, the precise mechanism of phosphorylated H3T45 (p-H3T45) - enhanced transcriptional termination is not yet understood. In this study, we show that human orthologs of yeast transcription termination factor Rtt103, RPRD1A and RPRD1B, interact with AKT1. RPRD1A, not RPRD1B, harbors well-conserved AKT phosphorylation site on its carboxy-terminal coiled-coil structure (Serine 285). Under the DNA damage conditions, *RPRD1A* knockdown had no effect on H3T45 phosphorylation, but caused impaired *CDKN1A* transcriptional induction. AKT1 mediated phosphorylation of H3T45 and RPRD1A-S285 both increased their binding affinity. Finally, S285A mutation impaired RNA polymerase II (Pol II) dissociation from the chromatin, resulting improper transcriptional termination and reduced transcription efficiency.

Taken together, we suggest a novel mechanistic insight that RPRD1A transmits H3T45 phosphorylation signal to transcriptional termination.

Keywords: RPRD1A, AKT1, Transcriptional termination, DNA damage

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LIST OF ABBREVIATIONS

Pol II	RNA polymerase II
p-PolII-S2	Phosphorylated RNA Polymerase II Carboxy-Terminal Domain 2nd Serine
p-PolII-S5	Phosphorylated RNA Polymerase II Carboxy-Terminal Domain 5th Serine
CTD	Carboxy-Terminal Domain
CID	Carboxy-terminal Interacting Domain
PH	Pleckstrin Homology
RPRD	Regulation of nuclear pre-mRNA domain-containing protein
H3T45	Histone H3 45th Threonine
p-H4T45	Phosphorylated Histone H3 45th Threonine
Rat1	RNA-trafficking protein 1
Rai1	Rat1-interacting 1
Rtt103	Regulator of Ty1 transcription protein 103
CF1	Cleavage Factor 1
RPAP2	RNA Polymerase II Associated Protein 2
WT	Wild Type
ADR	Adriamycin
CBB	Coomassie brilliant blue
TTS	Transcriptional termination Site
ChIP	Chromatin Immunoprecipitation
qRT -PCR	Quantitative real-time PCR

I. INTRODUCTION

1-1. C-terminal domain of RNA polymerase II and transcriptional termination.

Although transcriptional termination affects overall transcription efficiency (West and Proudfoot, 2009), termination process is less known than initiation or elongation. During transcription, a direct interaction forming a chromatin loop between 5' and 3' ends of a gene is observed for efficient transcription (Figure 1-1-1) (Kuehner et al., 2011, Ansari and Hampesy., 2005, O'Sullivan et al., 2004). Therefore transcription factors used for previous transcription can rapidly participate in the next transcription. For that reason, if transcriptional termination is performed improperly, transcription efficiency is reduced (Mapendano et al., 2010).

Transcriptional termination is closely related with the phosphorylation status of Pol II CTD (Carboxy-Terminal Domain) (Lunde et al., 2010). The CTD contains tandem heptapeptide repeats of consensus sequence '1-YSPTSPS-7', which varies from 26 repeats in Yeast to 52 repeats in Human (Corden, 1990; Lori A. Allison, 1985). The CTD is dynamically phosphorylated and dephosphorylated on Ser2, Ser5, and Ser7 by specific kinases and phosphatases depending on stage of the transcription cycle (Egloff and Murphy, 2008). This dynamic phosphorylation status is both temporally and functionally coupled to recruitment and activation of RNA processing complexes (Phatnani and Greenleaf, 2006). For example, the mRNA capping enzymes bind to p-PolII-S5 (Phosphorylated RNA Polymerase II Carboxy-Terminal Domain 5th

Serine) once transcription initiate (Fabrega et al., 2003), and the complexes responsible for cleavage and polyadenylation are recruited to p-PolII-S2 (Phosphorylated RNA Polymerase II Carboxy-Terminal Domain 2nd Serine) nearby the 3'end of the gene (Philip Komarnitsky, 2000; Seong Hoon Ahn and Buratowski, 2004)

There are two models about transcriptional termination in yeast (Kuehner et al., 2011). In poly(A)-dependent termination, which has been called the 'Torpedo' model(in case of protein coding region), the 5'-3' exoribonuclease Rat1 (RNA-trafficking protein 1) is recruited by Rai1(Rat1-interacting 1) and Rtt103 (regulator of Ty1 transposition protein 103) that binds to p-PolII-S2 via CID (C-terminal interacting domain) (Kim M, 2004; Pearson and Moore, 2013). Poly(A) site cleavage by CF1(cleavage factor I) provide an uncapped RNA 5' end (Luo et al., 2006). Rat1 degrade the uncapped RNA via its 5'-3' exonuclease activity, and thus induce dissociation of Pol II from DNA (Figure 1-1-2. a) (Kuehner et al., 2011; West et al., 2004). In Sen1-dependent termination (in almost case of non-coding region), Sen1, the RNA-DNA helicase, is recruited to CTD of Pol II via proteins that recognize p-PolII-S5 (Steinmetz et al., 2006). Sen1 unwind RNA-DNA hybrid via its helicase activity, thus induce Pol II release from DNA (Figure 1-1-2. b) (Eric J. Steinmetz, 1996; Kuehner et al., 2011). In human, similar torpedo mechanisms with XRN2, human homologue of Rat1, was unraveled (Figure 1-1-2. c) (Kuehner et al., 2011; West et al., 2004).

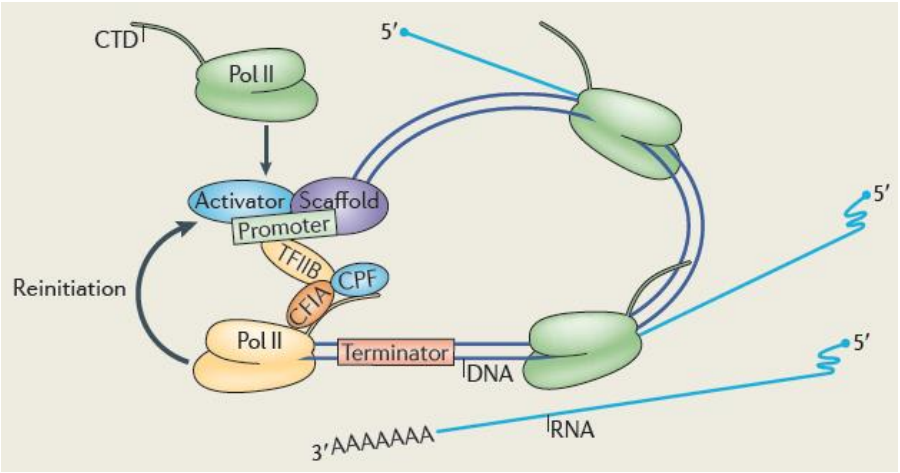

Figure 1-1-1. Chromatin forms loop for efficient transcription.

During transcription, 5’ and 3’ ends of gene interact by chromosomal looping. Thus transcription factors can be efficiently recycled, and overall transcription efficiency increased. This diagram is cited from (Kuehner et al., 2011).

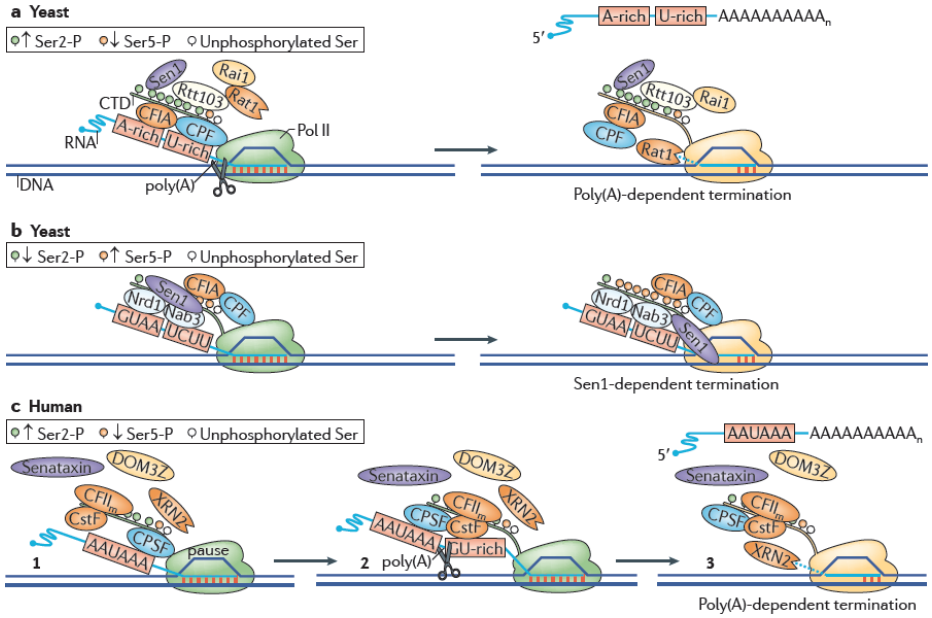

Figure 1-1-2. Schematic mechanism of transcriptional termination in yeast and human.

Counterparts of termination factors in yeast and human are shown in the same color. ‘a’ and ‘b’ are the mechanisms in yeast and ‘c’ is in human. ‘a’ is poly(A)-dependent termination called ‘torpedo model’. This mechanism is observed in most of protein coding regions. ‘b’ is Sen1-dependent termination which is observed in most of non-coding regions. Finally ‘c’ is poly(A)-dependent termination in human. This diagram is cited from (Kuehner et al., 2011).

1-2. Function of RPRDs (regulation of nuclear pre-mRNA domain-containing protein) in transcriptional termination and malignancy.

p-PolII-S2 is enriched at 3' end of genes and induces transcriptional termination by recruiting Rat1-Rai1-Rtt103 complex (Figure 1-2-1) (Kuehner et al., 2011; Philip Komarnitsky, 2000). RPRD1A and RPRD1B (known as P15RS and CREPT, respectively) are human homologs of Rtt103 (Lu et al., 2012). They share highly conserved amino acid sequence (over 65%) and contain CID on their N-terminus, which preferentially interacts with p-PolII-S2. They also have coiled-coil structure on the C-terminus (Ni et al., 2011), which is required for homo-or hetero dimerization (Ni et al., 2014). Both are components of Pol II complex (Mei et al., 2014; Patidar et al., 2016), which is recently identified as a scaffold for Pol II CTD-S5 dephosphorylation (Ni et al., 2014). RPRD1A and RPRD1B form homo- or hetero- dimer to interact with CTD repeats where phosphorylated-S2 and/or phosphorylated-S7 bracket a phosphorylated-S5 residue, serving as CTD scaffolds. Then RPAP2 (RNA Polymerase II Associated Protein 2) phosphatase which associates directly with the dimer, coordinates dephosphorylation of p-PolII-S5 (Figure 1-2-2) (Ni et al., 2014).

Although RPRD1A and RPRD1B have evolved from Rtt103, they seem to be function in opposite pattern in human malignancy. RPRD1A was first identified as P15INK4b-related gene, and inhibiting RPRD1A in human melano-

ma cell line resulted in up-regulation of cyclin D1 and cyclin E expression (Liu J, 2002; Wu et al., 2010). And also, expression of RPRD1A inhibited migration and invasion of human malignant melanoma cells (Zhang et al., 2012). RPRD1B, however, is preferentially expressed in diverse human tumors and accelerates tumorigenesis by regulating the transcription of cyclin D1 (Jung et al., 2009; Li et al., 2013; Lu et al., 2012). Regarding the evolutionary conservation and striking structural similarities between these two proteins, the factor making the different oncogenic characteristics has yet to be clarified.

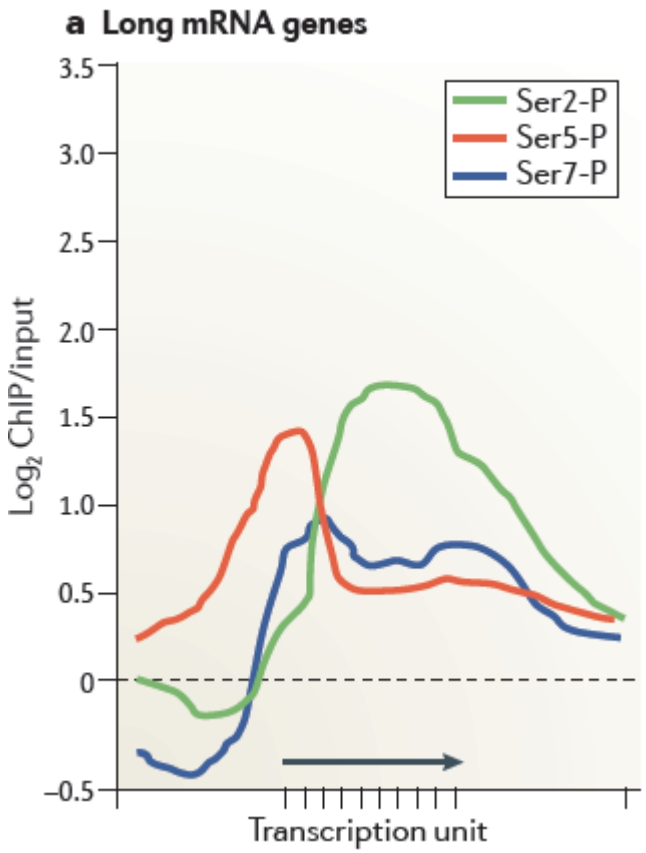

Figure 1-2-1. Genome-wide distribution of RNA polymerase II CTD phosphorylation.

The localization of Pol II CTD phosphorylation was mapped across the whole yeast genome by ChIP-chip. This diagram shows long mRNA-encoding genes (>2,000 bases, 128genes). Transcription unit indicates genome from the transcription start site to poly(A) site. Image is cited from (Kuehner et al., 2011).

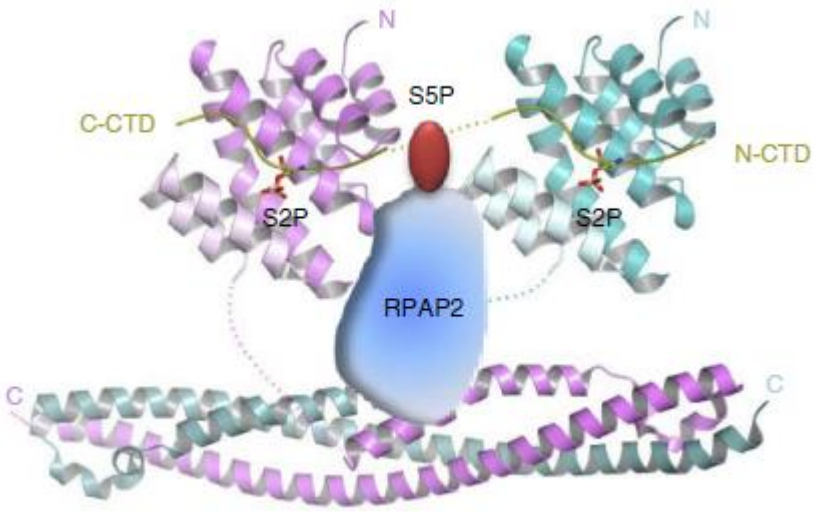

Figure 1-2-2. RPRD1A and RPRD1B serve as a scaffold for interaction of RNA polymerase II CTD and RPAP2.

RPRDs interact with RPAP2, the phosphatase of Pol II CTD-S5, and also interact with p-PolII-S2/7. Serving as a scaffold, RPRD1A and RPRD1B mediate RPAP2 interaction with Pol II CTD. This diagram is cited from (Ni et al., 2014).

1-3. AKT activation under DNA damage conditions and contribution to transcriptional termination.

AKT (Protein Kinase B / PKB) is a serine/threonine kinase regulated by various cellular stimulations. AKT consists of a PH (pleckstrin homology) domain at amino-terminal, central catalytic domain, and regulatory domain at carboxy-terminal (Downward, 1998). PI3K is a well-known upstream kinase of AKT by generating PIP_3 which recruits AKT to plasma membrane. At the membrane, another PH domain containing serine/threonine kinase PDK1 and PDK2 phosphorylate T308 and S473 of AKT, respectively (Dario R. Alessi and Piers R.J. Gaffney, 1997; David Stokoe et al., 1997). Most of the AKT signaling pathway has been studied in cancer, because AKT plays a central role in oncogenesis by promoting cell survival, proliferation, and growth (Vivanco and Sawyers, 2002). However, AKT is also activated under various genotoxic stresses via ATM/ATR or DNK-PK, the core factors of DNA damage response (Figure 1-3-1) (Liu et al., 2014). DNA-PK phosphorylates AKT-S473 under DNA damage conditions such as DNA damage agents, IR or UV irradiation, and phosphorylated AKT is involved in DNA repair signaling and *CDKN1A* induction (Bozulic et al., 2008). ATM is required for phosphorylation of AKT in radiation or insulin response (Viniegra et al., 2005).

Recently, we have identified 45^{th} threonine residue of core histone H3 (H3-T45) is phosphorylated by AKT, under DNA damage conditions (Lee et al., 2015). This particular modification occurs predominantly at the TTS (tran-

scriptional termination site) of DNA damage-inducible genes, and facilitates the 3’ end processing which is a critical step in transcriptional termination (Figure 1-3-2) (Kim M, 2004, Lee et al, 2015). However, molecular mechanism underlying p-H3T45 facilitated 3’ end processing remains largely elusive.

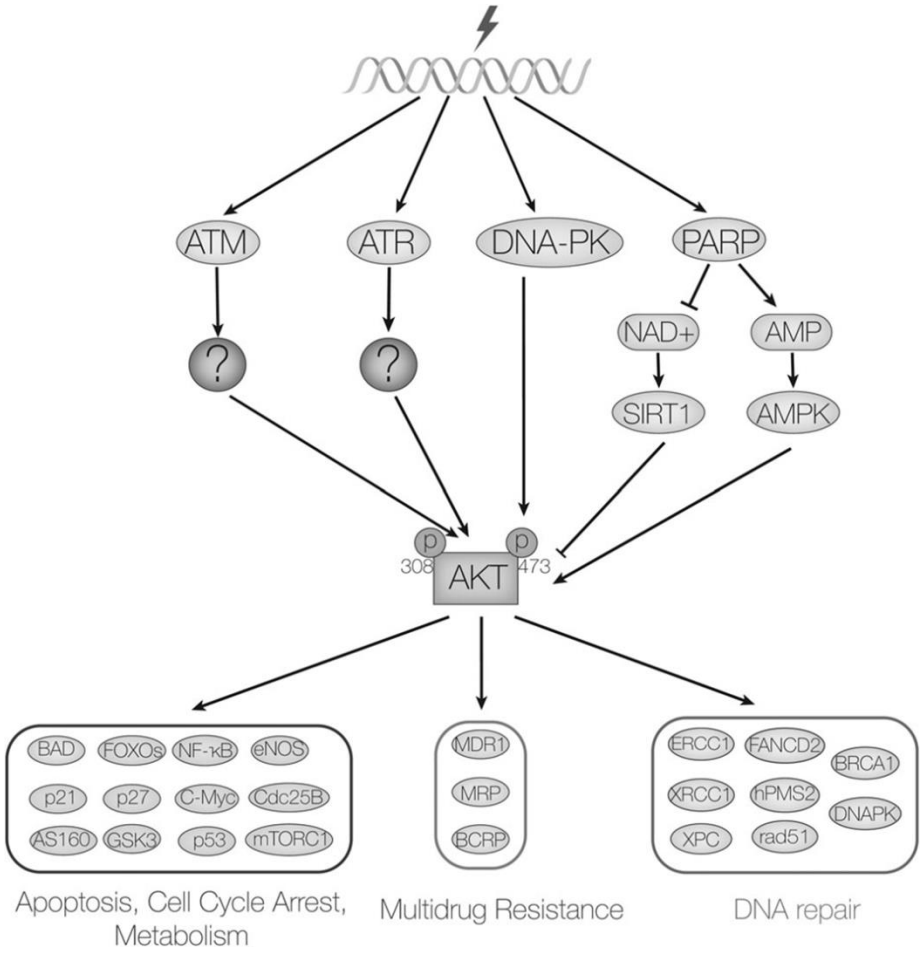

Figure 1-3-1. Functions of AKT under the DNA damage condition.

Under genotoxic stress, DNA damage sensors including ATM, ATR, and DNA-PK activate AKT. The mechanism how ATM and ATR activate AKT is not yet clearly clarified. DNK-PK phosphorylates AKT-S473. Activated AKT participates in various cellular responses to DNA damage, including cell cycle arrest, drug efflux, and DNA repair. This image is cited from (Liu et al., 2014).

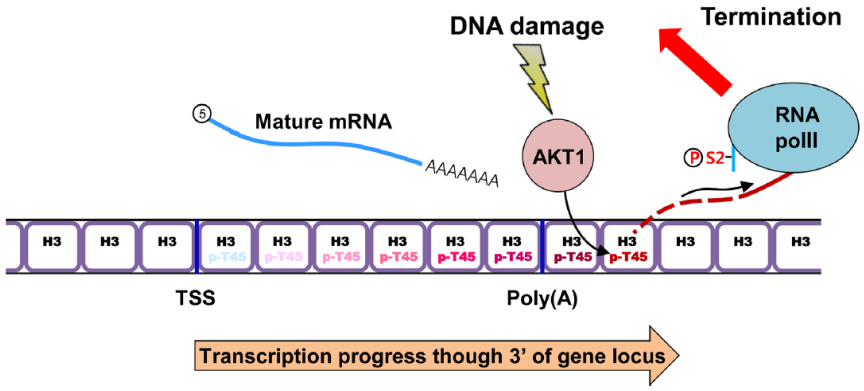

Figure 1-3-2. Phosphorylation of H3T45 by AKT facilitates transcriptional termination in response to DNA damage.

Under DNA damage condition, AKT phosphorylates H3T45 under DNA-damage response genes. p-H3T45 is predominantly increased at 3' of genes and it facilitates transcriptional termination by releasing Pol II from DNA. This diagram is cited from (Lee et al., 2015).

II. MATERIALS AND METHODS

2-1. Cell culture and transient expression

HEK293T and MCF10A cells were obtained from ATCC. HEK293T cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum (Gibco) and antibiotics. MCF10A cells were cultured in DMEM-F12 media, supplemented with 5% (v/v) horse serum (Gibco), antibiotics, insulin (10 μg/ml, Sigma), cholera toxin (0.1 μg/ml, Listbiological Labs), hydrocortisone (0.5 μg/ml, Sigma), and hEGF (0.02 μg/ml, PeproTech).

Polyethylenimine (PEI, Polysciences) was used to transfect HEK293T cells. Lipofectamine (Invitrogen) was used to transfect MCF10A cells per the manufacturer's instructions.

2-2. DNA constructs and purification of recombinant proteins

Full-length RPRD1A, RPRD1B cDNA was obtained by PCR from the MCF10A cell line. Full-length sequences were verified by sequencing analysis. The PCR products were inserted into pcDNA3.0-FLAG/HA (Invitrogen), pcDNA3.1-myc-His (Invitrogen) and pCAG-FLAG (Jang et al., 2012) for expression in mammalian cell lines. Expression vectors for WT, T45A/E H3, and AKT1 have been described (Lee et al., 2015)..

RPRD1A, RPRD1B and histone H3 proteins were generated by subcloning the corresponding PCR fragment into pGEX4T-1 (Amersham Biosciences) or pRSETB (Invitrogen). GST fusion proteins were expressed in the Escherichia-

coli strain DH5α, and proteins were isolated using Glutathione Sepharose™ 4B beads (Amersham Biosciences) per the manufacturer's instructions. His6 fusion proteins were expressed in the BL21DE3 strain and purified with Ni-NTA agarose (Qiagen) per the manufacturer's instructions.

2-3. Antibodies

Anti-actin was purchased from Sigma; anti-Myc (9E10) was obtained from Covance; anti-pan H3 and anti-RPRD1A were purchased from Abcam; anti-FLAG was purchased from Sigma; anti-Rpb1 was purchased from Santa Cruz; anti-RPRD1B was purchased from Bethyl Laboratories; and anti-AKT was acquired from Cell Signaling. Rabbit polyclonal H3-T45 phosphorylation-specific antibody was generated as described (Lee et al., 2015).

2-4. Lentiviral shRNA-mediated knockdown of RPRD1A

Lentiviral vectors that contained the human RPRD1A-targeting sequences pLKO.1-sh-RPRD1A #1 (TRCN0000002614), #2 (TRCN0000002616), #3 (TRCN0000002617), #4 (TRCN0000364070), and #5 (TRCN0000364071) were purchased from Sigma. As a control, the pLKO.1 vector was used. Lentivirus was produced per the manufacturer's protocol using the BLOCK-iT Lentiviral RNAi expression system (Invitrogen). Twenty-four hours after lentiviral infection, infected cells were selected with puromycin (1 μg/ml) for 2 weeks and then used in the experiments. Because pLKO.1-sh-RPRD1A-1 and pLKO.1-sh-RPRD1A-3 were the most effective, we used these vectors in most experiments.

2-5. AKT kinase assay

Active AKT1 (upstate) was mixed with substrate in 20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 75 mM MgCl2, 75 μM ATP, and 10 μCi 32P-ATP. The reaction mixture was incubated for 2 hours at 30℃ and loaded onto SDS-PAGE gels. The gels were dried for 1 hour at 80℃ and exposed to X-ray film overnight at -80℃.

2-6. Western blot assay

Cells were lysed with IP150 Buffer [150mM NaCl, 125mM Tris-Cl (pH 8.0), 0.1% NP-40, 10% glycerol, 1mM EDTA (pH 8.0)] and nuclear membrane was chopped by sonication (Bioruptor). Lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane for blotting using 25mM Tris-Cl, 250mM glycine, 15% (v/v) Methanol buffer at 4℃ for 2 hours. Membranes were blocked with 5% non-fat dry milk or BSA, 10mM Tris-Cl (pH 7.5), 150mM NaCl, 0.1% tween-20 for 1 hour. After blocking, membranes were washed 3 times and incubated with proper antibody at 4℃ for 16 hours. Membranes were washed 3 times and incubated with HRP conjugated antibody at room-temperature for 1 hour. For detection, blots were reacted with ECL solution and exposed to X-ray film.

2-7. ChIP (Chromatin Immunoprecipitation) assay

MCF10A cells($4x10^7$) were harvested and crosslinked with EGS [ethylene glycol bis (succinimidyl succinate); Thermo Scintific] to a final concentration of 5mM, and formaldehyde to a final concentration of 1%. The crosslinking reaction was stopped by adding glycine to a final concentration of 0.125 M. The cells were harvested and washed twice with cold PBS, and cytosolic fractions were eliminated with buffer A [5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP-40, protease inhibitors]. Nuclear pellets were resuspended in buffer B [100 mM Tris-Cl (pH 8.1), 1% SDS, 10mM EDTA, protease inhibitors], and the chromatin was digested with sonication (Bioruptor). Prepared chromatin fraction was diluted 1/10 in IP buffer [0.01% SDS, 1.1% TritonX-100, 1.2mM EDTA, 16.7mM Tris-Cl (pH 8.1), 167mM NaCl, and protease inhibitors cocktail] and incubated overnight at 4℃. Samples were incubated with salmon sperm DNA pre-coated protein A or G bead for 2~4 hours at 4℃, washed with TSE150 [0.1% SDS, 1% TritonX-100, 2mM EDTA, 20mM Tris-Cl (pH 8.1), 150mM NaCl], TSE500 [0.1% SDS, 1% TritonX-100, 2mM EDTA, 20mM Tris-Cl (pH 8.1), 500mM NaCl], BufferIII [0.25M LiCl, 1%NP40, 1% Sodium deoxycholate, 1mM EDTA, 10mM Tris-Cl (pH 8.1)], TE(pH 8.0) for two times. Washed beads were eluted with elution buffer (1% SDS, 0.1M NaHCO3), incubated overnight at 65℃ with NaCl for reversal of crosslinking. Samples were incubated 50℃ in addition of 10μl of 0.5M EDTA, 20μl of 1M Tris (pH 6.5), and 4μl of Proteinase K (20mg/ml), purified with phenol/chloroform/isoamyl alcohol. Nucleic acid was precipitated by centrifugation 30 min at 4°C with 1μl glycogen solution (20mg/ml), 20μl NaCl (5M),

and 500ul isopropanol. Pellet was washed with 70% EtOH, dried and eluted with pure water.

2-8. qRT -PCR (Quantitative real-time PCR) analysis of relative mRNA levels and ChIP products

Total RNA was extracted with TriZol® (Invitrogen) and reverse-transcribed using AMV Reverse Transcriptase (Sigma-Aldrich). mRNA and antibody-bound chromatin levels by chromatin immunoprecipitation assay were quantified by real-time qPCR with the SYBR® Green qPCR Kit (Finnzymes, F-410L) on the iQ5 and CFX Connect Real-time PCR Detection System (Bio-Rad) and then normalized to actin or 1% input chromatin using the 2-ΔΔ**C**T2-ΔΔCT calculation method. The sequences of the primers were described in Table 1, 2.

2-9. Statistics

Data are presented as means ± standard deviations, and P-values were calculated using the student's t-test calculator (http://www.physics.csbsju.edu/stats/t-test.html). A value of $p < 0.05$ was considered to be statistically significant. All data are representative of at least 3 independent experiments.

	Gene	5’ primer	3’ primer
Real-time PCR	18s	GCTTAATTTGACTCAACAC-GGGA	AGC-TATCAATCTGTCAATCCTGTC
	CDKN1A	GCAGACCAG-CATGACAGATTT	GGATTAGGGCTTCCTCTT-GGA
	RPRD1A	GCCTAGGAA-GCGAACTTATG	TCCAGATCTT-GTAATGCTCTAAC
	RPRD1B	CCCTCTTGACTGAGGAAC-TA	GCTGCCTCTTTGTCTGTTAT

Table 1. qRT-PCR primer list

	Gene	5’ primer	3’ primer
ChIP	*CDKN1A* #1	TGAGGCAGAATTGCTT-GAACCTGG	TTCACACAGGCTCCCAA-GAAGTGA
	CDKN1A #2 (Promoter)	CTCCCACCCCTAC-CTGGGCT	CAGAACCCAGGCTTGGAG-CA
	CDKN1A #3	CAGTTCCTTGTG-GAGCCGGA	TGTGAACGCAG-CACACACCC
	CDKN1A #4	TGGG-GATGTCCGTCAGAACCC	CTCCCAGGCGAAGTCACC
	CDKN1A #5	TGTCCTTTCCCTTCAG-TACCCTCT	TAGGGTGCCCTTCTTCTT-GTGTGT
	CDKN1A #6	CCTCTGCAAAGATCAC-CAAT	CCAGTTGCTCCATAACCTT-GCC
	CDKN1A #7 (TTS)	TGCACAGGG-CAGAGCTTTCTACTA	AAACAGGTGCAGGC-TATGGGACAA
	CDKN1A #8	TGGTTAACATTCAGGCCTT GCTGC	ATGGCCAC-TGTCGATGGAGACATT

Table 2. ChIP primer list

Ⅲ. RESULTS

3-1. AKT interacts with RPRD1A and phosphorylates 285th serine.

To find mediator which transmits p-H3T45 signal to transcriptional termination, we searched termination factors which is substrate of AKT. Among termination factors, we focused on RPRD1A. Despite structural similarities between RPRD1A and RPRD1B, only RPRD1A harbors well-conserved AKT target site on the 285th serine residue, in C-terminal coiled coil structure (Figure 3-1-1). We tested the interaction of RPRD1A and RPRD1B with AKT1 by co-immunoprecipitation assay. As a result, both RPRD1A and RPRD1B interacted with AKT1 (Figure 3-1-2). To test if these are direct interactions, we performed in vitro GST-pulldown assay using recombinant proteins of RPRD1A, RPRD1B and AKT1. As a result, both RPRD1A and RPRD1B interacted directly with AKT1 protein. However, RPRD1A showed greater affinity to AKT1, compared to RPRD1B (Figure 3-1-3). Since the binding affinity of RPRD1A and RPRD1B to AKT1 from co-immunoprecipitation assay showed no apparent differences, we assumed that RPRD1B might interact with AKT1 by dimerizing with endogenous RPRD1A. In vitro binding assay confirmed the direct interaction between RPRD1A and RPRD1B (Figure 3-1-4), and in vitro binding affinity of RPRD1B to AKT1 was greatly increased by addition of RPRD1A protein (Figure 3-1-5). These data suggest that RPRD1B interact with AKT1 by forming hetero-dimers with RPRD1A.

Next, to investigate whether RPRD1A is a true substrate of AKT1, we performed in vitro AKT1 kinase assay using radiolabeled ATP and proteins

which are purified from bacteria. In vitro kinase assay confirmed that only RPRD1A, not RPRD1B, was phosphorylated by AKT1 (Figure 3-1-6). To determine the precise phosphorylation site on RPRD1A, we substituted 285th serine to alanine, using site-directed mutagenesis. Substitution of predicted 285th serine to alanine completely hindered AKT1 mediated phosphorylation of RPRD1A, indicating that 285th serine of RPRD1A is indeed a substrate of AKT1 (Figure 3-1-7).

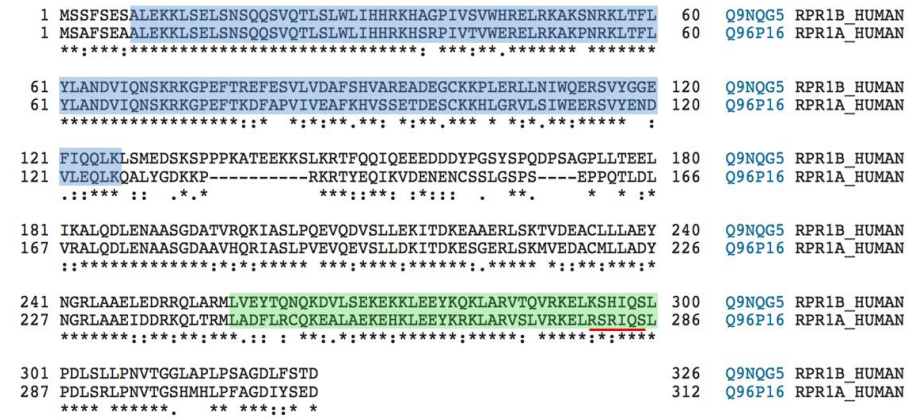

Figure 3-1-1. RPRD1A harbors putative AKT phosphorylation site.

Amino acid sequence alignment of human RPRD1A and RPRD1B. Blue box indicates Pol II CID. Green box indicates predicted coiled-coil structure and red underline represents putative AKT substrate sequence.

Sequence alignment was performed using UniProt (http://www.uniprot.org).

* indicates positions which have a single, fully conserved residue.

: indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

. indicates conservation between groups of weakly similar properties - scoring $\leq$ 0.5 in the Gonnet PAM 250 matrix.

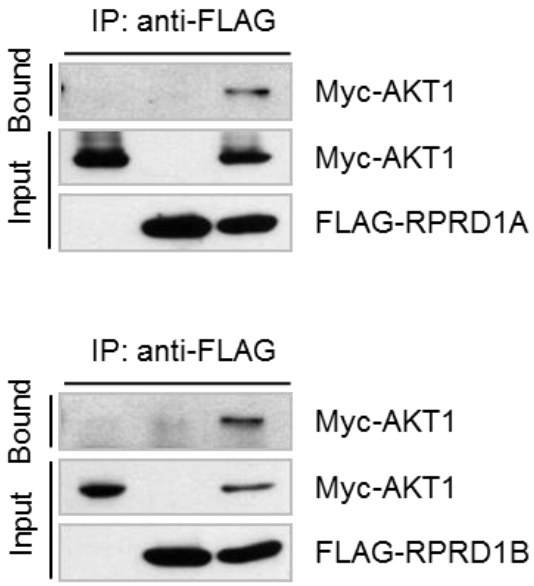

Figure 3-1-2. RPRD1A and RPRD1B bind to AKT1 in HEK293T.

Myc tagged AKT1 and FLAG tagged RPRD1A or RPRD1B were co-transfected in HEK293T cells. Total cell lysates were immunoprecipitated with FLAG antibody and probed with anti-Myc antibody.

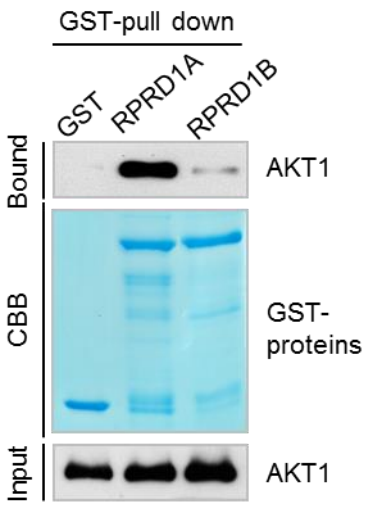

Figure 3-1-3. RPRD1A has greater affinity than RPRD1B to AKT1 in vitro.

AKT1 was incubated with GST-RPRD1A or RPRD1B. GST-sepharose bead was used to pull down GST proteins and probed with AKT antibody. CBB (Coomassie brilliant blue) staining show an amount of protein used in this assay.

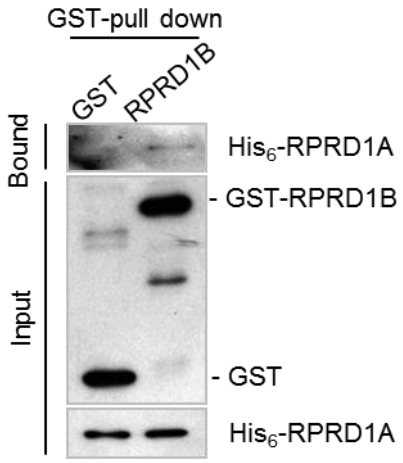

Figure 3-1-4. RPRD1A and RPRD1B dimerize directly.

GST pulldown assay with GST and GST-RPRD1B incubated with His-RPRD1A. GST-proteins were pulled down with GST-sepharose bead and probed with RPRD1A antibody. GST was used as control.

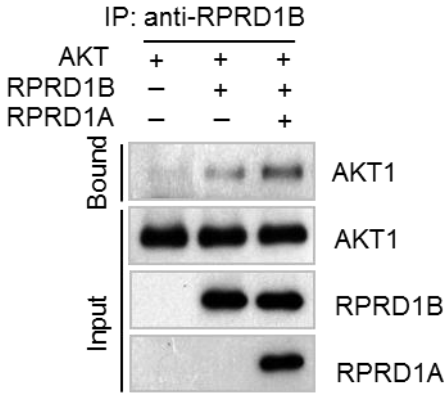

Figure 3-1-5. Interaction between RPRD1B and AKT1 is enhanced by RPRD1A.

In vitro binding assay of AKT1 and RPRD1B. AKT1 and GST-RPRD1B were incubated with/without GST-RPRD1A. Incubated proteins were immunoprecipitated with RPRD1B antibody and probed with AKT antibody.

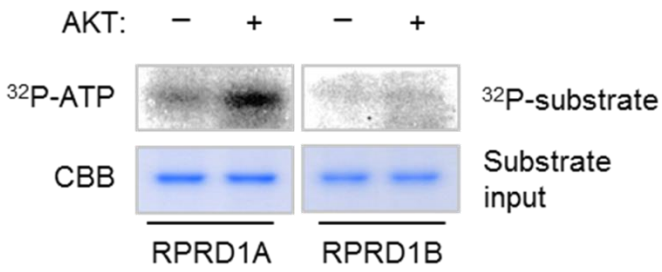

Figure 3-1-6. AKT1 directly phosphorylates RPRD1A.

AKT1 in vitro kinase assay of GST-RPRD1A and RPRD1B. GST-RPRD1A or RPRD1B incubated with/without active AKT1 and with [^{32}P]-ATP. CBB staining show an amount of substrate used in this assay.

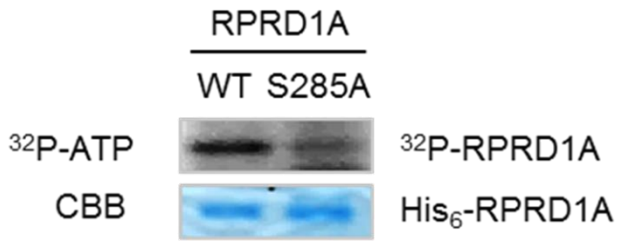

Figure 3-1-7. AKT1 phosphorylates RPRD1A at Serine 285.

AKT1 in vitro kinase assay of His-RPRD1A wild type (WT) and mutant, which 285th serine was substituted into alanine (S285A). His-RPRD1A WT and mutant incubated with active AKT1 and [^{32}P]-ATP. CBB staining show an amount of substrate used in this assay.

3-2. Phosphorylated 285thserine of RPRD1A induces *CDKN1A* transcription under the DNA damage.

To investigate whether RPRD1A is involved in DNA damage-induced *CDKN1A* transcription activation, we generated *RPRD1A* knockdown MCF10A using lentivirus clones. Successful knockdown of *RPRD1A* was confirmed by qRT-PCR and western blot (Figure 3-2-1). In order to make DNA-damage condition, we treated ADR (adriamysin) to control and *RPRD1A* knockdown MCF10A and confirmed mRNA level of *CDKN1A* by qRT-PCR. Interestingly, knockdown of *RPRD1A* showed a defect in induction of *CDKN1A* mRNA (Figure 3-2-2). According to our previous research, p-H3T45 under the DNA damage condition regulates *CDKN1A* mRNA induction (Lee et al., 2015). Thus, we wondered if knock-down of RPRD1A affects p-H3T45. We performed ChIP assays targeting *CDKN1A* promotor and TTS locus in control and *RPRD1A* knockdown cell lines (Figure 3-2-3). As a result, RPRD1A inhibition showed no effect on p-H3T45 level under the DNA damage condition. These results mean impaired *CDKN1A* mRNA induction under the DNA damage condition was caused by *RPRD1A* knockdown.

Next, to confirm the role of phosphorylated S285 of RPRD1A in *CDKN1A* mRNA induction, we generated WT and S285A mutant FLAG-RPRD1A backup MCF10A cell lines. Since lentivirus clone #3 targeted 3'UTR of RPRD1A mRNA, we were able to use plasmid DNAs containing human WT and S285A mutant RPRD1A. We observed that backup proteins were compa-

rable to endogenous RPRD1A of normal cell (Figure 3-2-4). We treated ADR to control cell line (Mock), *RPRD1A* knockdown cell line (shRPRD1A-3), WT RPRD1A backup cell line (shRPRD1A-3+WT), and S285A mutant RPRD1A backup cell line (shRPRD1A-3+SA). As a result, *CDKN1A* mRNA fold induction was restored in WT RPRD1A backup cell line, but not completely in S285A mutant RPRD1A backup cell line (Figure 3-2-5). This data suggests that phosphorylated S285 of RPRD1A under the DNA damage condition contributes to *CDK1NA* mRNA induction.

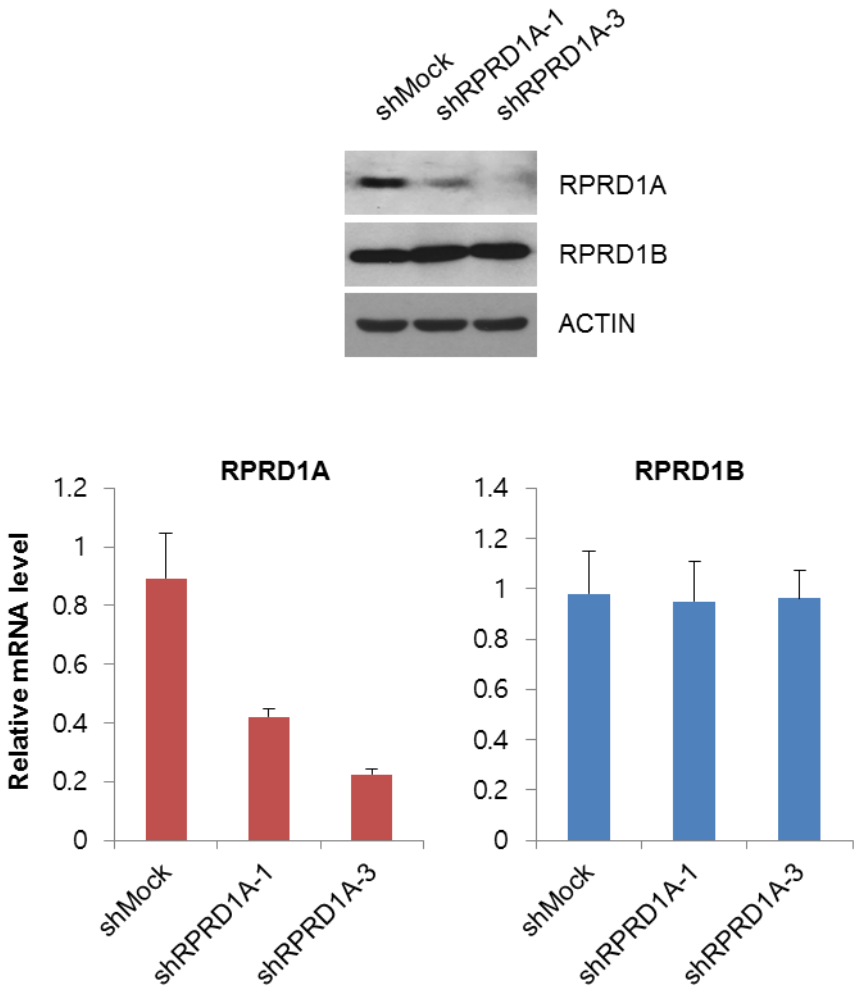

Figure 3-2-1. Generation of *RPRD1A* knockdown cell lines.

Endogenous *RPRD1A* knockdown MCF10A is generated using lentivirus containing shRNA. Whole cell lysates were detected by indicated antibodies. RPRD1B and ACTIN were used as control. RPRD1A and RPRD1B mRNA levels were analyzed with qRT-PCR and normalized to 18S ribosomal RNA.

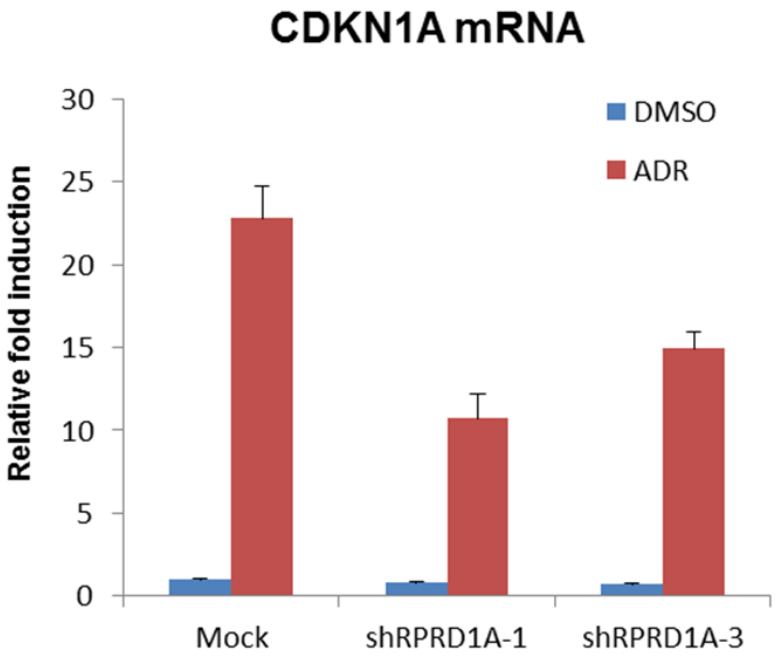

Figure 3-2-2. *RPRD1A* Knockdown inhibits *CDKN1A* mRNA induction under the DNA damage condition.

0.4 μg/ml ADR was treated to each MCF10A cell lines which contain scrambled shRNA (Mock), shRPRD1A-1, and -3 for 16 hours. DMSO was treated to each cell lines as control for ADR. Relative mRNA level was normalized to DMSO treated Mock cell line.

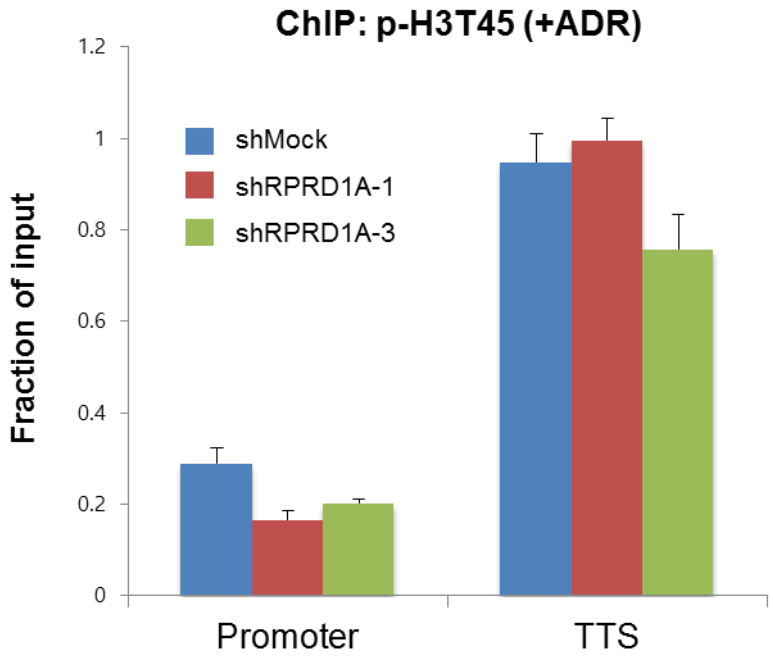

Figure 3-2-3. RPRD1A has no effect on distribution of p-H3T45.

ChIP assay with p-H3T45 antibody in normal (shMock), and *RPRD1A* knock-down cell lines (shRPRD1A-1, -3) against *CDKN1A* promoter and TTS. 0.4μg/ml ADR was treated for 18 hours. The result was normalized with 1% input of whole chromatin.

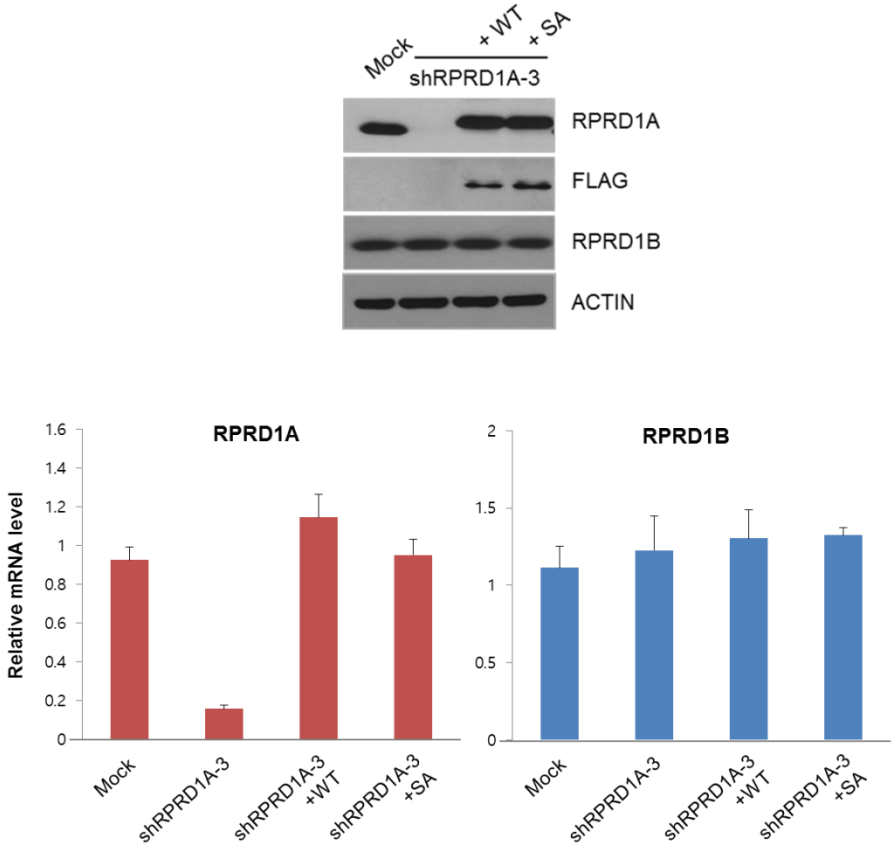

Figure 3-2-4. Generation of wild type and S285A mutant RPRD1A backup cell lines.

FLAG tagged WT and S285A mutant RPRD1A backup in *RPRD1A* knock-down-3 cell line. Each cell lines are selected using blasticidin(10 ㎍/ml) for 4 weeks. Whole cell lysates were probed with indicated antibodies. RPRD1B and ACTIN is used as control. RPRD1A and RPRD1B mRNA level of each cell lines are analyzed by qRT-PCR and are normalized to 18S ribosomal RNA.

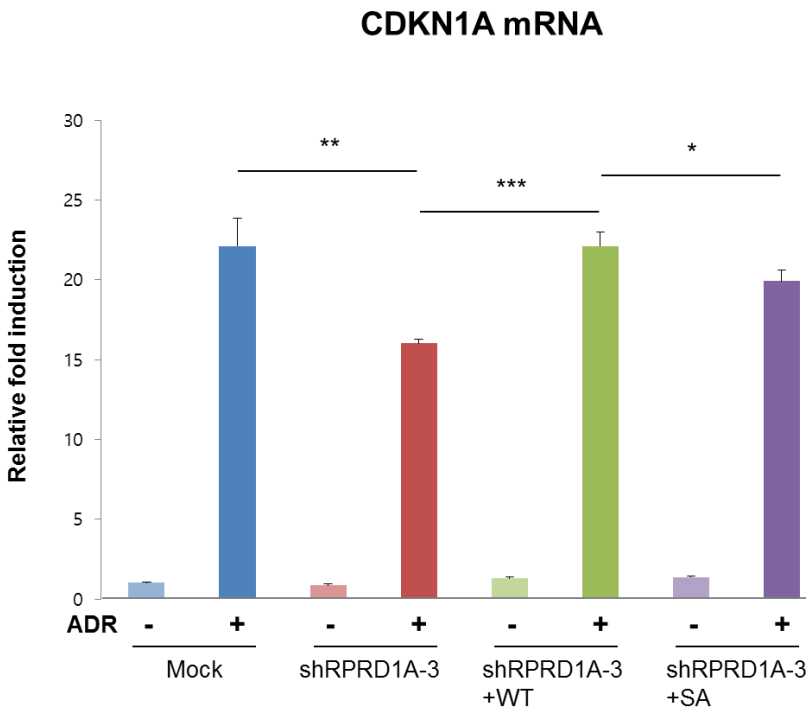

Figure 3-2-5. Wild type RPRD1A backup cell line restores *CDKN1A* mRNA induction under the DNA damage condition.

0.4 μg/ml ADR is treated to normal cell(Mock) and each knockdown and rescued cell lines (shRPRD1A -3, shRPRD1A-3+WT, shRPRD1A-3+SA) for 18 hours. DMSO is treated to each cell lines as control for ADR. Relative mRNA level is analyzed with qRT-PCR and is normalized to Mock cell line, which is treated with DMSO. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

3-3. Phosphorylation of RPRD1A S285 and histone H3-T45 by AKT1 enhances binding affinity to each other.

On our previous report, we verified that H3T45 is phosphorylated by AKT1 under the DNA-damage conditions and its importance to transcriptional termination (Lee et al., 2015). But how p-H3T45 contributes to transcriptional termination needs to be clarified. ChIP-sequencing results showed that p-H3T45 and p-PolII-S2 co-occupy at the TTS region of DNA damage response genes (Lee et al., 2015). Since RPRD1A and RPRD1B are well known binding partners of p-PolII-S2 (Lunde et al., 2010; Ni et al., 2014), we assumed that there might be an interaction between p-H3T45 and RPRD1A/B. Therefore, we performed running-ChIP assay with RPRD1A/B antibody under the DNA-damage conditions. As expected, both RPRD1A and RPRD1B peaked at the TTS region of *CDKN1A* gene locus likewise p-H3T45 (Figure 3-3-1). Next, we tested whether RPRD1A physically interact with histone H3. Mammalian expression vector cloned histone H3-WT, 45th threonine substituted to alanine (TA, non-phosphorylated form) or glutamate (TE, phosphor mimic form), were co-transfected with RPRD1A into HEK293T cells to check for interactions (Figure 3-3-2). Interaction between H3 and RPRD1A was greatly abolished when 45th threonine was substituted to alanine (TA), while it was even more enhanced when 45th threonine was substituted to glutamate (TE). Compared to RPRD1A, interaction of RPRD1B and histone H3 was not affected by substitution of 45th threonine. To test further that the interaction of H3 and RPRD1A is direct, we used in vitro AKT1 kinase assay coupled bind-

ing assay (Figure 3-3-3). His_6-H3 and GST-RPRD1A (WT/S285A) were mixed in a buffer and in vitro kinase assay was performed with/without AKT1. Basal binding level of RPRD1A and H3 wasn't observed, which was increased upon addition of AKT1 protein. The interaction was greatly increased by additional ATP, which showed no difference using RPRD1A S285A mutant. In conclusion, AKT1 mediated phosphorylation of RPRD1A and histone H3 enhances binding affinity to each other and contributes to tight transcriptional termination complex formation.

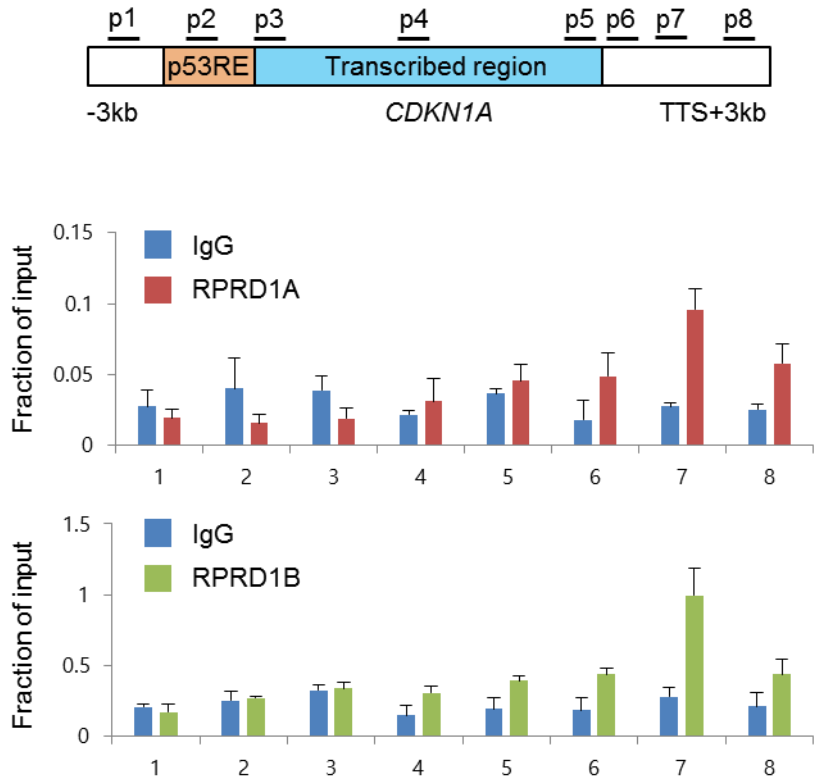

Figure 3-3-1. RPRD1A and RPRD1B show major distribution on TTS locus.

Schematic diagram of *CDKN1A* genomic locus. p1~p8 are primers used in ChIP assay. Localization of RPRD1A and RPRD1B are analyzed by ChIP assay with indicated antibodies and primers at *CDKN1A* genomic locus. In order to induce *CDKN1A* gene, 0.4 μg/ml ADR was treated to MCF10A cells. Primer information is described in Table 2.

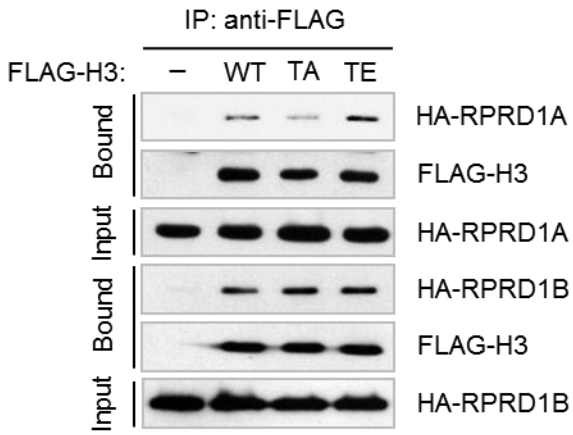

Figure 3-3-2. Binding affinity between histone H3 and RPRD1A depends on substitution of H3T45.

FLAG tagged wildtype H3 or H3-T45A/E was co-transfected with HA tagged RPRD1A/B into HEK293T cells. Whole cells lysate was immunoprecipitated with anti-Flag antibody and probed with anti-HA or anti-Flag antibody.

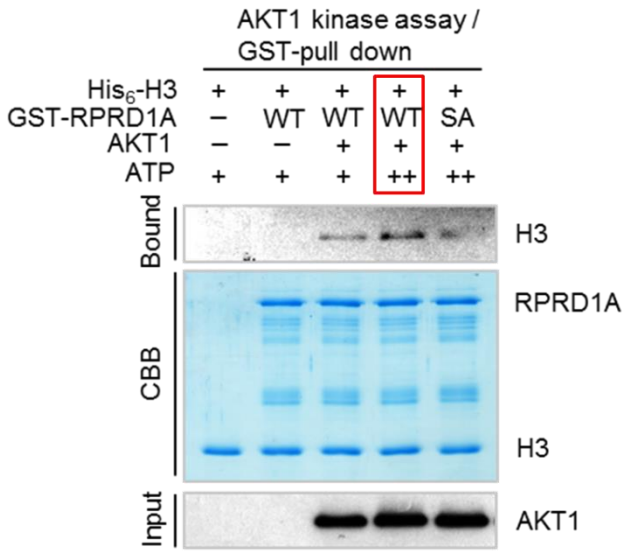

Figure 3-3-3. Phosphorylation of RPRD1A by AKT1 enhances affinity to histone H3.

AKT1 in vitro kinase assay was performed with His-H3 and GST-WT/S285A RPRD1A, with or without AKT. After AKT1 kinase assay, each sample was mixed with GST-sepharose bead and pulled down, and probed with anti-H3 antibody. CBB staining show an amount of substrate used in this assay.

3-4. Ser285 of RPRD1A is critical to transcription termination complex formation at TTS.

To confirm the complex formation of H3 and RPRD1A at TTS, we performed Re-ChIP assay. FLAG tagged WT or S285A RPRD1A was overexpressed in MCF10A cells and their expression was confirmed by western blot analysis (Figure 3-4-1, top). After ADR treatment, RPRD1A overexpressed MCF10A cells were used for binding with FLAG antibody. First bound chromatin products were bound again with p-H3T45 or RPB1 antibody. As a result, RPRD1A co-occupied with p-H3T45 at the TTS of *CDKN1A* genomic locus, while it was significantly abolished by RPRD1A S285A mutant (Figure 3-4-1, bottom, left). Moreover, more Pol II remained at TTS with S285A mutant compared to WT (Figure 3-4-1, bottom, right). These results mean phosphorylated S285 of RPRD1A is important for forming termination complex with p-H3T45 and facilitates dissociation of Pol II from TTS. In other words 285^{th} serine of RPRD1A is important to transcriptional termination.

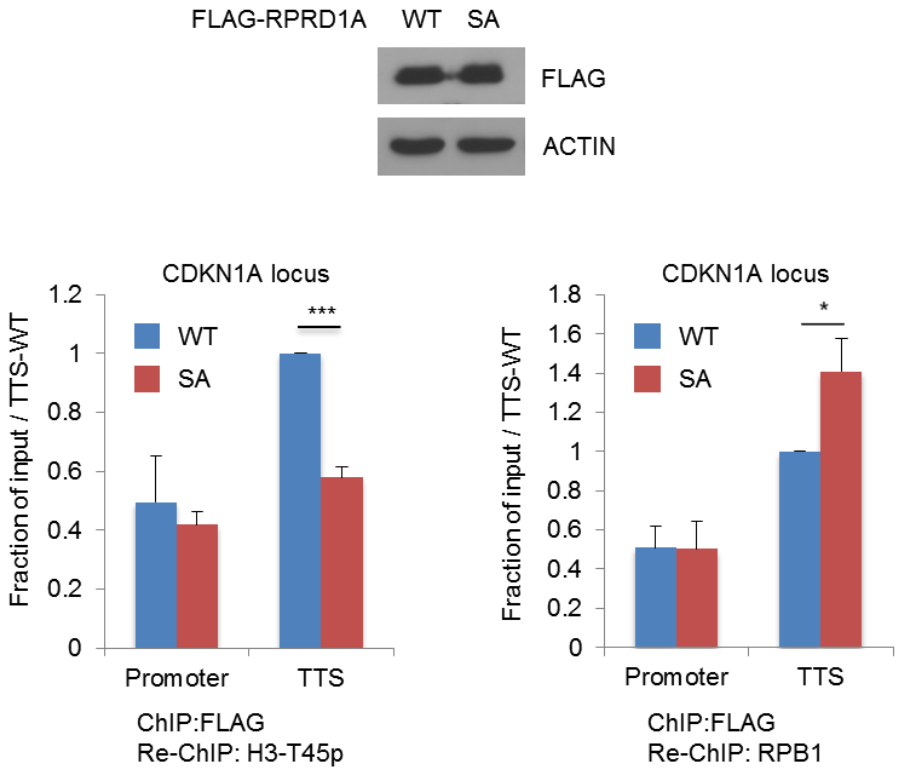

Figure 3-4-1. RPRD1A S285A mutant impedes transcriptional termination complex at TTS.

WT or S285A RPRD1A overexpression in MCF10A cells probed with western blot (top). ACTIN was used as control. ChIP assay was performed with FLAG antibody using chromatin from WT or S285A RPRD1A overexpressed MCF10A cells. FLAG-precipitated chromatin was eluted by competing with FLAG peptides, and re-immunoprecipitated using p-H3T45 (bottom, left), or RPB1 (bottom, right) antibody. ChIP products were analyzed by qRT-PCR on promoter or TTS region of *CDKN1A* genomic locus. The result was normalized with value of TTS in WT cell line.

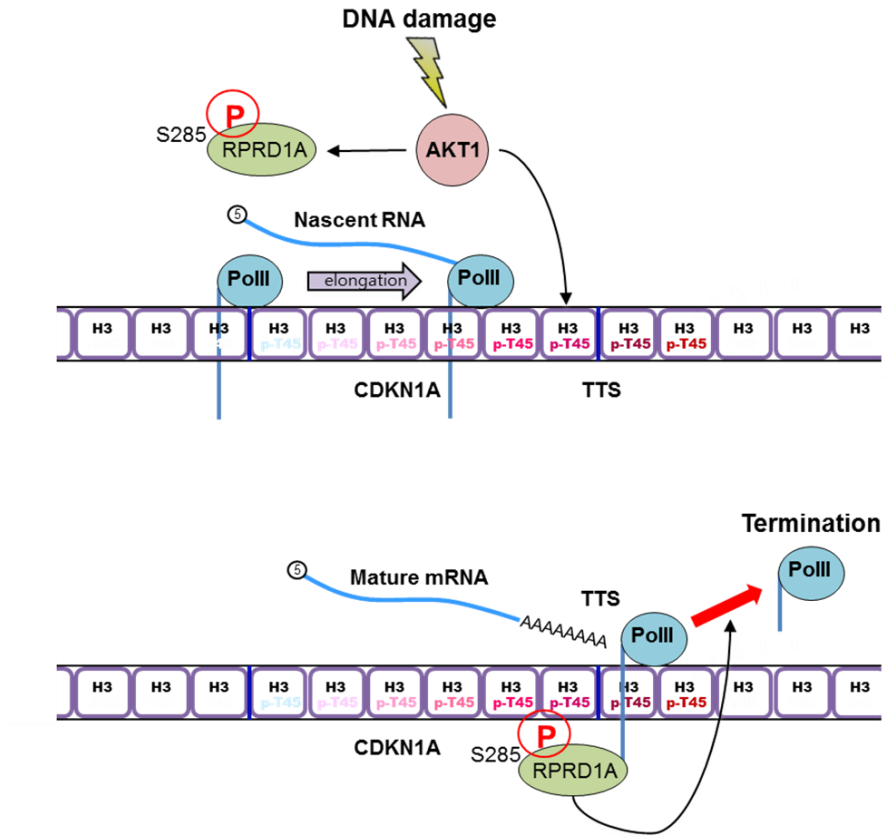

Figure 3-4-2. A schematic model.

Under DNA-damage condition, AKT1 phosphorylates of RPRD1A and H3. Phosphorylated RPRD1A interacts with phosphorylated H3 intensively, thus can be accumulated at TTS. Accumulated RPRD1A facilitates termination, therefore enhances transcription of *CDKN1A*.

IV. DISCUSSION

Diverse post-translational modifications of core histones regulate transcription process (Karlic et al., 2010). But histone modifications related to transcriptional termination are not yet understood. In our previous report, we demonstrated that AKT-mediated H3T45 phosphorylation regulates transcriptional termination of DNA damage response genes under the DNA damage (Lee et al., 2015). However, we could not elucidate how p-H3T45 contributes to transcriptional termination.

In our effort to find the factor which transmits p-H3T45 signal to termination, we focused on transcription termination factors. Among them, we found RPRD1A, a protein which harbors AKT1 target motif. Rtt103, the ancestor of RPRD1A and RPRD1B, is a transcription termination factor which recruits exonuclease Rat1 in yeast (Kim M, 2004). RPRD1A and RPRD1B show alternative functions in human. They function as termination factors by serving as a scaffold for transcription termination factors including RPAP2, the p-PolII-S5 phosphatase (Ni et al., 2014).

To confirm the possibility that RPRD1A functions as a mediator between p-H3T45 and transcriptional termination, we demonstrated that RPRD1A directly interacts with both AKT1 and H3 (Figure 3-1-3 and Figure 3-3-3). RPRD1A was a substrate of AKT1, and the 285th serine was a target site (Figure 3-1-6 and Figure 3-1-7). Although RPRD1A inhibition had no effect on p-H3T45 (Figure 3-2-3), it caused apparent decrease on the mRNA induction of *CDKN1A* (Figure 3-2-2). And WT RPRD1A backup completely restored *CDKN1A* fold induction (Figure 3-2-4). These results mean that decrease in *CDKN1A* mRNA fold induction in *RPRD1A* knockdown cell line is

not caused by variation of p-H3T45, but caused by *RPRD1A* knockdown. However, non-phosphorylated mutant RPRD1A backup cell line showed less effect compared to WT RPRD1A backup cell line (Figure 3-2-4). It means phosphorylated S285 of RPRD1A contributes to *CDKN1A* transcription. We found RPRD1A and p-H3T45 showed similar distribution patterns at *CDKN1A* genomic locus (Figure 3-3-1). Therefore, we focused on the relation of both proteins. We checked interaction of both proteins by using the non-phosphorylated mutant and phosphor-mimic mutant. Phosphorylation of RPRD1A and H3 (or phosphor-mimic mutant) enhanced binding affinity to each other (Figure 3-3-2, 3-3-3). Non-phosphorylated mutant RPRD1A over-expressed cell line showed reduced ability to form termination complex with p-H3T45, and abolished dissociation of Pol II from TTS (Figure 3-4-1). It suggests that phosphorylated RPRD1A is a key factor to form transcriptional termination complex.

In summary, under the DNA damage, AKT1 phosphorylates not only 45^{th} threonine of H3, but also 285^{th} serine of RPRD1A (Figure 3-4-2, top). Phosphorylation of these proteins enhances binding affinity to each other leading RPRD1A accumulation at TTS where p-H3T45 is abundant. It facilitates transcriptional termination, and finally enhances overall transcription of *CDKN1A* (Figure 3-4-2, bottom).

In this study, we reveal novel function of RPRD1A related to transcriptional termination. For efficient transcription, balance between transcription initiation and termination is important. Therefore, when transcription is induced, quick recruiting of transcription termination factors to TTS region is

needed. According to previous research, RPRD1A serves as a scaffold for transcription termination factors (Ni et al., 2014). Accumulation of RPRD1A at TTS region is likely to enhance recruitment of transcriptional termination complex to TTS region. The termination complex might include RPAP2, because RPRD1A interacts with RPAP2 via its CID (Ni et al., 2014). Tight termination complex could provide more chance for RPAP2 to dephosphorylate p-PolII-S5. Elimination of p-PolII-S5 makes turnover from transcriptional elongation to termination. Maybe it could be an answer to the question how p-H3T45 regulates transcriptional termination.

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Ⅵ. ABSTRACT IN KOREAN

국문 초록

핵심 히스톤 단백질에는 전사과정을 조절하기 위해 다양한 전사 후 변형이 일어난다. 본 단체의 사전 연구결과에 따르면, DNA 손상 상황에서 AKT1 은 히스톤 H3 의 45 번 threonine 잔기를 인산화시키고, 이는 전사종결을 빠르게 일어나게 함으로써 해당 유전자의 전사를 촉진시킨다. 하지만 인산화된 히스톤 H3 가 어떻게 전사종결을 증가시키는지에 대한 정확한 기작은 아직 밝혀지지 않았다. 이 연구에서는 효소에서 전사종결 인자인 Rtt103 의 인간 orthologs 인 RPRD1A 와 RPRD1B 가 AKT1 과 결합한다는 것을 확인했다. 또한 RPRD1A 는 RPRD1B 와 달리 C 말단의 coiled-coil 구조 내부에 AKT1 이 인산화 시킬 수 있는 잘 보존된 위치(285 번 Serine)가 있다. *RPRD1A* 를 knockdown 시킨 세포주에서 DNA 손상을 유도하였을 때, 히스톤 H3 45 번 threonine 잔기의 인산화에는 영향이 없었지만, *CDKN1A* 의 전사증폭은 감소하였다. AKT1 에 의한 히스톤 H3 45 번 threonine 잔기와 RPRD1A 285 번 serine 잔기의 인산화는 서로에 대한 결합세기를 증가시켰다. 또한 285 번 serine 잔기에 인산화가 되지 않도록 돌연변이를 일으켰을 때, RNA 중합효소 II 가 chromatin 으로부터 잘 분리되지 않는 다는 사실을 확인 할 수 있었고, 이는 정상적이지 않은 전사종결을 일으키고 그로 인한 전사효율 감소를 일으키는 것을 확인하였다.

위의 사실들을 종합하면, 전사종결과정에서 RPRD1A 가 히스톤 H3 45 번 threonine 잔기의 인산화 신호를 전사종결과정에 전해준다는 새로운 기작을 제안할 수 있다.

주요어: RPRD1A, AKT1, 전사종결, DNA 손상

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