이학박사학위논문

5′-3′ exoribonuclease Rat1 에 의한 RNA 중합효소 II 의 전사 종결 기작의 특징 규명

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2015 년 8 월

서울대학교 대학원

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Unraveling mechanistic features of RNA polymerase II termination by the 5´-3´ exoribonuclease Rat1

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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To the Faculty of
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by

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ABSTRACT

Unraveling mechanistic features of RNA polymerase II termination by the 5´-3´ exoribonuclease Rat1

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The 5'-3' exoribonuclease Rat1 promotes termination of RNA polymerase II (RNAPII) on protein-coding genes, but its underlying molecular mechanism is still poorly understood. Using *in vitro* transcription termination assay, I have found that RNAPII is prone to terminate more effectively by Rat1/Rai1 when its catalytic site is disrupted due to NTP misincorporation, proposing that paused RNAPII often found *in vivo* near termination sites might adopt similar configuration for Rat1 to trigger termination. Intriguingly, Rat1 does not terminate *E. coli* RNAP, implying that specific interaction between Rat1 and RNAPII may also contribute to termination. Furthermore, the efficiency of termination increases as the RNA transcript being degraded by Rat1 gets longer. It suggests that Rat1 may generate a driving force for dissociating RNAPII from the template while degrading the nascent transcripts to

catch up the polymerase. These results indicate that multiple mechanistic features contribute to Rat1-mediated termination of RNAPII.

Keywords: Transcription, RNAPII, Termination, 5´-3´ exoribonuclease, Rat1/Rai1, Rtt103

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LIST OF ABBREVIATIONS

TAP	Tandem affinity purification
ChIP	Chromatin Immuno Precipitation
RNAP	RNA polymerase
EC	Elongation complex
CTD	Carboxyl-terminal domain
CID	Carboxyl-terminal domain-interacting domain
Ser2P, Ser5P, Ser7P	Serine 2 phosphorylation, Serine 5 phosphorylation, Serine 7 phsophorylation
PIC	Pre-initiation complex
OC	Open-complex
p(A) site	Polyadenylation site
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetate
EtBr	Ethidium bromide
IPTG	Isopropyl-β-D-thiogalactopyranoside
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethanesulfonylfluoride
SDS	Sodium dodesyl sulfate
PCR	Polymerase chain reaction
5-FOA	5-fluoroorotic acid

1. INTRODUCTION

1.1. RNA Polymerase II and transcription process

In eukaryotes, there are three classes of RNA polymerases: RNAPI transcribes rRNAs, RNAPII transcribes mRNAs and majority of non-coding RNAs (sn/snoRNA, miRNA, CUTs, SUTs), and RNAPIII transcribes 5S rRNA and tRNAs. The RNA polymerases are multisubunit proteins and structurally well conserved each other (Fig.1.1) (1).

RNAPII consists of ten-subunit core and peripheral heterodimer of subunits Rpb4 and Rpb7 which are dissociable from core subunits (Table 1.1) (1). RNAPII has four different mobile elements: core, clamp, shelf, and jaw lobe. The cleft lies in the center of the enzyme where incoming DNA penetrates into the active site (2) (Fig 1.2). The structural study reveals that double-stranded DNA enters the main cleft but cannot proceeds further, rather RNAPII unwinds DNA and single-stranded DNA enters deep into the cleft and reaches active site. It shows 8-9 base pairs of DNA-RNA hybrid lies in the active site and 3' end of RNA is positioned above a pore liked to funnel, implying the backtracking of RNAPII for proofreading of RNA extension (3) .

The core RNAPII has two different states: pre-initiation complex (PIC) and open-complex (OC). PIC is analogous to the bacterial closed complex and with all general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) bind to the promoter to initiate transcription at the start site (Fig1.3) (4). Recognition of the promoter elements by transcription machinery is essential for recruitment of general transcription factors and assembly of PIC. TFIIB (TBP) binds minor groove of an 8bp-TATA element and serve as a platform for binding other components of PIC.

After PIC is assembled, it is poised before the conversion into transcriptionally active state and moves on to the next step using ATP-dependent helicase activity (5). ATP-dependent helicase (XBP), a subunit of TFIIH, binds to the downstream DNA and initiates unwinding of DNA, which is known for promoter melting. Since

structure of XPB-DNA interaction is now solved yet, the detailed underlying mechanism is not elucidated.

Open-complex escaped from promoter starts to elongate mRNA moving along the DNA template. Elongation is achieved by repetitive cycles of NTP additions. The Elongation-complex (EC) consists of short duplex DNA and single strand RNA emerging from the active site which forms transcription bubble inside of RNAPII.

Table 1.1. RNA polymerase subunits

RNA polymerase	RNAP I	RNAP II	RNAP III
Ten-subunit core	A190 A135 AC40 AC19 A12.2 Rpb5 (ABC27) Rpb6 (ABC23) Rpb8 (ABC14.5) Rpb10 (ABC10α) Rpb12 (ABC10β)	Rpb1 Rpb2 Rpb3 Rpb11 Rpb9 Rpb5 Rpb6 Rpb8 Rpb10 Rpb12	C160 C128 AC40 AC19 C11 Rpb5 Rpb6 Rpb8 Rpb10 Rbp12
Rpb4/7 subunits	A14 A43	Rpb4 Rpb7	C17 C25
TFIIF-like subcomplex	A49 A34.5	(Tfg1/Rap74) (Tfg2/Rap30)	C37 C53
Pol III-specific subcomplex			C82 C34 C31
Number of subunits	14	12	17

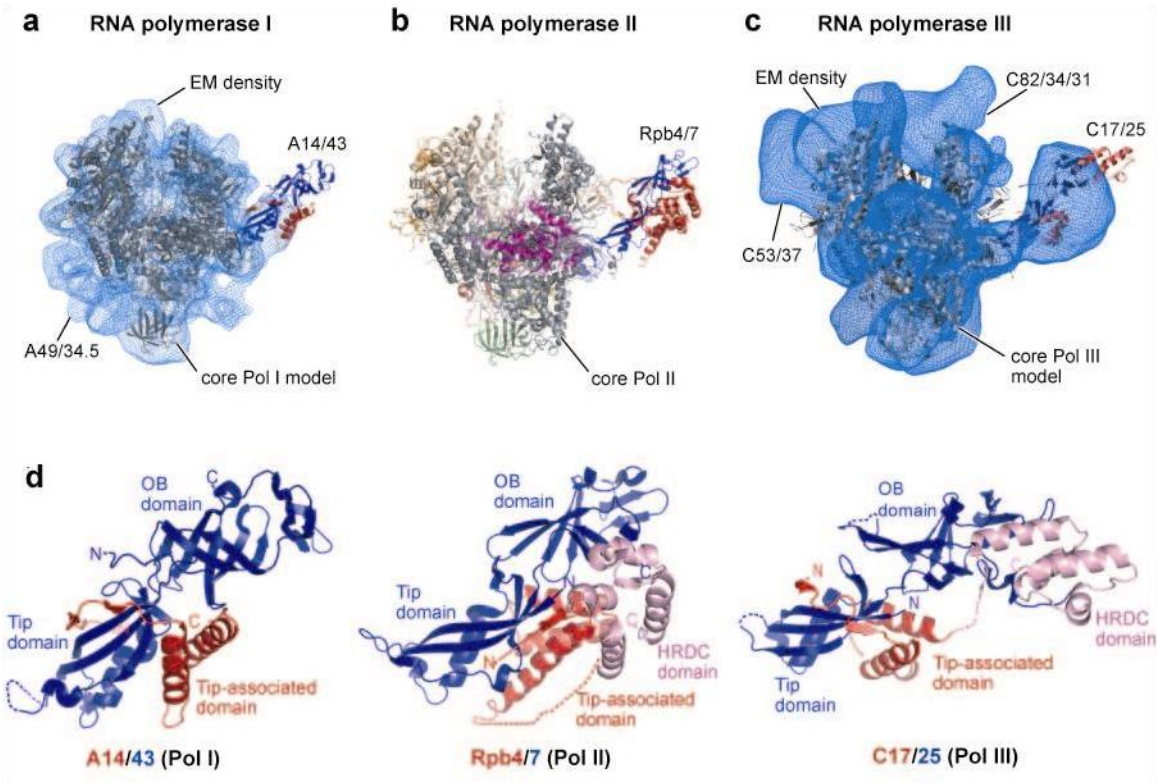


Figure 1.1. Structurally conserved ten core subunits of eukaryotic RNA polymerases (adapted from Cramer *et al*, 2008) (1)

Structures of multisubunit RNAPI, II and III composed of 14, 12 and 17 subunits in yeast, respectively. There are ten conserved core subunits and additional subunits located on the periphery. (a) RNAPI hybrid structure. (b) Ribbon model of complete RNAPII crystal structure. (c) RNAPIII EM structure. (d) Rpb4/7 subcomplex structures: A14/43 (left), Rpb4/7 (center), C17/25 (right)

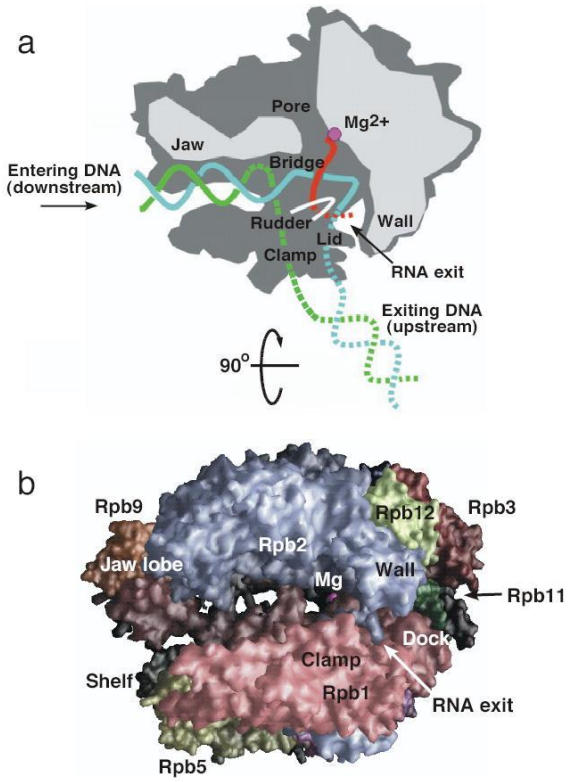


Figure 1.2. Structure of RNAPII elongation complex (adapted from Hahn, 2004) (2)

(a) Top view of RNAPII with the DNA template strand (blue), nontemplate strand (green), RNA (red) and active site Mg (Magenta). (b) Side view of RNAPII looking into the active site cleft.

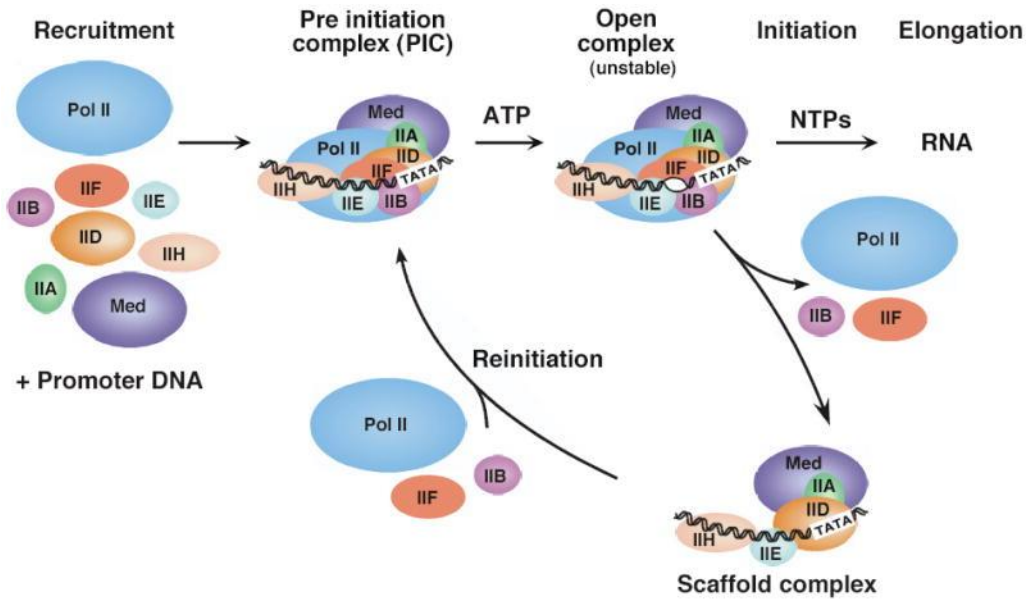


Figure 1.3. Cycle of transcription initiation and reinitiation for RNAPII (adapted from Hahn, 2004) (2)

RNAPII and transcription general factors are recruited to promoter regions. Once preinitiation complex (PIC) is assembled, it undergoes transition state before conversion into open complex. Using ATP, unstable open complex is formed and escapes from promoter region to initiate transcription. General transcription factors remaining behind at the promoter after initiation keep scaffold complex and bind next RNAPII to reinitiate transcription cycle.

An EM analysis reports that the transition between open-complex and PIC depends on the movement of jaw domain of RNAPII (6).

1.2. Importance of transcription termination study

The definition of transcription termination is that RNAPII is dissociated from DNA template after the elongating RNAPII recognizes termination signal such as poly(A) site.

Accurate transcription termination is crucial because early or late termination may disrupt normal gene regulation and produce abnormal RNAs, which may be harmful to cellular fitness. Recent studies show that when human non-coding RNA termination factor senataxin is mutated, severe neurological defects such as recessive disorder ataxia with oculomotor apraxia type 2 (AOA2) are observed in mouse and human (7). MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) patients show mutations occurred within the mtDNA binding site of mTERF protein which promotes transcription termination at 16S rRNA/tRNA (8). Also, mutations in anti-terminator factor *Tat* are responsible for postintegration latency of Human Immunodeficienty Virus (HIV) (9). These results suggest that fine tuning of transcription termination is essential for the life of organisms.

1.3. Two distinct termination pathways: for mRNAs vs non-coding RNAs

In *Saccharomyces cerevisiae*, there are at least two distinct pathways for RNAPII termination (10). One pathway involves Nrd1/Nab3/Sen1 complex to terminate non-coding RNA transcription and is independent of cleavage of nascent RNA transcripts (11,12). In contrast, the other termination pathway for most protein-coding genes requires cleavage of nascent transcripts by cleavage/polyadenylation factors, and RNA degradation from the newly formed 5’ phosphorylated end by 5’- 3’ exoribonuclease Rat1 which promotes RNAPII termination (13,14) (Fig 1.4.a). Rat1 itself strongly supports Torpedo model which postulates an essential role for RNA

cleavage and thereby generating an entry site for 5'-3' exoribonuclease to trigger termination. Supporting this notion, previous study shows that RNA cleavage factors (Rna14, Rna15 and Pcf11) but not polyadenylation factors (Pap1, Fip1 and Yth1) are required for termination (15).

When elongating RNAPII recognizes poly(A) signal, it slows down and polyadenylation and cleavage factors (CPF, CF1A/CF1B complex and Pap1) are recruited to the polymerase. After cleavage and polyadenylation of premature RNA, it is exported to cytoplasm to be translated. Then, the remaining 5' phosphorylated nascent RNA is removed not to create defective mRNAs by Rat1 which rapidly degrades RNA from 5'-3' direction catching up the slow-moving RNAPII. Once Rat1 approaches the surface of RNAPII, it somehow docks on polymerase and dissociates it from the DNA template. The detailed mechanism about how Rat1 dissociates RNAPII is still uncovered (Fig 1.4.b)(13).

1.4. The role of Rat1 and characterization of its interacting partners

The yeast Rat1 is an essential nuclear protein and evolutionally well conserved from yeast to human (Xrn2 in human) (14,16,17). Rat1 is 116kDa-dize and recognizes monophosphate of single-strand RNA and processively degrades it. Rat1's human homologue Xrn2 is also nuclear exoribonuclease and specifically localizes in nucleolar. It plays a role in not only termination of mRNA but also both maturation of rRNA and degradation of various discarded pre-rRNA species (18).

Rat1's cytoplasmic counterpart Xrn1 in yeast is also a processive 5'-3' exoribonuclease. Xrn1 regulates RNA turnover by degrading wide range of cytoplasmic RNAs, including noncoding RNAs and Nonsense-Mediate Decay (NMD) substrates. Yeast Xrn1 especially degrades long non-coding RNAs (lncRNAs), called Xrn1-senstivie unstable transcripts (XUTs) (19). Interestingly, a study shows that engineered Xrn1 having nucleus localizing signal (NLS) can enter nucleus and complements growth defect of *rat1-1* mutant. Rat1 which has lost NLS signal is

localized to cytoplasm instead of nucleus. This suggests that Rat1 and its counterpart Xrn1 are functionally interchangeable and its localizations are tightly linked to its function in transcription termination (20).

Rat1 acts as a complex with Rai1 that confers stability on Rat1 and helps to target 5' monophosphate RNA by its pyrophosphohydrolase activity (21) (Fig 1.5). Rai1 in yeast is homologous to Dom3Z in human and Dxo1 in *Kluyveromyces lactis*. Interestingly, unlike Rai1, hDom3Z and Dxo1 show decapping and 5'-3' exoribonuclease activities. Especially, hDom3Z preferentially degrades defectively capped pre-mRNAs *in vivo* (22,23). Generally, 5'-end capping was believed to proceed to completion and does not require quality-control mechanisms to maintain the fidelity of the 5' end cap. However, recent structural studies clearly show that termination factor is involved in quality control function of incomplete 5'end capping of mRNA, thereby providing possible link between 5'end RNA processing and transcription termination.

Not only possible role of Rai1 homologue at 5'end of gene but also localization of Rat1 at the region implies that Rat1-dependent transcription termination could occur upstream the polyadenylation sites (13). Supporting this, rat1-dependent termination is observed in the capping-deficient *ceg1-63* strain (24) and, in mammals, Xrn2 contributes to the maturation of 5'end of rRNAs by degrading improperly processed intermediates (18). All of these results suggest that Rat1-mediated termination could be linked to not only properly processed mRNA but also improperly processed pre-mature RNAs in yeast and mammals.

Another interacting protein Rtt103 has a CID (RNAPII C-terminal domain-interacting domain) which might facilitate the access of Rat1 to RNAPII via interaction with Ser2-phosphorylated CTD (13,25). Numerous studies, over the past decades, have reported the tight relationships between CTD phosphorylation and transcription process (Fig 1.6). The largest subunit of RNAPII (Rpb1) has C-terminal domain which consists of multiple repeats of the heptamer sequence YSPTSPS. The

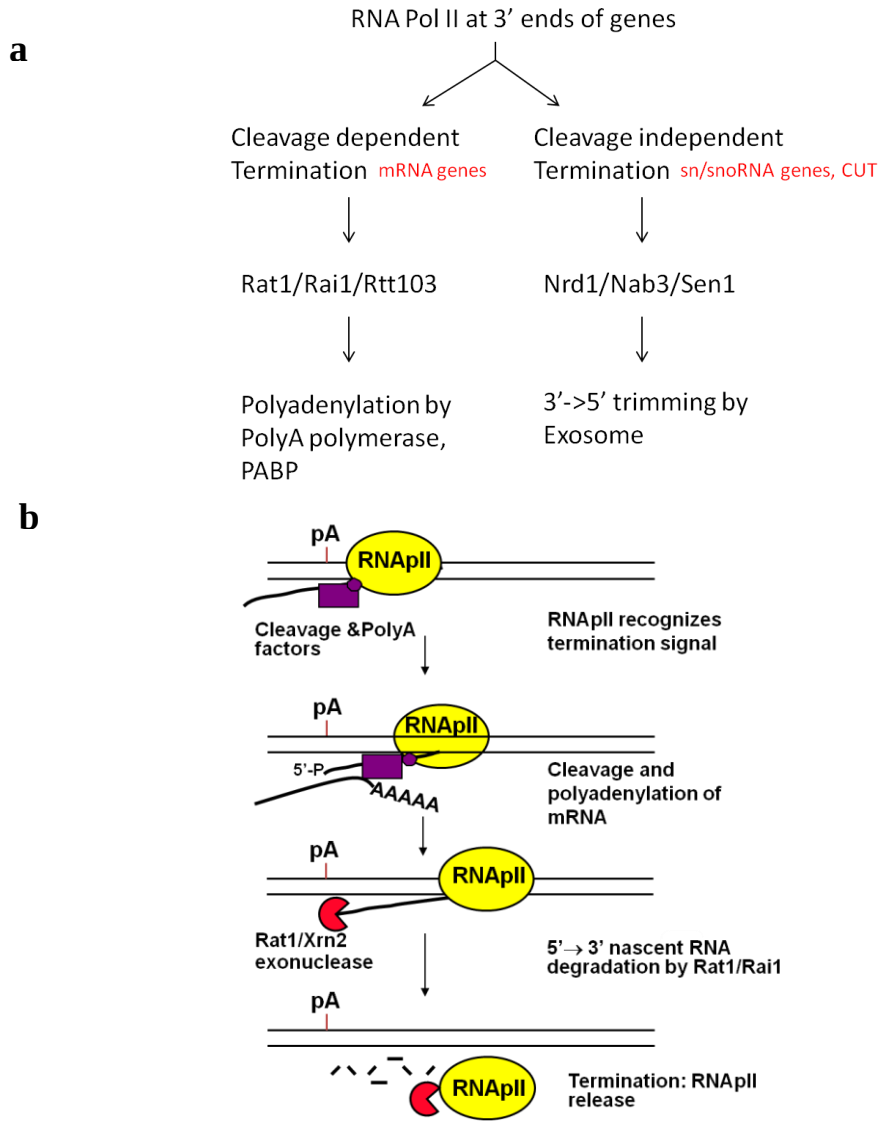


Figure 1.4. Decision tree of termination pathway and Torpedo model (13)

(a) Two distinct termination pathways for mRNAs and non-coding RNAs. mRNA genes are terminated cleavage dependently whereas non-coding RNAs are terminated cleavage independently. Rat1/Rai1/Rtt103 plays a role in cleavage-dependent pathway and Nrd1/Nab3/Sen1 does in cleavage-independent pathways. (b) Rat1 acts like a torpedo catching up slow-moving RNAPII degrading 5' phosphorylated nascent RNA, and dislodge RNAPII from DNA template.

number of repeats varies from organism to organism: 26 (all consensus) repeats and 52 (21 consensus and 31 non-consensus) repeats of YSPTSPS in yeast and mammals. Serine2, Serine 5 and Serine 7 are the major sites of CTD phsohporylation. Generally, towards 3' end of gene body, Ser5P level drops and Ser2P level increases, and consequently recruitment of termination and RNA processing factors are increased, too. Ser2P-binding protein Rtt103 is co-purified with termination complex Rat1/Rai1 through TAP purification (13). Rtt103 interacts with another CID-harboring polyadenylation factor Pcf11 and their cooperative binding to CTD repeats is facilitated when the Ser2P level is the highest near at 3' end of gene. This Rtt103-Pcf11 interaction provides another level of tight regulation for Rat1-mediated termination confined only to proper termination and/or 3' processing sites (26).

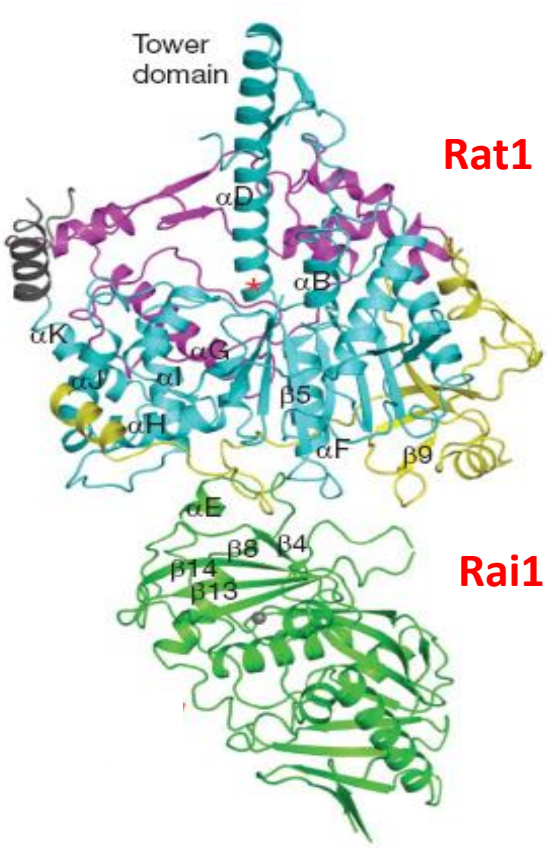


Figure 1.5. Structure of Rat/Rai1 complex *in S.pombe* (adapted from Xiang *et al*, 2009) (21).

Crystal structure of *S.pombe* Rat1/Rai1 complex at 2.2Å resolution. Solved Rat1 structure contains residues from 1-885 amino acids, missing the last 106 residues that does not affect cell viability. Rai1 is solved as full-length structure. Active site of Rat1 is indicated with the red star and a bound divalent cation in the active site of Rat1 is shown as a grey sphere. Helix αD domain is called tower domain contributes several conserved residues to the active site. Since Xrn1 does not have this domain, the tower domain may be unique feature for Rat1 for RNAPII termination.

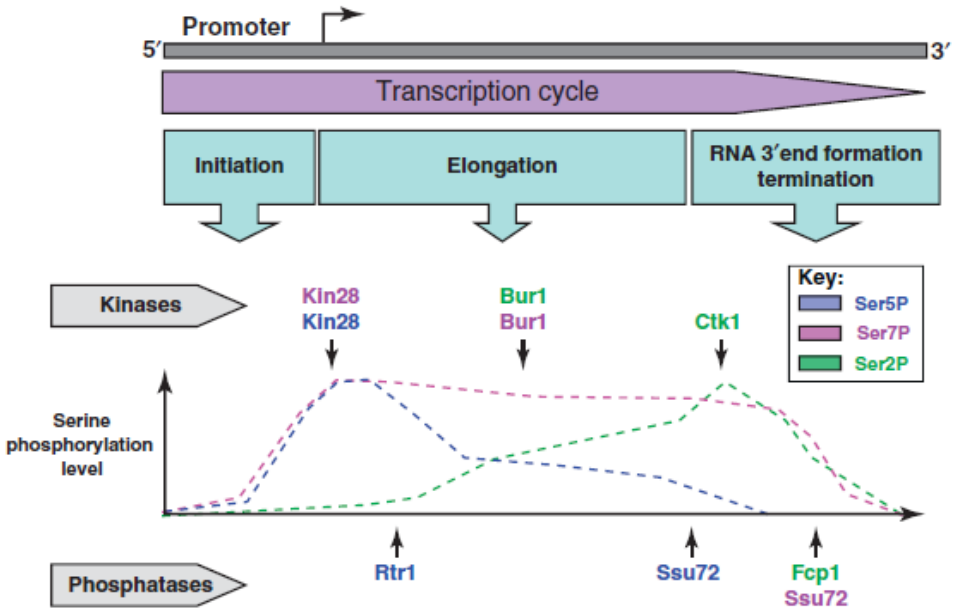


Figure 1.6. Coupling of transcription and CTD phosphorylation pattern (adapted from Egloff *et al*, 2012) (27).

The carboxyl-terminal domain (CTD) phosphorylation pattern across yeast genes. The average level of Ser2P, Ser5P and Ser7p relative to RNA Polymerase II level detected on protein-coding gene during the different steps of the transcription cycle is represented. The kinases and phosphatases responsible for establishing these patterns are noted above and below, respectively.

1.5. Previous studies on Rat1/Rai1/Rtt103 complex regarding transcription termination in-vitro

Although there is a big emphasis on the role of Rat1/Rai1/Rtt103 in transcriptional termination *in vivo*, its molecular mechanism of how Rat1 actually dissociates RNAPII from DNA template is still unclear. A recent study adapting scaffold transcription elongation complex insisted that Rat1/Rai1 itself is not sufficient to terminate RNAPII *in vitro* It also shows that Rtt103 does not influence the exoribonuclease activity of Rat1 arguing that the role of Rtt103 would be dispensable for termination *in vivo* (28).

However, another *in vitro* study using promoter-driven elongation complex showed that Rat1/Rai1 released stalled RNAPII in the absence of other factors. The study agrees with that Rtt103 is not essential for termination but it probably plays a redundant role in mediating interaction between Rat1 and RNAPII based on the result that Rtt103 restores termination capability in exonucleolytically deficient rat1 D235A mutant (25). Thus, what Rat1 requires to promote termination and how it triggers RNAPII dissociation still remain obscure.

1.6. Characteristics of RNAPII movements: pausing, backtracking and reactivation

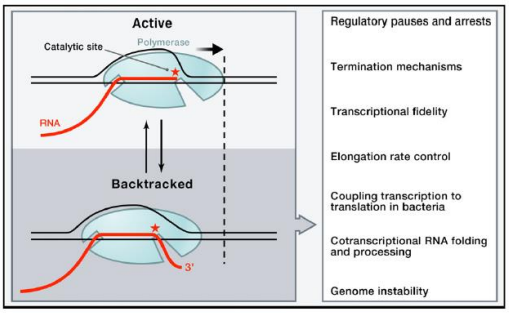
To elucidate the molecular mechanism of Rat1-mediated RNAPII termination, studying mechanistic features of RNAPII movement in depth is necessary. Old study adapting systematic analysis of transcription elongation complexes reveals that irregular DNA footprinting (29), suggesting that RNAP may adopt inchworm movement during the elongation. However, the later study shows that irregular footprints occur only at certain DNA sites, whereas the majority of DNA positions display relatively monotonic movement of RNAP in *E.coli* (30). Since then it has been uncovered that RNAPII moves by Brownian motion rather than by ATP-driven

power-strokes, and thereby frequent pausing, backtracking and transcriptional arrest are commonly observed.

Promoter-proximal pauses are one class of RNAP backtracking events. It is often observed in widespread, especially in highly active and regulated genes (31-34). The long-lasting pausing is crucial for transcriptional regulation of many genes and in RNA processing.

Besides promoter-proximal pausing, eukaryotic RNAPII often shows sequential events of pausing, backtracking and reactivation during elongation. This phenomenon is closely related to the transcriptional fidelity, elongation rate control and termination mechanisms (Fig 1.7.a). Backtracking of bacterial and eukaryotic RNAPs is triggered by weak DNA-RNA hybrid. A recent study solves crystal structure of backtracked yeast RNAPII and revealed that arrested polymerase is reactivated by transcription factor IIS (TFIIS), which promotes intrinsic RNA cleavage by RNAPII itself (Fig 1.7.b) (35). In the arrested state, eight nucleotides of backtracked RNA bind backtrack site beyond the gating tyrosine in the pore and funnel, trapping the active center trigger loop and inhibiting mRNA elongation. In the reactivation state, TFIIS locks the trigger loop away from the backtracked RNA, dislodges RNA from the backtrack site and complements the active site with a basic and two acidic side chains. This promotes intrinsic cleavage of RNAPII and consequently release backtracked RNA creating a new RNA 3’ end at the active site. Therefore, RNAPII gets ready to elongate.

a



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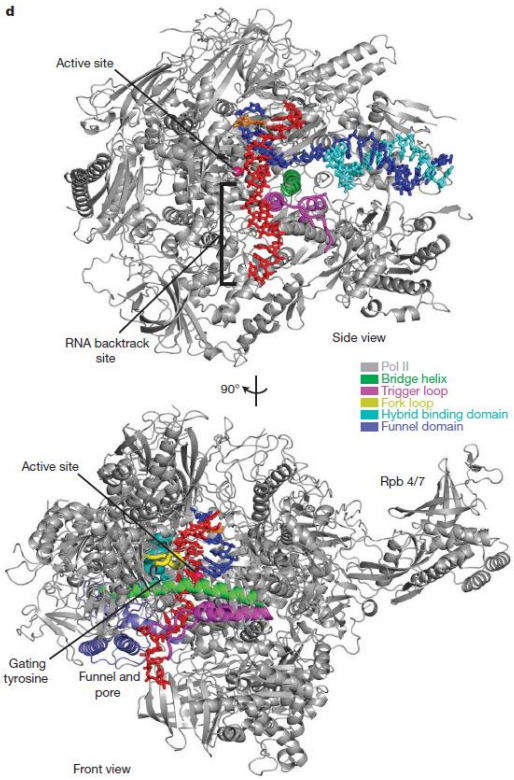

Figure 1.7. RNAP backtracking scheme and crystal structure of backtracked yeast RNAPII (adapted from Cheung *et al*, 2011 and Nudler, 2012) (35,36).

(a) Schematic illustration of the ternary elongation complex in active and backtracked configurations. The catalytic site (star) looses the 3'-OH end of RNA (red), which is extruded through the secondary channel during backtracking. Backtracking is always happened with bacterial and eukaryotic RNA polymerases. (b) Backtracking is triggered by a weak DNA/RNA hybrid. Side and front views of the arrested RNAPII crystal complex structure refined at 3.3 Å resolution with functional elements highlighted. 13-bp of downstream DNA, 6-bp hybrid and 9 nucleotides of single-stranded 3' RNA that is extruded through the pore and the funnel in secondary channel. Color code for each domain is used.

1.7. RNAPII pausing induced by NTP misincorporation via template misalignment.

The next question now will be how RNAPII pausing, backtracking and reactivation are achieved. One of major factors for RNAPII pausing is NTP-misincorporation via template misalignment. As previously described, nucleotides are incorporated into active site of polymerases in repetitive cycle. NTP misincorporation leads to slow addition of next nucleotides and cleavage of mismatched RNA 3'end by RNA polymerase stimulated by Gre factors or TFIIS in bacteria and yeast, respectively. In other words, to maintain fidelity, RNA polymerase discriminates nucleotides against the wrong one and recognizes and removes mismatched nucleotides through pausing and backtracking. During this process, pausing and backtracking is inevitable steps. In bacterial EC, a mismatched RNA 3'end is removed by cleavage-stimulatory Gre factors (37). In human EC, a mismatched RNA 3' end nucleotides cause slow addition of the next nucleotides and cleaved by TFIIS (38).

According to recent works on T7 RNAP, *E.coli* RNAP and yeast RNAPII, a non-cognate NTP complementary to the n+1 template DNA base (n+1 NTP) can be incorporated through template misalignment, leading to temporary flipping-out of n DNA base to extrahelical position. With mismatched nucleotides, RNA transcript can be extended after realignment of template DNA (Fig.1.8.). This misalignment mechanism seems universal for all DNA-dependent RNAPs including bacterial RNAP (39,40).

The average frequency of NTP misincorporation keeps relatively low for eukaryotic RNAPII ($\sim 10^{-5}$) (41), since Rpb9 and TFIIS stimulate the proofreading cleavage activity of RNAPII (42-45). Supporting these results, deletion of *RPB9* or TFIIS (*DST1*) gene significantly decreases transcriptional fidelity of yeast RNAPII (46). NTP misincorporation would arrest elongation complex and/or induce transient catalytic inactivation of elongation complex (38,47).

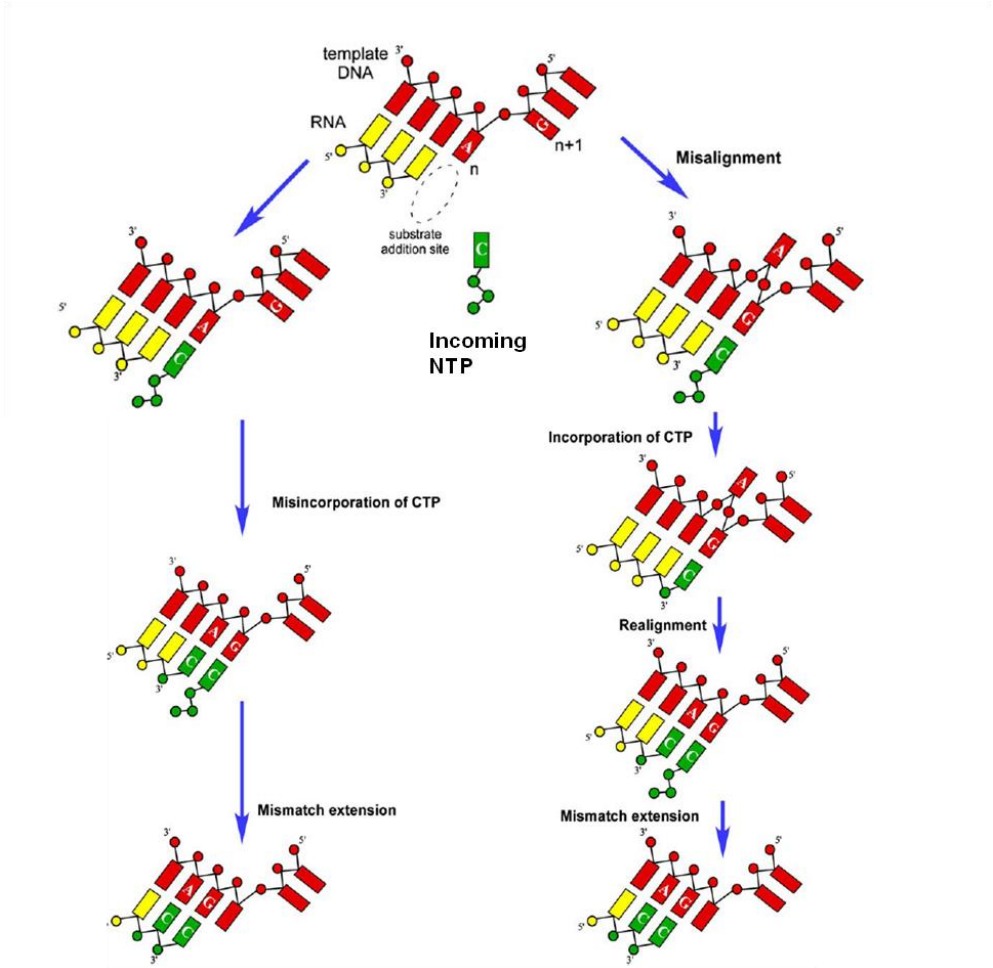

Figure 1.8. NTP misincorporation via template misalignment scheme (Kashkina *et al*, 2006) (39)

The template base that is paired with the 3' end of the RNA in designated as n-1, and the template base in the substrate site as n; template bases further downstream are designated n+1, n+2 *etc*. The noncognate substrate complementary to the n+1 base is termed the n+1 NTP. In the old misincorporation model (left), the n+1 NTP (CTP) is misincorporated opposite the n base, resulting in a mismatch at the 3' terminus of the RNA; this is followed by mismatch extension by correct incorporation of CTP opposite the n+1 base. In the new NTP misincorporation via template misalignment model (right), misalignment of the T strand allows incorporation of CTP opposite the n+1 base; this is followed by realignment of the primer/template and mismatch extension as above. In both cases, the result is a substitution error in the RNA product.

1.8. Sequence-specific RNAPII pausing

Not only template misalignment-induced pausing, but also sequence-specific pausing occurs in various RNAPs. In case of yeast RNAPII, mismatched T·U wobble base pair of DNA/RNA hybrid induces disruption of the catalytic site, resulting in RNAPII pausing and backtracking (Fig 1.9) (47).

In addition, bacterial and *T. thermophilus* RNAPs also show sequence-specific pausing, depending upon the incoming NTP in active sites (48,49). So far, several studies report that RNAPII pausing is linked to and stimulates termination by Rat1/Xrn2 (50,51), but other than its collaborative feature with a poly (A) signal, how the RNAPII pausing contributes to Rat1-mediated termination remains unknown.

1.9. Aims of this study

Transcription termination is tightly regulated process and numerous factors are involved to complete accurate dissociation of RNAPII from the DNA template. In *Sacchromyces cerevisiae*, mRNA transcription termination pathway involves Rat1/Rai1/Rtt103 complex, which supports Torpedo model requiring cleavage of premature RNA and subsequent degradation of nascent RNA by 5'-3' exoribonuclease. ChIP assays and several *in vitro* studies reveal that Rat1/Rai1/Rtt103 is required to promote transcription termination but the detailed molecular mechanism is still not fully understood.

Here, I show using *in vitro* assays that when NTP misincorporates, RNAPII becomes catalytically disrupted and is more efficiently terminated by Rat1/Rai1, proposing that pausing of RNAPII often found *in vivo* near termination site might generate a similar configuration to facilitate termination by Rat1/Rai1. Although NTP misincorporation also catalytically disrupts *E. coli* RNAP, Rat1 does not terminate it, indicating that specific interaction between Rat1 and RNAPII may be also important to induce termination. Additionally, I found that RNAPII termination efficiency depends upon the length of RNA transcript being degraded by Rat1, which suggests

that Rat1 may generate a driving force to dislodge RNAPII while degrading the nascent transcripts to catch up the polymerase.

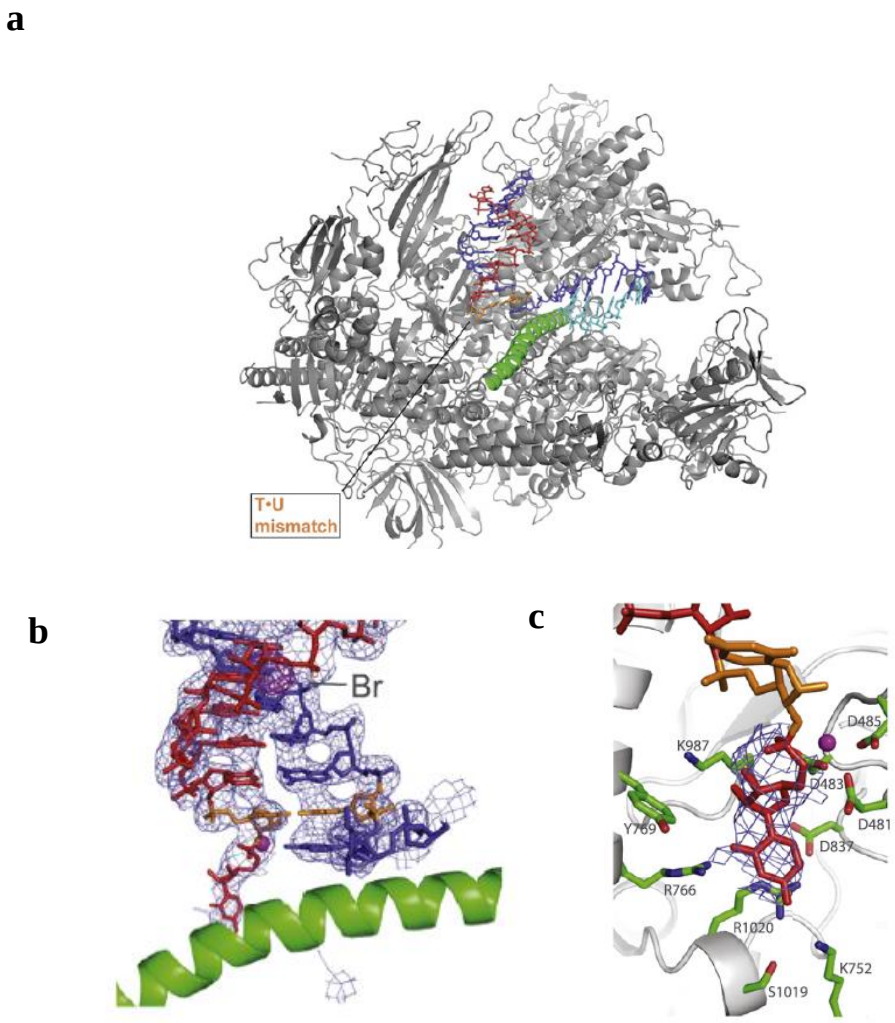

Figure 1.9. T·U wobble base pairing and frayed 3'end nucleotides of RNA (Sydow *et al*, 2009) (52)

(a) Structure of the T·U mismatch-containing RNAPII EC. RNAPII is shown from the side as a ribbon model in silver, with the bridge helix highlighted in green and a portion omitted for clarity. The nucleic acids are shown as stick models using color code. The T·U mismatch is shown in orange. (b) Structure of RNAPII EC with frayed 3' terminal RNA uridine at 3.6 Å resolution. The electron density is shown as a blue mesh. The location of the bromine atom at position -5 defines the register. (c) Detailed view of frayed site. RNAPII residues contact with the frayed 3' terminal RNA uridine.

2. MATERIALS AND METHODS

2.1. Strains and plasmids construction

The gene encoding for *Saccharomyces cerevisiae* Rat1, Rai1, and Rtt103 were cloned into pET21b (Novagen) tagged with hexahistidine (6xHis) (gifts from P. Cramer Ludwig-Maximilians Universität, Munich, Germany). Human Xrn2 (hXrn2) was cloned from cDNA (Harvard DNA Resources) into pET21b tagged with 6xHis. Catalytic mutant of rat1 (E203A, D233A, D235A) and rai1 (E221A, D223A) were prepared by site-directed mutagenesis. Xrn1 was purchased from NEB (cat# M0338S).

The rat1 E203A/D233A/D235A (rat1EDD) mutant was generated by PCR mutagenesis and cloned into either pET21b or pRS41H. Rtt103 was cloned into pRS415 or pRS423 and transformed into a yeast strain (rat1Δ::KanMX/pAJ202-Rat1/pRS41H-rat1EDD) to test whether extra copies of Rtt103 gene can rescue the rat1EDD lethal phenotype when wild-type Rat1 was shuffled out by FOA selection.

2.2. Protein expression and purification

The recombinant proteins with 6xHis tag of Rat1, Rai1, Rtt103, and TFIIS were over-expressed in BL21 CodonPlus (DE3) RIL (Stratagene) strain via IPTG induction (0.25 mM IPTG) at 25℃ for 6 hr.

Cells expressing Rat1/Rai1 or Rat1 were lysed by sonication in freezing buffer A (50 mM Tris-HCl, pH 7.9 at 24℃, 150 mM NaCl, 10% glycerol, 10 mM β-ME, 1.8 μM leupeptin, 5.46 μM pepstatin A, 6.33 mM benzamidine, 37.5 μg bestatin, 3 mM PMSF). The lysates were cleared by centrifugation and sequentially applied to Ni-NTA agarose column (Qiagen), HiTrap Heparin HP affinity column (5 ml, GE Healthcare), MonoQ 10/100 GL anion exchange column (GE Healthcare), and Superose 6 10/300 GL size-exclusion chromatography. The purified proteins were eluted in final buffer (25 mM Tris-HCl, pH 7.9 at 24℃, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol), quick frozen in liquid nitrogen and stored at -80℃. The Rat1 variants were purified by the same procedure. For Rtt103, cells were lysed

by sonication in freezing buffer B (50 mM Tirs-HCl, pH 7.3 at 24℃, 150 mM NaCl, 10% glycerol, 10 mM β-ME, 1.8 µM leupeptin, 5.46 µM pepstatin A, 6.33 mM benzamidine, 37.5 µg bestatin, 3 mM PMSF) and the cell lysate was applied to Ni-NTA, MonoQ and Superose 6 columns, respectively. The purified protein was eluted in final buffer B (25 mM Tris-HCl, pH 6.8 at 24℃, 125 mM NaCl, 1.25 mM DTT, 10%(v/v) glycerol). For TFIIS, purification procedure was performed as described elsewhere (53).

Rat1/Rai1/Rtt103 and rat1EDD/Rai1/Rtt103 complex were purified using the same procedures but with slightly different buffer conditions. In affinity and ion-exchange chromatographies, Tris-HCl, pH 7.6 at 24ºC was used. In Superpose 6 size-exclusion chromatography step, protein complexes were eluted with low-salt storage buffer (25 mM Tris-HCl, pH 7.6 at 24 ºC, 80 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 10% (v/v) glycerol).

For *Saccharomyces cerevisiae* RNAPII purification, BJ5464 Rpb3 His-Bio strain (a gift from P. Cramer) was fermented and purified as described previously (54). Rpb4/7 subunit was over-expressed in BL21 CodonPlus (DE3) RIL (Stratagene) strain via IPTG induction (0.25 mM IPTG) at 25℃ for 6 hr. It was purified as described in elsewhere (55).

2.3. *in- vitro* transcription termination assay

Transcription-competent elongation complexes were assembled as previous described (28). Template/non-template DNAs and RNAs used to assemble an elongation complex (EC) were listed in Supplementary Table 2.1. Briefly, 3 pmol of RNAPII was incubated with 2-fold molar excess of DNA/RNA hybrid, 4-fold molar excess of 5'-biotinylated non-template DNA and 5-fold molar excess of Rbp4/7 to form EC. Streptavidin-coated magnetic beads (Dynabeads MyOne streptavidin T1, Invitrogen) were pre-blocked O/N with blocking buffer (50 mM Tris-HCl, pH 8.0 at 25℃, 150 mM NaCl, 2 mM EDTA, 0.1% (w/v) Triton X-100, 5% (w/v) glycerol, 0.5% (w/v)

BSA, 0.2 mg/ml insulin, 0.1 mg/ml heparin, 0.5 mM DTT) to prevent nonspecific binding of ECs. After ECs were bound to beads, 3'-end of RNA was labeled with [α-^{32}P] UTP by RNAPII.

For RNA digestion, 6 pmol of Rat1/Rai1 was added to EC and incubated for 1 hr at 30℃ in the presence or absence of each single NTP. After the reaction is completed, the nuclease and NTP were removed with washing buffer (20 mM Tris-HCl, pH 8.0 at 30℃, 500 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT), and EC bound beads were resuspended in reaction buffer (20 mM Tris-HCl, pH 8.0 at 30℃, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT). Mixture of four NTPs was added to EC and incubated for 30 min at 28℃ to allow RNAPII elongation. The reactions were stopped and RNA samples were analyzed by 7 M Urea-PAGE. The radioactively labeled RNA was detected by phosphorimager (BAS-5000, Fujifilm).

Table 2.1. RNA and DNA oligonucleotides sequence used in in-vitro transcription termination assay

Oligonucleotide	**Sequences**
EC1 Template DNA	5'-CCAGTCATCGTCGACTGACTCCAAGCTCAAGTACTTGAGCCTGGTCATTACTAGTACTGCCTTGACCTAGCGTCGG-3'
EC1 Non-Template DNA	5'BIOTIN-GGCTACCGACGCTAGGTCAAGGCAGTACTAGTAATGACCAGGCTCAAGTACTTGAGCTTGGAGTCAGTCGACGATGACTGG-3'
EC2 Template DNA	5'-CCAGTCATCGTCGACTGACTCCAAGCTCAAGTACTGGAGCCTGGTCATTACTAGTACTGCCTTGACCTAGCGTCGG -3'
EC2 Non-Template DNA	5'BIOTIN-GCTACCGACGCTAGGTCAAGGCAGTACTAGTAATGACCAGGCTCCAGTACTTGAGCTTGGAGTCAGTCGACGATGACTGG -3'
RNA 19	5'P-AUAUGCAUAAAGACCAGGC -3'
RNA 22	5'P-UAUAUAUGCAUAAAGACCAGGC -3'
RNA 24	5'P-CAUAUAUAUGCAUAAAGACCAGGC -3'
RNA 30	5'P-UAAUCCCAUAUAUAUGCAUAAAGACCAGGC -3'
RNA 40	5'P-UACAUAAUCAUAAUCCCAUAUAUAUGCAUAAAGACCAGGC -3'

2.4. ATPase activity assay

ATPase activity assay was performed as previously described (56). Each 20 µl reaction contains 0.66 pmol of [γ-^{32}P] ATP, 6 pmol of nucleases, 5.6 pmol of DNA or RNA and 0.2 mM of $MgCl_2$. The reaction mixture was incubated for 45 min at 37℃ and terminated by addition of EDTA to 0.5 mM and cold ATP to 0.6 mM. About 2 µl from each reaction was spotted onto TLC PEI plate (Merck) and developed in 0.6 M KH_2PO_4 (pH 3.4). The extent of ATP hydrolysis was detected by phosphorimager.

2.5. Helicase assay

RNA/DNA duplex helicase assay was performed as previously described(57). The substrate for the assay consisted of an RNA/DNA duplex formed by 5' radiolabeled 65bp of DNA oligonucleotide annealed to 3' end of 31bp of RNA. The oligonucleotides are purchased from IDT. The RNA/DNA duplex was incubated with the desired enzyme at a molar ration of 1:30 (duplex:protein) in a reaction buffer used in in-vitro transcription termination assay. Aliquots were taken at each time point and mixed with 1 volume of stop/loading buffer containing 50 mM EDTA, 1% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue and 20% glycerol. Samples were separated by electrophoresis on 11% native PAGE gel.

2.6. Western blotting analysis

Western blotting analysis is performed as described somewhere else. Purified polymerase fractions were boiled at 95'C with 1X SDS buffer for 10 min and separated by electrophoresis on 12% PAGE gel. α-Ser2P antibody and α-Rpb3 antibody are purchased from Santa Cruz and Millipore, respectively.

3. RESULTS

3.1. *in-vitro* transcription termination assay

To unravel the mechanism of RNAPII termination, I adopted *in vitro* transcription termination assay that was previously developed by Cramer and colleagues (Fig 3.1) (28). This simplified system mimics an elongation complex (EC), consisting of double stranded DNA, 31 nt 5'-phosphorylated RNA and purified RNAPII. The ECs were immobilized to streptavidin-coated magnetic beads by the use of biotin at the 5'-end of the non-template DNA strand. The 3'-end of RNA was labeled via [α-^{32}P] UTP incorporation by RNAPII. After washing out unincorporated [α-^{32}P] UTP, Rat1/Rai1 was added with or without other factors to EC, and RNAPII termination was monitored. In this set-up, Rat1/Rai1 would initially degrade RNA up to the surface of RNAPII (~17/18nt). Once being hit by Rat1/Rai1, if RNAPII is terminated, the RNA would be no longer protected by the polymerase and degraded by Rat1. In contrast, if RNAPII is not terminated, the template bound polymerase would continue to elongate in the presence of NTPs, generating ~54/55 nt run-off transcript (Fig 3.1).

3.2. Purification of RNAPII and Rat1/Rai1 recombinant proteins

To perform the *in-vitro* transcription termination assays, RNAPII and Rat1/Rai1 were expressed and highly purified from *Saccharomyces cerevisiae* and *Escherichia coli*, respectively (Figure 3.2). The detailed procedure is described in materials and methods section.

3.3. Rat1/Rai1 terminates efficiently RNAPII in the presence of ATP *in-vitro*

When Rat1/Rai1 was added to ECs, the vast majority of RNAs were degraded up to ~17/18 nt but subsequently extended to ~54/55 nt by RNAPII. RNA signals from the Rat1/Rai1-treated ECs were about 60% of the control lacking nuclease (no nuclease or NN), indicating that Rat1/Rai1 alone somehow terminate RNAPII but still very

inefficiently as previously reported (28) (Fig 3.3.a and b, left panel) However, I surprisingly found that the remaining RNA level was dramatically decreased by Rat1/Rai1 upon adding ATP (2 mM) (Fig 3.3.a and b, right panel), indicating that ATP significantly enhances Rat1/Rai1-mediated RNAPII termination. Titration experiments obviously detected the improved termination at as low as 0.5 mM ATP (Fig 3.4). This ATP-dependent effect seems specific to Rat1/Rai1 only because no nuclease control or RNase I do not lead to similar decrease in the RNA level after ATP treatment (Fig 3.3.c).

3.4. Rat1/Rai1 does not have an ATPase activity

I first postulated that ATP-dependent RNAPII termination might occur via ATP hydrolysis by Rat1/Rai1. Since 5'-pyrophosphohydrolase activity of Rai1 is limited only to RNA substrates (21), I tested whether Rat1 may possess an ATP hydrolyzing activity. But several fractions of highly-purified Rat1/Rai1 from size-exclusion chromatography (Superose 6) did not show ATPase activity (Fig. 3.5.a). Similarly, Xrn1 does not have ATPase activity either (Fig 3.5.a), as predicted from amino acids sequences of XRN family proteins. However, when non-hydrolyzing ATP analogs were used in termination assay, RNAPII termination was significantly reduced, as judged by the increase of remaining RNA level (Fig. 3.5.b), indicating that ATP hydrolysis may be crucial to promote RNAPII termination but not driven by Rat1/Rai1.

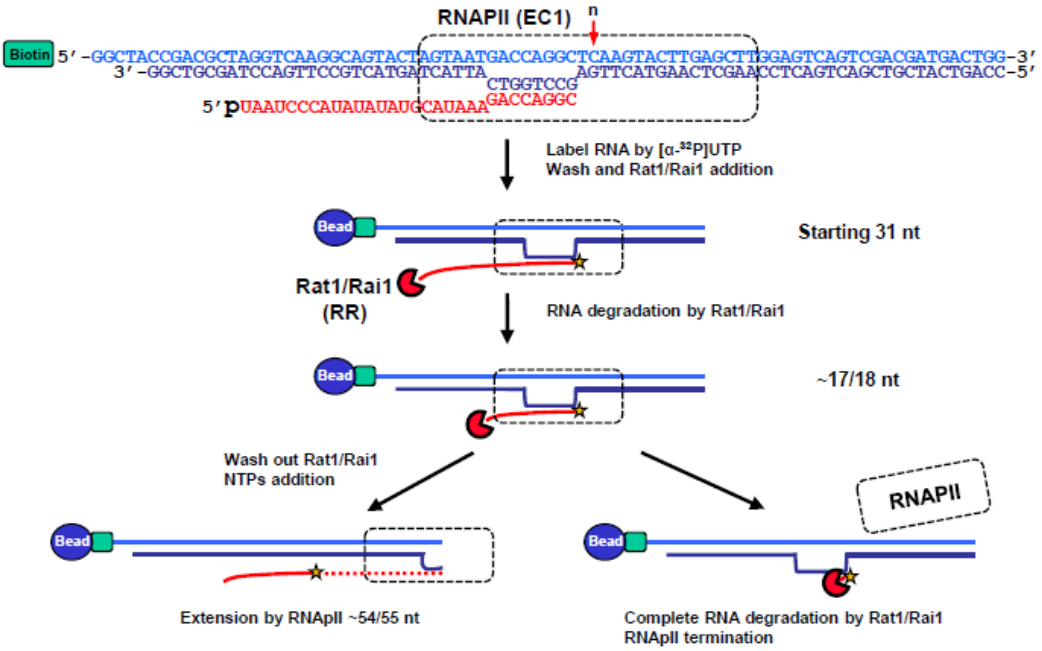

Figure 3.1. *in-vitro* transcription termination assay scheme

The EC was assembled with double-stranded DNA (EC1), 5’ phosphorylated RNA and purified RNAPII, and subsequently coupled to magnetic beads. The 3’-end of RNA is radioactively labeled by RNAPII. Rat1/Rai1 digests RNA from 5’ to 3’ direction up to the surface of RNAPII. If Rat1/Rai1 fails to terminate RNAPII, the polymerase would elongate further using NTPs. But if Rat1/Rai1 terminates RNAPII, the remaining RNA protected by RNAPII would be completely degraded by Rat1/Rai1. Red arrow specifies the position (n) of the first incoming NTP.

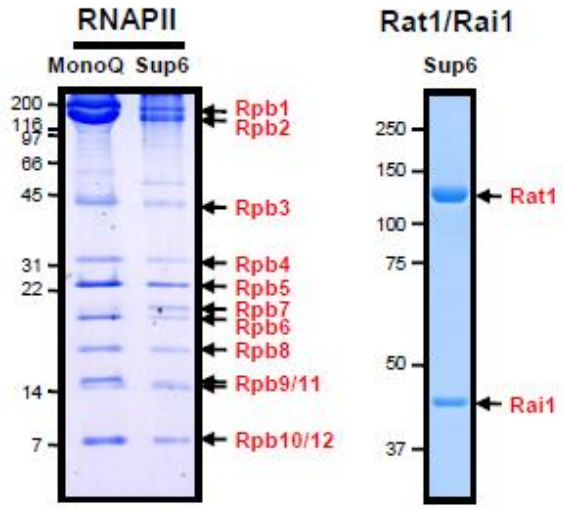

Figure 3.2. Purification of RNAPII and Rat1/Rai1 recombinant proteins

Purified RNAPII complex and Rat1/Rai1 on gels stained with Coomassie.

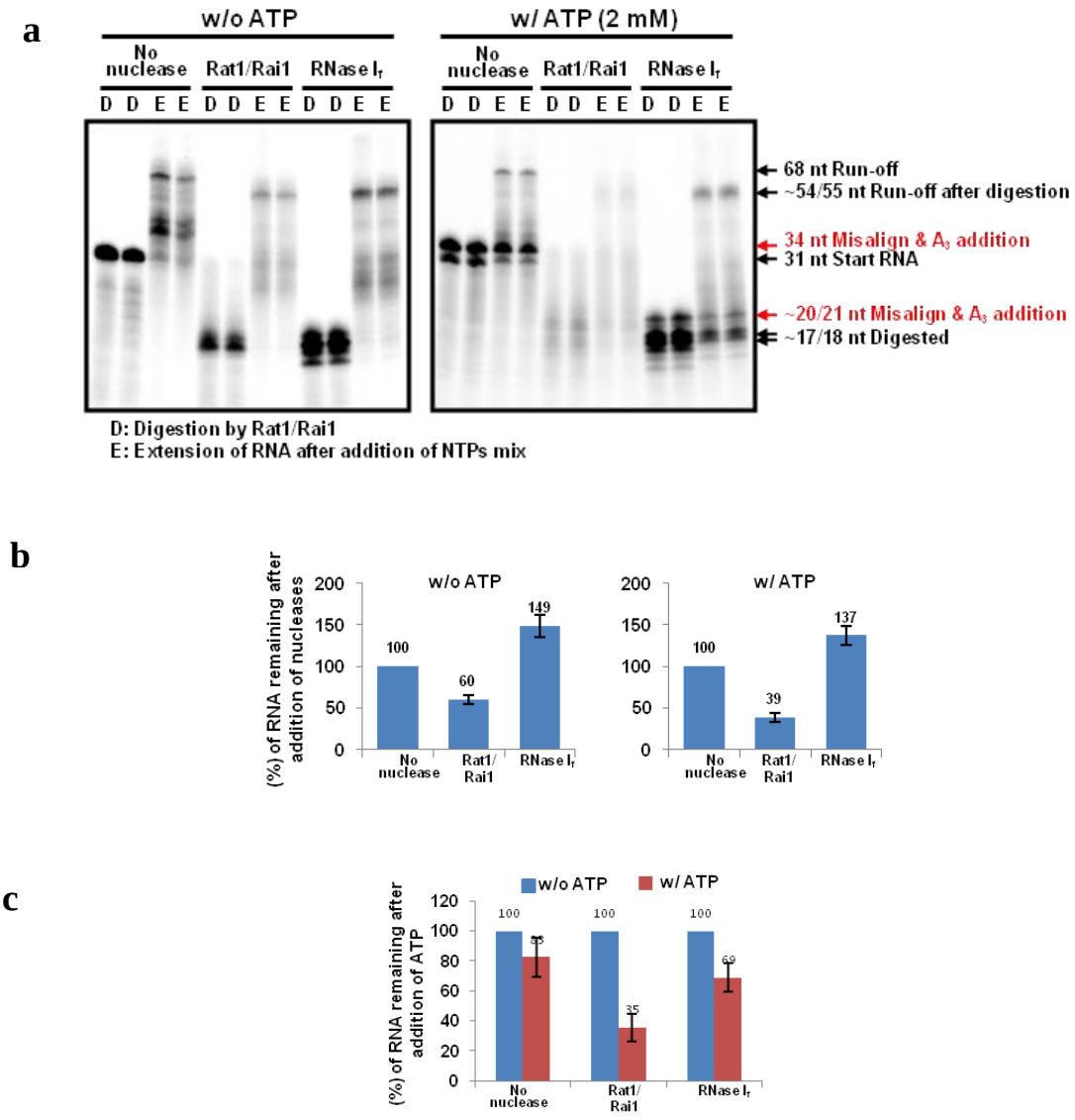

Figure 3.3. Termination of RNAPII by Rat1/Rai1 in the presence of ATP *in-vitro*

(a) Representative gel images of *in vitro* transcription termination assay with Rat1/Rai1 treatment. Rat1/Rai1 does not terminate RNAPII by itself but it can in the presence of ATP. Black arrows indicate RNAs predicted in (Fig 3.1). Red arrows show ATP-misincorporated RNAs. D, digestion by Rat1/Rai1. E, Extension of RNA after addition of NTPs mix. (**b**) Quantification of the remaining RNAs compared to the control lacking nuclease (set to 100%). (**c**) Quantification of the remaining RNAs after ATP treatment compared to no ATP control (set to 100%).

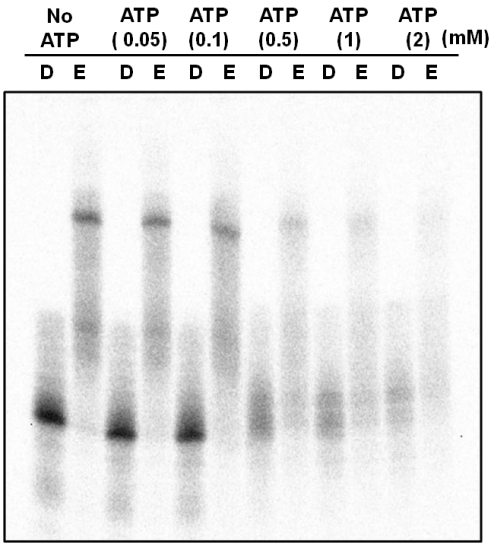

Figure 3.4. ATP titration assay shows minimal concentration of ATP for ATP-dependent Rat1-mediated RNAPII termination

ATP-dependent Rat1-mediated RNAPII termination occurs when the concentration of ATP is higher than 0.5 mM.

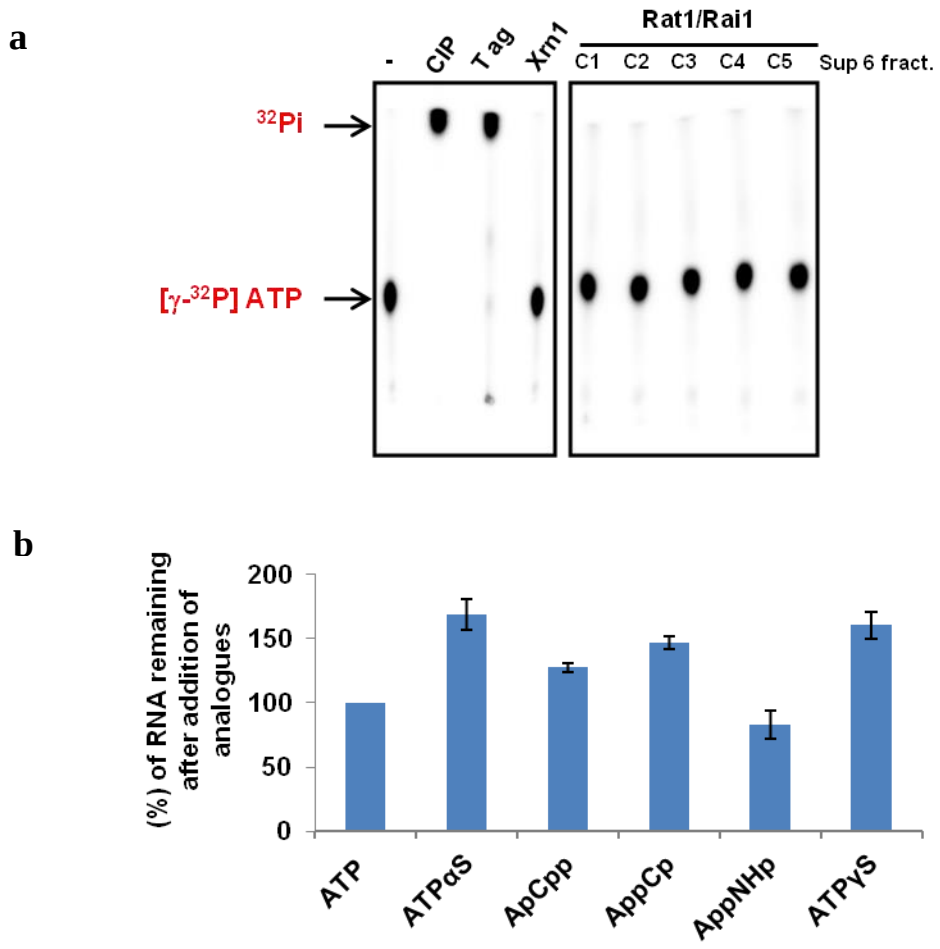

Figure 3.5. Rat1/Rai1 does not have an ATPase activity. ATP hydrolysis is still important for RNAPII termination.

(**a**) ATPase activity assay of Rat1/Rai1. Calf intestinal phosphatase (CIP), SV40 T antigen (T ag) and Xrn1 were used for controls. Highly purified Rat1/Rai1 fractions from size-exclusion chromatography (Superose 6) show no ATPase activity. (**b**) Quantification of the remaining RNAs from *in vitro* transcription termination assay with various non-hydrolysable ATP analogues.

3.5. NTP misincorporation induces RNAPII pausing and enhances termination efficiency by Rat1/Rai1

Intriguingly, I saw additional RNA band when ATP was added in the assay: ~34 nt in case of no nuclease control, and ~20/21 nt in RNase I-treated group (Fig 3.3.a, right panel). These RNAs seem to be generated by ATP misincorporation via template misalignment (39,40), since ATP is complementary to thymine at n+1 and n+2 positions of the template DNA strand (Figure 3.1). When ATP misincorporates into RNA transcripts, I observed that elongation of RNAPII was significantly blocked (Fig 3.3.a), presumably due to disruption and/or rearrangement of RNAPII active center (52,58). I hypothesized that RNAPII with disrupted active center might be more effectively terminated by Rat1/Rai1.

To investigate whether NTP misincorporation could induce RNAPII pausing and enhance subsequent termination by Rat1/Rai1, other NTPs were separately added to RNAPII assembled in the same EC1 scaffold (Fig 3.6). In fact, non-cognate GTP addition also resulted in longer (~34/35 nt) RNA transcripts via template misalignment and lesser RNAPII elongation (Fig 3.6. left gel image). When GTP was co-treated with Rat1/Rai1, it caused RNAPII termination as efficiently as ATP (Fig 3.6. right quantification graph). Another non-cognate UTP produced ~32 nt RNA transcripts and strongly blocked RNAPII elongation, probably because UTP misincorporation results in a UU pause sequence at RNA 3'-end that is shown to adopt a frayed position in the pore below the active center (58). Upon adding with Rat1/Rai1, UTP induced RNAPII termination, albeit it was far less efficient than ATP (Fig 3.6). However, the cognate CTP did not significantly cause RNAPII pausing nor termination, compared to non-cognate ATP and GTP (Fig 3.6).

To further verify this NTP misincorporation effect, a different DNA sequence template (EC2 scaffold) was tested in the *in-vitro* system (Fig 3.7). Again, non-cognate NTPs (ATP, UTP, and CTP) led to RNAPII termination much more

efficiently than cognate GTP does (Fig 3.7), confirming that NTP misincorporation indeed affects RNAPII termination by Rat1/Rai1. Although the incoming UTP is non-cognate to both EC scaffolds tested and generates a mismatching UU sequence, its effect on RNAPII pausing and termination by Rat1/Rai1 significantly varies at each EC scaffold (Fig 3.7), suggesting that sequence context may also affect the efficiencies of NTP misincorporation, RNAPII pausing and subsequent termination by Rat1/Rai1.

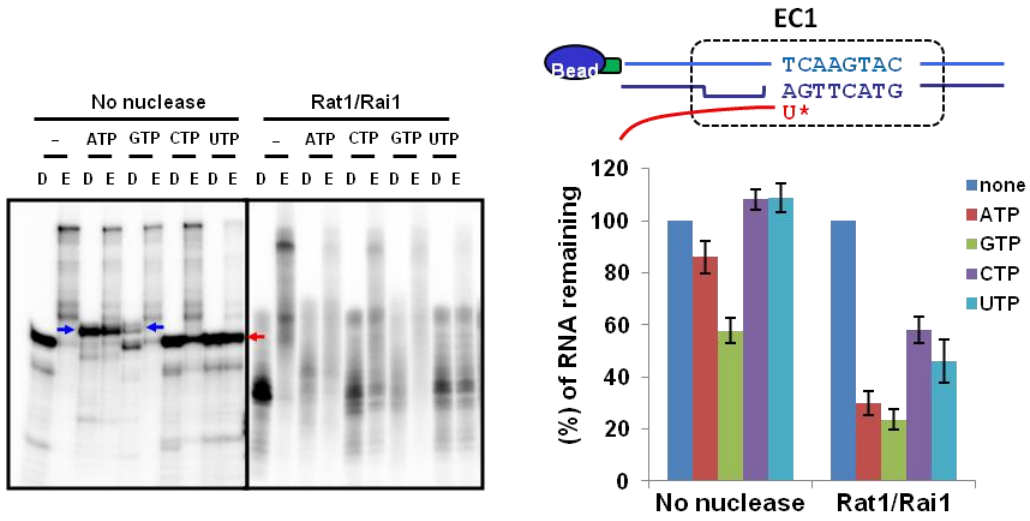

Figure 3.6. NTP misincorporation induces RNAPII pausing and enhances termination efficiency by Rat1/Rai1. EC1 is tested.

DNA sequences are shown on top of the quantification graph. Asterisk specifies the incorporation site of an incoming NTP (n position). EC1 Scaffold tested. In a control group lacking nuclease, addition of non-cognate ATP or GTP generates misincorporated RNA bands via template misalignment (blue arrows, ~34/35 nt) whereas cognate CTP does not. Notably, Rat1/Rai1 more efficiently terminates RNAPII when non-cognate NTP (ATP or GTP) rather than cognate CTP was added. Addition of UTP to EC1 induces strong pausing of RNAPII that does not support further elongation (red arrow), which results in slight inhibition of termination by Rat1/Rai1.

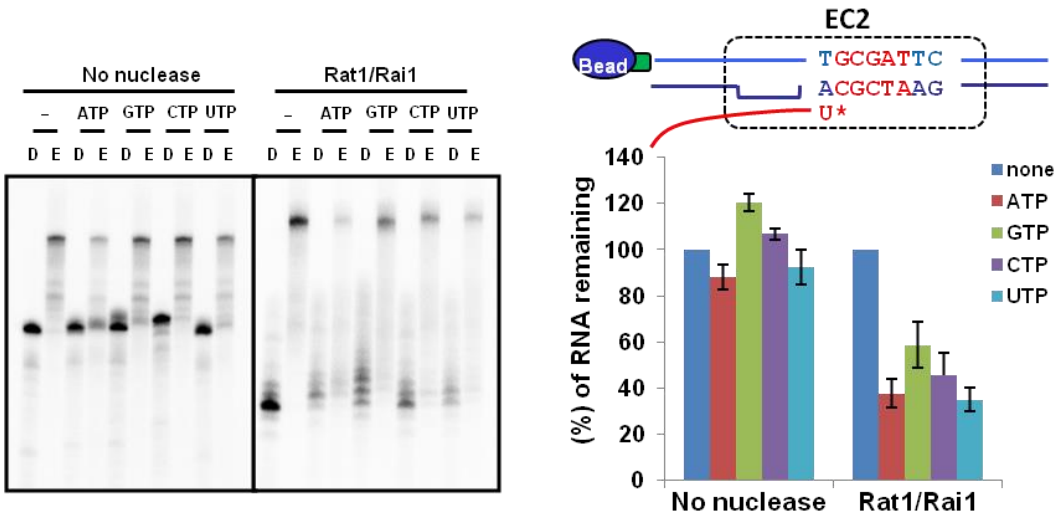

Figure 3.7. NTP misincorporation induces RNAPII pausing and enhances termination efficiency by Rat1/Rai1. EC2 is tested.

EC2 scaffold tested. Different sequences are shown in red. Rat1/Rai1 more effectively terminates RNAPII when non-cognate NTP (ATP, CTP or UTP), rather than cognate GTP was added.

3.6. Other yeast 5'-3' exoribonucleases can terminate RNAPII efficiently at least *in-vitro*. Rat1/Rai1 cannot terminate *E. coli* RNAP *in-vitro*.

Since the human homologue of Rat1, hXrn2, also plays a key role in RNAPII termination (14,59), I examined whether NTP-misincorporation could enhance termination by hXrn2. Indeed, hXrn2 better terminated RNAPII in the presence of ATP (Fig 3.8.a), demonstrating that this mechanism is conserved between yeast and human. In fact, paused RNAPII over the G-rich region of human β-actin gene was shown to be efficiently terminated by hXrn2 along with Senataxin (60).

We also tested a cytoplasmic 5'-3' exoribonuclease, Xrn1. In contrast to Rat1, Xrn1 terminates RNAPII very effectively even without ATP (Fig 3.8.b). It is surprising because nuclear-localized Xrn1 was previously shown to be incapable of rescuing the termination defect in *rat1-1* mutant (61). But my assay shows that degradation of RNA by 5'-3' exonuclease activity of Xrn1 is sufficient to terminate RNAPII at least *in vitro*. It implies that Xrn1 may have a higher processivity than Rat1 which would be useful to translocate proteins ahead of it (e.g. RNAPII). Supporting this hypothesis, Xrn1 was shown to processively degrade RNA substrates containing stem-loop structure (62,63) and employ an unwinding mechanism via substrate translocation past a steric barrier that excludes double-stranded regions (64). Based on this, I tested if Rat1 has any helicase activity, thereby leading to termination or not, and turns out Rat1/Rai1 does not show helicase activity as expected (Fig 3.9).

The reason why nuclear-localized Xrn1 fails to terminate RNAPII *in vivo* would be probably because it cannot make the same interaction as Rat1 with RNAPII and/or other key factors necessary for termination.

To investigate whether Rat1 can terminate other polymerases, *E. coli* RNAP was tested. Although Rat1/Rai1 successfully reached *E. coli* RNAP by degrading RNA, it was not able to terminate the polymerase. As previously reported (39), NTP misincorporation occurred in the bacterial RNAP as similarly as the yeast RNAPII, and reduced the elongation of the polymerase (Fig 3.10).

But it did not significantly enhance the termination of *E. coli* RNAP by Rat1/Rai1 (Fig 3.11). This result suggests that specific protein-protein interaction(s) between Rat1/Rai1 and RNAP may also be critical to trigger termination

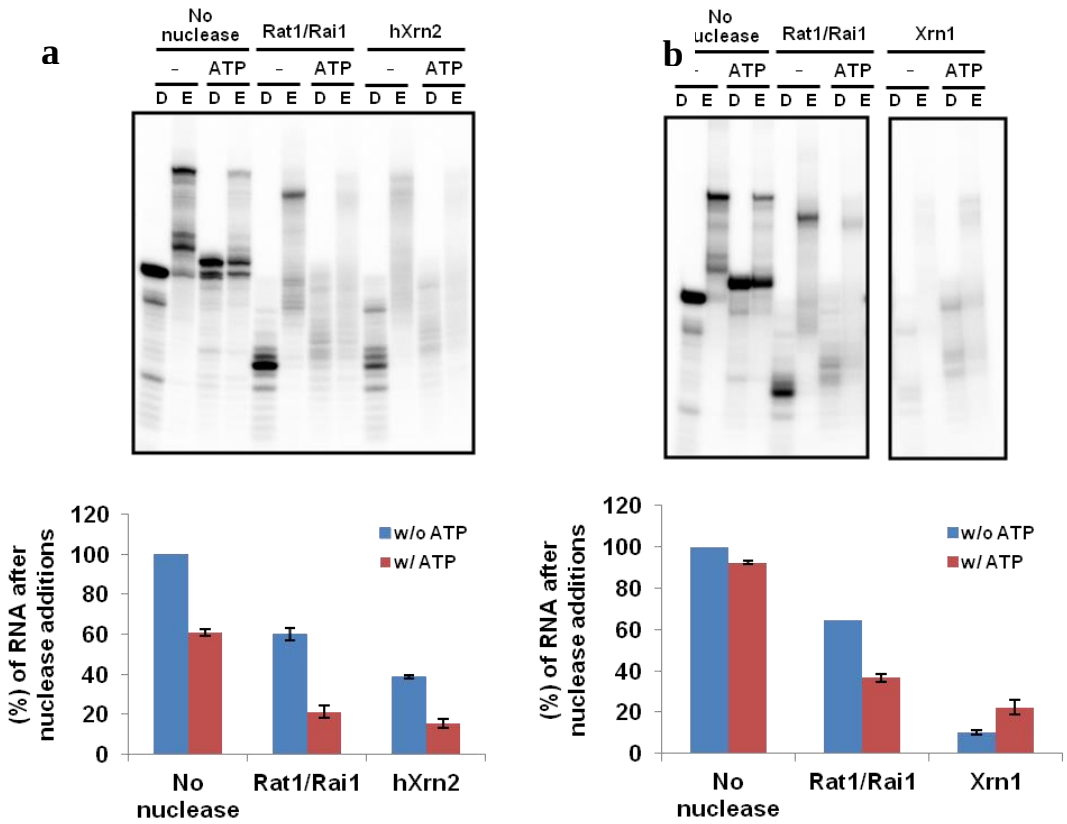

Figure 3.8. Other 5'-3' exoribonuclease also terminates RNAPII at least *in vitro*.

(a) hXrn2 terminates yeast RNAPII more efficiently in the presence of ATP. Quantification of the remaining RNAs was shown below. (**b**) Yeast Xrn1, Rat1's cytoplasmic counterpart, terminates RNAPII regardless of ATP *in vitro*. Quantification of the remaining RNAs was shown below.

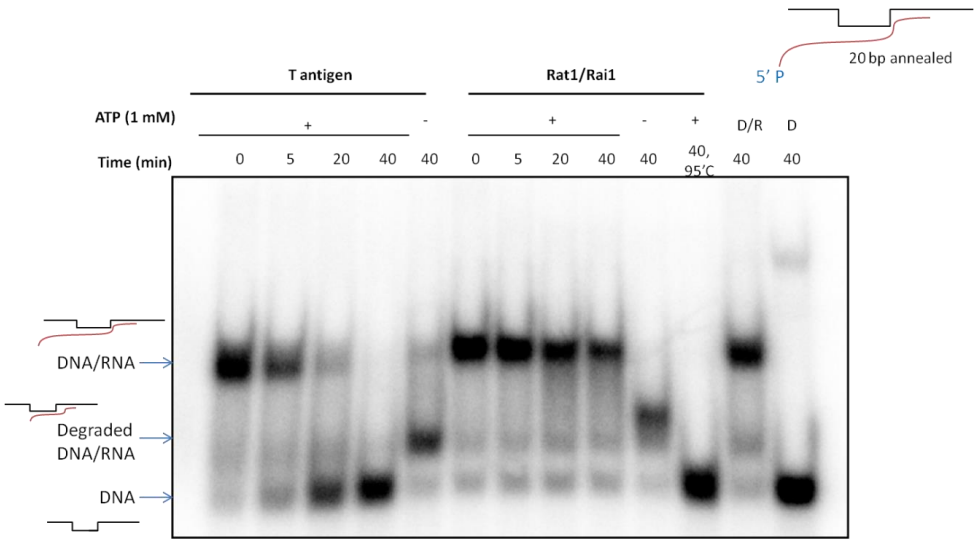

Figure 3.9. Rat1/Rai1 does not have helicase activity

Rat1/Rai1 cannot unwind single-stranded DNA/RNA hybrid. Strand scheme is shown up on the top right corner. 31bp of single strand RNA is annealed to 65bp of single-strand DNA, resulting 20bp of DNA/RNA hybrid. Assay was done in the presence or absence of 1mM of ATP. A known ATP-dependent helicase SV40 T antigen is used for a control. Enzymes are incubated with DNA/RNA hybrid for up to 40 minutes and the reaction was stopped at each designated time point.

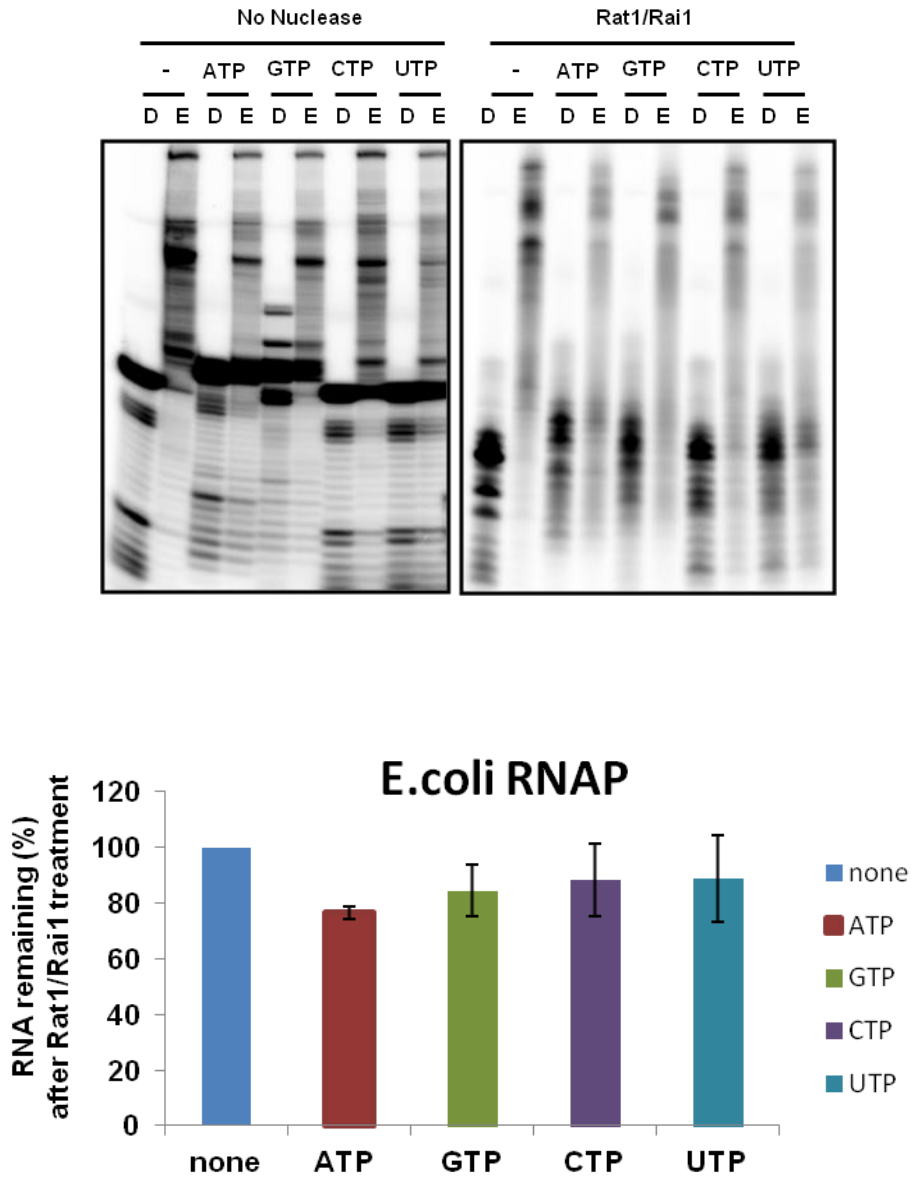

Figure 3.10. Rat1/Rai1 cannot terminate *E.coli* RNAP even though the polymerase is paused due to NTP misincorporation

(a) *E.coli* RNAP is treated with 1mM of each NTP for 30 min, and shows NTP misincorporation pattern same as observed in yeast RNAPII experiment (**b**) Although *E.coli* RNAP shows similar pattern of NTP misincorporation as yeast RNAPII does, Rat1/Rai1 cannot terminate *E.coli* RNAP *in vitro*, and addition of each NTP had no effect either, which implies that specific protein-protein interaction would be required for termination.

3.7. The length of RNA degraded by Rat1 affects RNAPII termination

To analyze whether the length of RNA within EC might affect loading of Rat1/Rai1 and subsequent RNAPII termination, we tested ECs having variable lengths of RNA (20, 23, 25, 31 and 41 nt). The 20 nt RNA was readily degraded by Rat1/Rai1 up to ~17/18 nt, indicating that extra 2 or 3 nt would be sufficient to be recognized by Rat1/Rai1 (Fig 3.11).

Surprisingly, I found that termination efficiency increases as the RNA within EC gets longer (Fig 3.11). In the presence of ATP, the remaining RNA level after Rat1/Rai1 treatment drastically decreases from 74.6% (20 nt RNA) to 26.9% (41 nt RNA) (Fig 3.11.a and b). But in a control group lacking nuclease, the remaining RNA level did not significantly altered after ATP addition (Fig 3.11.c). I also observed this RNA length effect even in the absence of ATP after Rat1/Rai1 treatment [from 68.7% (20 nt) to 46.7% (41 nt)], although little weaker than in the presence of ATP (Fig 3.12.b). These results indicate that RNA degradation step is critical for Rat1/Rai1 to trigger RNAPII termination. One plausible explanation would be that, while degrading RNAs, Rat1/Rai1 might accumulate a driving force to mechanically dissociate RNAPII from the template.

To determine which protein induces the RNA length effect observed, the same experiment was performed without Rai1. Similarly to Rat1/Rai1, Rat1 terminates RNAPII more effectively as it degrades longer RNA (Fig 3.12), implying that the RNA length effect derives from Rat1. But compared to Rat1/Rai1 (Fig 3.11.b), Rat1 alone terminates RNAPII more efficiently (Fig 3.12.b), and reached the highest level of termination (~25%) at 41 nt RNA, regardless of ATP addition (Fig 3.12.a). Consistently, hXrn2 also terminates RNAPII better than Rat1/Rai1 (Fig 3.8.a). These results implies that Rai1 may act negatively on or fine-tune the ‘RNAPII-dislodging’ function of Rat1 at the final step, although it helps Rat1 to degrade structured RNAs more effectively and by so doing allows access of Rat1 to the polymerase (21).

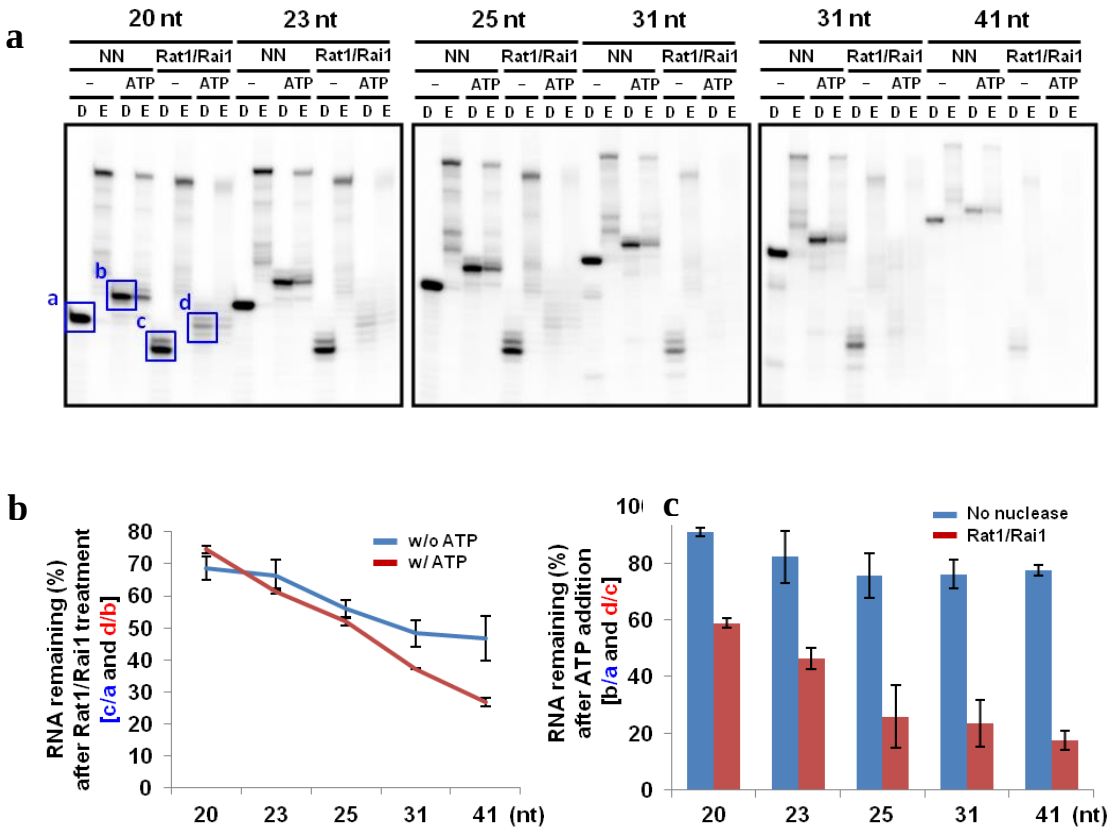

Figure 3.11. The length of RNA degraded by Rat1/Rai1 affects RNAPII termination.

Five ECs harboring different lengths of RNA were tested in the assay. (**a**) Representative gel images of Rat1/Rai1-treated *in vitro* transcription termination assay. (**b**) Quantification graph of the remaining RNAs after Rat1/Rai1 treatment without or with ATP addition. The remaining RNA amounts without or with ATP addition in no nuclease (NN) groups (a or b, respectively; blue box in gel images) were set to 100% at each EC, and the percentage of remaining RNA amounts in Rat1/Rai1-treated groups without or with ATP treatment (c/a or d/b, respectively) were calculated for each EC. In the presence of ATP, the remaining RNA level after Rat1/Rai1 treatment rapidly decreases as the RNA gets longer. But it moderately decreases in the absence of ATP. (**C**) Quantification of the remaining RNAs after ATP addition in no nuclease (NN) or Rat1/Rai1-treated groups. The remaining RNA amounts without ATP addition (a or c, respectively) were set to 100%, and the percentage of remaining RNA amounts after ATP addition (b/a or d/c, respectively) were calculated for each EC. The remaining RNA amounts specifically and gradually decreases by Rat1/Rai1 as the length of RNA increases.

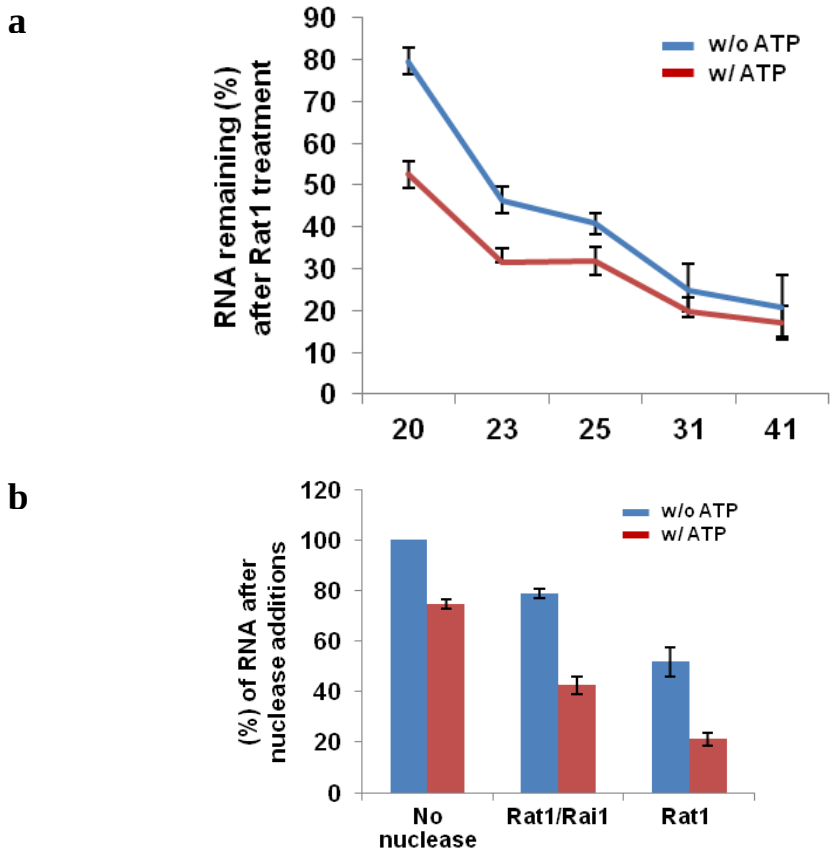

Figure 3.12. RNA length effect to termination is derived from Rat1

(a) Quantification graph of the remaining RNA amounts without or with ATP addition after Rat1 treatment. It shows dramatic decrease in the remaining RNA level as the RNA length increases, but there is little difference in the remaining RNA levels, regardless of ATP addition, at the ECs harboring 31 nt and 41 nt RNAs. (**b**) Rat1 terminates RNAPII as similarly as Rat1/Rai1. Addition of ATP also enhances termination by Rat1 alone.

3.8. 5’-3’ exoribonuclease activity of Rat1 is essential for RNAPII termination

To validate the role of 5’-3’ exoribonuclease activity in termination, I generated a catalytically inactive (exo-) form of Rat1 by mutagenizing three conserved acidic residues in the active site (E203A, D233A, and D235A). This rat1 mutant (referred to as rat1EDD) was co-expressed and purified as a complex with Rai1 in *E. coli* cells. Since rat1 EDD does not have 5’-3’ exoribonuclease activity, the elongation efficiency (run-off RNAs to initial starting RNAs) rather than the remaining RNA level was measured to determine the extent of RNAPII termination. rat1EDD/Rai1 did not degrade RNAs nor decrease RNAPII elongation (Fig 3.13), confirming that RNA degradation by exonuclease activity is critical to RNAPII termination.

A recent study claimed that Rtt103 allowed exonucleolytic-deficient Rat1 (D235A) to access and terminate RNAPII, suggesting that exonucleolytic activity of Rat1 may not be a key feature that triggers termination (25). However, I did not observe a rescue of termination defect of rat1EDD by adding Rtt103 in the assay (Fig 3.14).

Consistently, the lethality of rat1EDD mutation was not suppressed by introducing a multi-copy plasmid of Rtt103 gene (Fig 3.15). Furthermore, gel filtration profiles show that only a small portion of rat1EDD/Rai1 is bound to Rtt103, whereas the majority of wild-type Rat1/Rai1 is complexed with Rtt103 (Fig 3.15). It indicates that rat1EDD significantly lost the binding affinity for Rtt103, arguing against the role of Rtt103 in bridging exo- rat1 to RNAPII CTD to complement the defective exonuclease activity. The discrepancy could be partially due to additional mutations in the Rat1 active site (D235A vs. E203A, D233A, D235A), but not likely due to the lack of RNAPII CTD Serine 2 phosphorylation in our assay system, since I detected significant levels of CTD phosphorylation at Serine 2 (Fig 3.17). Taken together, my results clearly show that the exoribonuclease activity is required for Rat1 not only to approach RNAPII but also to get a sufficient driving force to dislodge the polymerase from the DNA template.

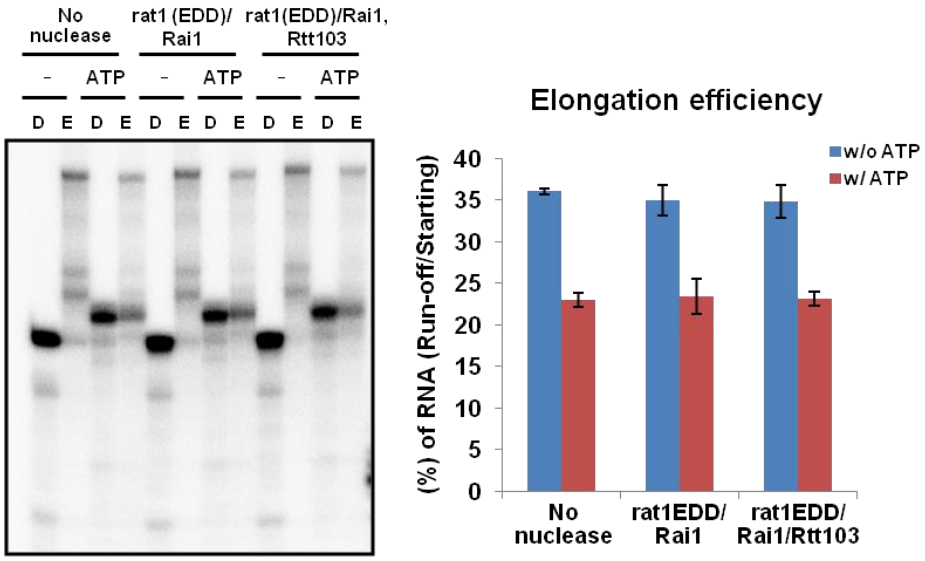

Figure 3.13. Exonucleolytic-deficient mutant rat1EDD/Rai1 cannot terminate RNAPII, and Rtt103 cannot rescue the termination defect of rat1EDD/Rai1

(Left) Representative gel image. rat1EDD/Rai1, regardless of Rtt103 addition, does not terminate RNAPII. (Right) Quantification of the extended run-off RNA amounts relative to the initial starting RNAs without or with ATP addition. rat1EDD does not reduce the RNA elongation efficiency by RNAPII, compared to no nuclease control.

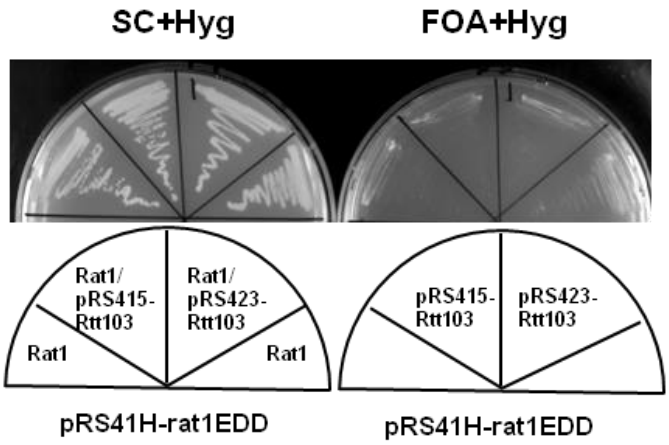

Figure 3.14. Exoribonuclease function is essential for cell viability and Rtt103 cannot restore the lethality of rat1EDD mutant

Multiple copies of Rtt103 gene cannot rescue the lethality of rat1EDD mutation. pRS415-Rtt103 produces single copy of Rtt103 and pRS425-Rtt103 does multiple copies of Rtt103. Wild type pAJ202- Rat1 is shuffled out by FOA selection (FOA + Hyg).

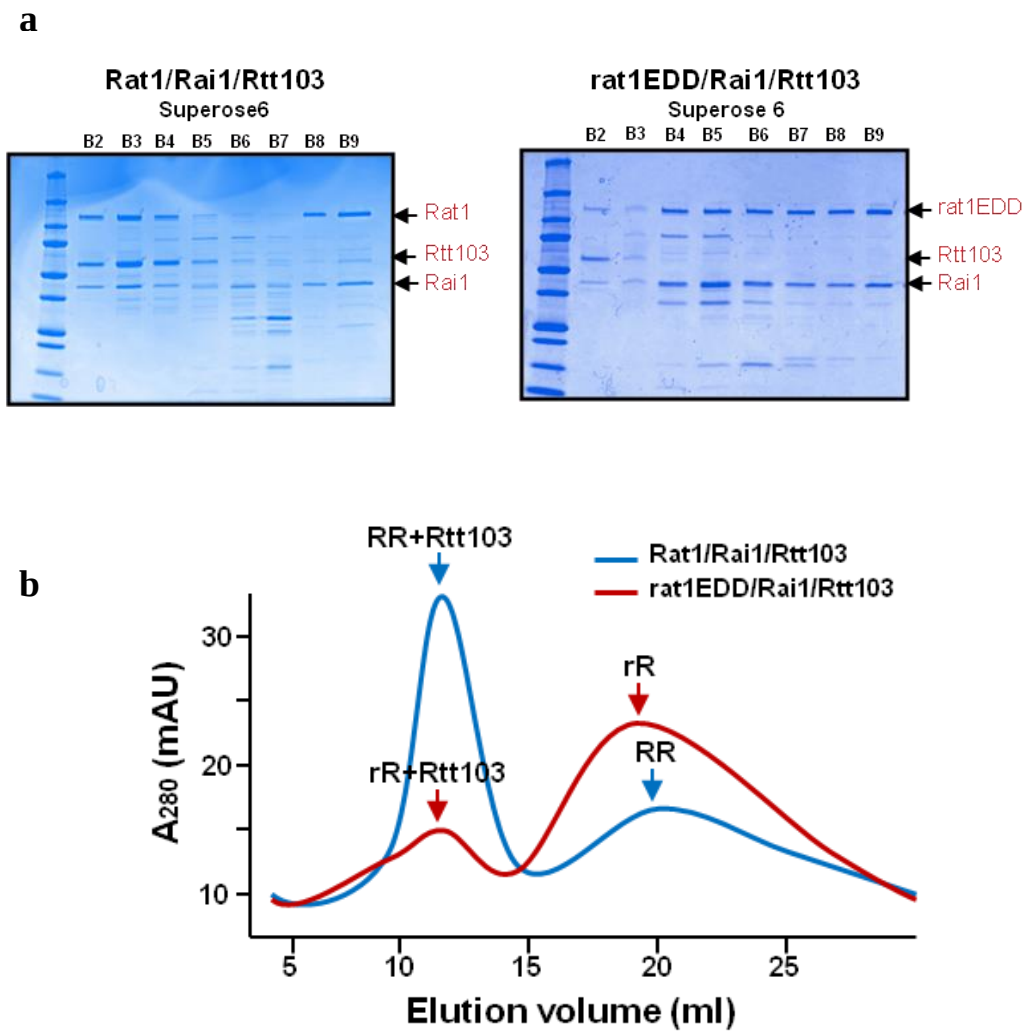

Figure 3.15. Exonucleolytic-deficient rat1EDD/Rai1 shows reduced interaction with Rtt103 relative to Rat1/Rai1.

(a) Coomassie stained gel images of highly purified Superpose 6 fractions (**b**) Elution profiles of size-exclusion chromatography (Superose 6) of Rat1/Rai1/Rtt103 (RRR) and rat1EDD/Rai1/Rtt103 (rRR) show that most of Rat1/Rai1 is co-eluted with Rtt103, whereas most of rat1EDD/Rai1 is not. It indicates that rat1EDD shows reduced binding with Rtt103, compared to wild-type Rat1. The peak (height x elution volume) for RRR (blue) is 1^{st}:2^{nd} peak = 1.5:1. The peak area for rRR (red), peak area 1^{st}: 2^{nd} peak=1:10

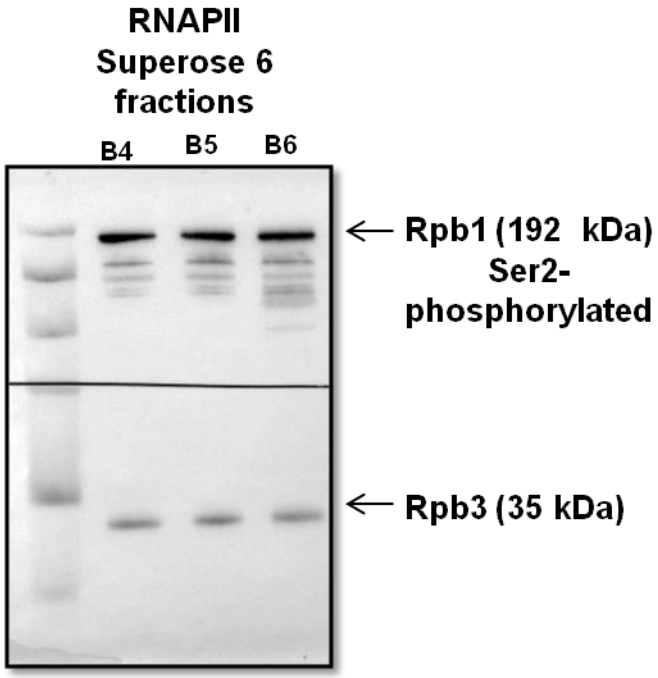

Figure 3.16. Ser2 phosphorylation level of RNAPII is detected by western blot

The phosphorylation level of highly purified RNAPII is detected by western blot. Ser2-phosphorylated Rpb1 (192 kDa) and Rpb3 (35 kDa) were detected by monoclonal α-Ser2P Ab (3E10) (Millipore) and α-Rpb3 Ab (Santa Cruz).

4. DISCUSSION

In this work, I discovered novel mechanistic features of RNAPII termination by Rat1/Rai1 using *in vitro* system (Fig 4.1). Firstly, Rat1/Rai1 more efficiently terminates RNAPII when NTP misincorporates and induces pausing of the polymerase. Secondly, Rat1/Rai1 may need to make direct interaction with target RNAPs to trigger termination since it is unable to terminate *E. coli* RNAP, presumably due to lack of specific contacts with subunits of bacterial RNAPs. Lastly, the length of RNA degraded is positively correlated with the efficiency of termination. Thus, degradation of RNA by 5'-3' exoribonuclease activity is not only crucial for Rat1 to gain access to RNAPII, but it may be also important to build up a driving force to dissociate the polymerase.

NTP misincorporation impairs RNA extension in several ways. These include disruption of RNAP active site conformation and generation of an offline state of the EC with a frayed RNA 3'-end (52). Paused RNAPII in these states may have subtle changes in the structure near the RNA exit channel and/or in the stability of the DNA-RNA hybrid that can be more easily accessed by Rat1/Rai1. Mismatches often facilitate RNAP backtracking and RNA cleavage, which rescues the paused polymerase to elongate. TFIIS stimulates a weak intrinsic cleavage activity of RNAPII (53), and helps to maintain transcriptional fidelity during transcription (46). However, TFIIS occupancy greatly reduces at 3'-untranslated region of genes (65), indicating that RNAPII approaching the termination sites might not have TFIIS within the complex. It may contribute, at least in part, to the occurrence of mismatches near the termination sites that make RNAPII more vulnerable to Rat1/Rai1.

Although non-cognate NTPs stimulate Rat1-mediated termination, termination efficiencies affected by each non-cognate NTP were not the same (Figure 3). It is presumably because the extent of misincorporation and mismatch extension apparently differs at each mismatch pair, as demonstrated previously (52). It is also noteworthy that simultaneous addition of Rat1/Rai1 and non-cognate NTPs to ECs

resulted in 20-30% better termination than pre-treatment of non-cognate NTPs to ECs before Rat1/Rat1 addition (data not shown), suggesting that NTPs play an extra role in Rat1-mediated termination besides misincorporation. Co-existing NTPs may increase a processivity of Rat1 via as yet unknown allosteric mechanism. Alternatively, it may activate a cryptic RNase H activity to degrade RNAs within the DNA-RNA hybrid, leading to dissociation of the polymerase as previously hypothesized (28,66).

The bacterial RNAP from *E. coli* was not terminated by Rat1/Rai1 regardless of that pausing phenomenon occurs due to the NTP misincorporations. This result can suggest that direct contact between Rat1/Rai1 and the polymerase at the final step of termination would be specific to dislodge the polymerase. Supporting this idea, another RNAPII termination factor, Sen1 was not able to terminate *E. coli* RNAP, either (67). These results indicate that both Rat1 and Sen1 could recognize unique features of eukaryotic RNAPII despite the structural similarities between eukaryotic and bacterial polymerases. I would suggest that Rat1 may interact with RNAPII near the RNA exit channel and/or the CTD, but for the precise prediction of interfacing region between Rat1/Rai1 and RNAPII, further structural studies will be required.

Termination efficiency enhances as the length of RNA degraded by Rat1 increases, proposing that multiple rounds of processive RNA hydrolysis would lead to more effective termination. We postulate that RNA hydrolysis may allow Rat1 to gradually accumulate a driving force to trigger termination, but the underlying mechanism is currently not understood. Alternatively but not exclusively, Rat1 may need a minimal length of RNA to engage in the termination process. Along the same line, Sen1 was recently shown to require at least ~15 nt RNA protruding from RNAPII to elicit termination (67), although the reason is not known. Since the highly conserved catalytic center of Xrn family proteins accommodates only the 5'-terminal trinucleotides (64), the rest of nascent RNA transcript may somehow transiently contact Rat1 outside of the active site in this model. This interaction could assist in

pulling out the nascent transcript from the RNAPII active center. In either scenario, the RNA length effect manifests an essential role of exonuclease activity in Rat1-mediated termination. Notably, the rat1EDD mutant was not able to terminate RNAPII even when it was put next to the polymerase using the shortest 20 nt RNA (~3 nt protruding from RNAPII, which allows recognition by Rat1) in the assay (data not shown). It elucidates that RNA degradation process is probably more important to termination than direct interaction between Rat1 and RNAPII, although we cannot rule out a possibility that the rat1EDD mutant fails to induce anticipated conformational changes of RNAPII as well when contacted the polymerase.

It would be interesting to see how multiple mechanistic features differentially contribute to RNAPII termination at individual genes. For genes whose transcription termination sites (TTS) are relatively far from p(A) sites, degradation of the downstream RNA transcript and subsequent specific RNAPII interaction by Rat1 would be sufficient to trigger termination. However, if p(A) sites and TTS are in close proximity, pausing of RNAPII by either NTP misincorporation or specific pausing sequences might help Rat1 to displace the polymerase. But needless to say, all these features would be valuable tools of Rat1 to terminate the transcription at the 3'-end of genes.

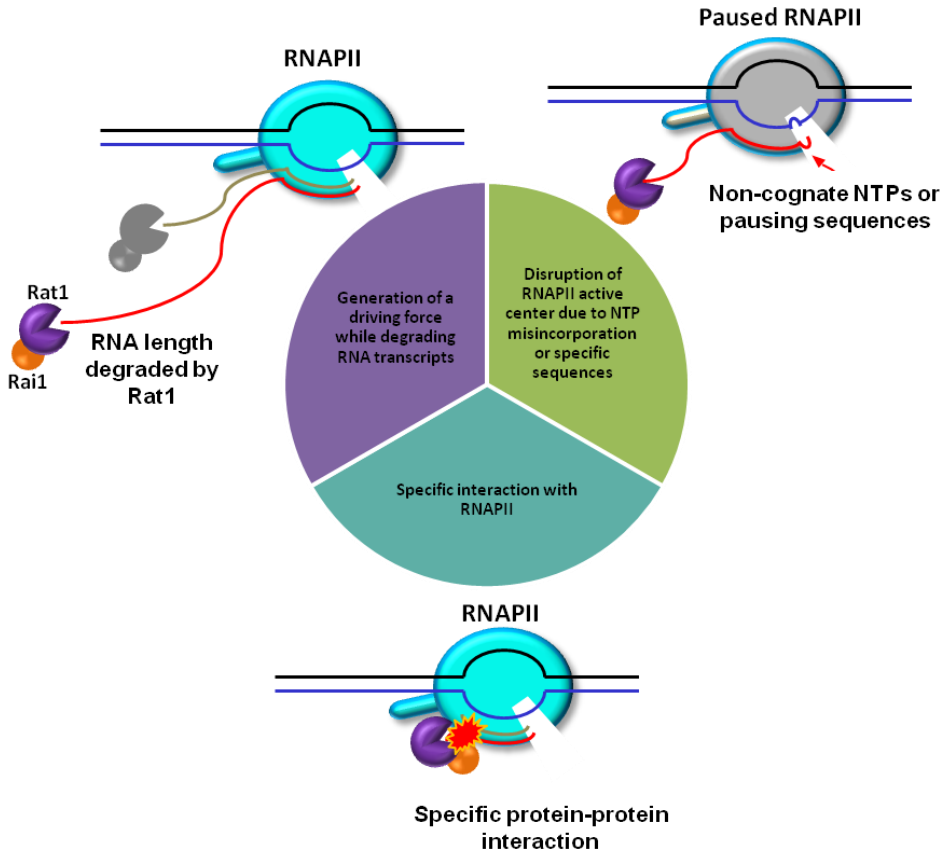

Figure 4.1. Multiple mechanistic features contribute to Rat1-mediated RNAPII termination

Disruption of the RNAPII active center due to NTP misincorporation or specific sequences facilitates termination by Rat1/Rai1 *in vitro*. Specific interaction between Rat1/Rai1 and RNAPII is shown to be critical, since Rat1/Rai1 cannot terminate *E. coli* RNAP. Furthermore, Rat1 needs to degrade RNA transcripts to build up a driving force for termination. Thus, 5'-3' exonuclease activity is essential for Rat1 not only to gain access to RNAPII, but also to accumulate sufficient driving forces to execute termination. Active RNAPII (cyan), paused RNAPII (grey).

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6. Abstract in Korean

5′-3′ exoribonuclease Rat1 에 의한 RNA 중합효소 II 의 전사 종결 기작의 특징 규명

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5′-3′ exoribonuclease 인 Rat1 은 coding gene 을 위한 RNA 중합효소 II 의 전사 종결을 촉진하는 것으로 알려져 있지만 그 자세한 분자 생물학적 기작은 아직 알려져 있지 않다. 본 연구는 *in-vitro* 시스템을 이용하여 전사중 NTP misincorporation 이 일어났을 때, RNA 중합효소 II 의 active site 가 망가지고 이로 인해 Rat1/Rai1 이 더 효과적으로 전사 종결을 일으킨다는 것을 밝혀내었다. 흥미롭게도 Rat1/Rai1 은 같은 현상이 *E.coli* RNAP 에 일어났을 때는 전사 종결을 시키지 못함으로 인하여 단백질간 특이 결합이 전사 종결에 있어 중요한 역할을 한다는 것을 발견하였다. 또한, Rat1 에 의한 전사 종결 효율이 Rat1 이 분해하는 RNA 의 길이가 길어짐에 따라 증가하는 것을 확인하였다. 이는 Rat1 이 RNA 를 분해 하는 과정에서 RNAPII 를 DNA template 로부터 떨어뜨리는, 즉 전사 종결을 일으킬 수 있는 충분한 driving force 를 축적할

것이라는 점을 의미한다. 본 연구는 위와 같은 발견들을 통하여 Rat1 을 매개로 한 RNAPII 전사 종결 기작의 다양한 성질을 밝혀내었다.

키워드: 전사, RNAPII, 전사 종결, 5′-3′ exoribonuclease, Rat1/Rai1, Rtt103

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