TRANSCRIPTION
2018, VOL. 9, NO. 1, 52–60
https://doi.org/10.1080/21541264.2017.1333558

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POINT-OF-VIEW

Sub1 and RNAPII, until termination does them part

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ABSTRACT
Sub1 was initially identified as a coactivator factor with a role during transcription initiation. However, over the last years, many evidences showed that it influences processes downstream during mRNA biogenesis, such as elongation, termination, and RNAPII phosphorylation. The recent discover that Sub1 directly interacts with the RNAPII stalk adds new insights into how it achieves all these tasks.

ARTICLE HISTORY
Received 2 May 2017
Revised 16 May 2017
Accepted 18 May 2017

KEYWORDS
RNAPII transcription; Rpb1-CTD phosphorylation; Rpb4/7; Sub1; Spt5

Introduction

In eukaryotes, transcription by RNA polymerase II (RNAPII) is strictly regulated by general transcription factors, regulators, cofactors, and chromatin regulators that include histone modifiers and chromatin remodeling complexes.[1,2] Additionally, the interactions of the polymerase is tightly regulated by post-transcriptional modifications of the carboxy-terminal domain (CTD) of its largest subunit, Rpb1. Being, for instance, the regulation of Rpb1-CTD phosphorylation crucial for the biogenesis of mRNAs.[3,4]

Eukaryotic RNAPII contains 12 subunits, Rpb1 to Rpb12, that in *Saccharomyces cerevisiae* dissociate into a 10-subunit core and a Rpb4/Rpb7 heterodimer that forms the stalk domain.[5] Rpb4 and Rpb7 are conserved from yeast to humans, and orthologs found in archaea also function in transcription.[6] Rpb4/7 participates in a broad range of activities under a variety of conditions.[7,8] In *S. cerevisiae*, Rpb4/7 is required for promoter-dependent transcription *in vitro*, is involved in elongation and termination,[8] and is important for co-transcriptional recruitment of factors required for 3′-end formation of mRNA and snoRNA genes.[9] Rpb4/7 may also function in mRNA quality control and translation, where it is thought to bind co-transcriptionally to nascent transcripts and promote nuclear export. It was proposed that, once in the cytoplasm, it stimulates translation initiation and subsequent deadenylation and mRNA decay.[10,11] Moreover, we showed that Rpb4/7 plays a key role in modulating Rpb1-CTD phosphorylation.

Among RNAPII regulators, Sub1, in *S. cerevisiae*, was originally characterized as a coactivator due to its homology with human Positive Coactivator 4 (PC4), its genetic and physical interaction with the general transcription factor IIB, and its capacity to activate transcription.[12,13] Later, *in vitro* assays demonstrated that in fact Sub1 is a functional component of the Pre-initiation complex (PIC),[14] and has a role in the selection of transcription start site (TSS).[15] Accordingly, Sub1 is predominantly associated with gene promoters,[14,16] and therefore, its function has been primarily linked to processes that take place at the 5′-end region of coding genes. Nevertheless, a number of evidences showed that Sub1 also plays a significant role in transcription elongation.[17,18] It can be localized within coding regions in a transcription-dependent manner and affects the levels of total RNAPII associated to genes during the entire transcription cycle. Furthermore, it co-purifies with the elongation factor Spt5, influences the elongation rate and promotes splicing.[17] Likewise, Sub1 has been found to play an anti-terminator role at 3′-end regions, while interacting with the termination factor Rna15.[16,19,20] More recently, we have shown that Sub1 directly interacts with the RNAPII stalk domain formed by the Rpb4

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and Rpb7 subunits, and is genetically related to the polymerase clamp domain.[21] This may help to explain how this multifaceted factor may influence different processes during the whole transcription cycle in addition to globally modulating Rpb1-CTD phosphorylation.[18] Thus, in the course of the transcription cycle, Sub1 could exert its function at different stages through the association with specific factors that directly interact with Rpb4/7 and/or the clamp domain.

Sub1 has been also described as a regulator of RNAPIII transcription. It stimulates transcription initiation and reinitiation *in vitro*, and it is required for optimal transcription by RNAPIII in exponentially growing cells.[22,23] Similarly, PC4 stimulates human RNAPIII transcription.[24,25] Sub1 and PC4, as ssDNA binding proteins, have been also implicated in DNA-dependent processes other than transcription, such as DNA repair, replication and in the maintenance of genome stability.[26-31] Here, the focus will be on the implications of Sub1 interaction with Rpb4/7 during the biogenesis of mRNAs.

Some features of Sub1 and Rpb4, keys to understand the current working model

The Rpb4/7 heterodimer is a stalk-like protrusion extending from the main body of the RNAPII complex.[32] Although Rpb4 is required for stable interaction of Rpb7 with the RNAPII core, most contacts between Rpb4/7 and the core complex occur between Rpb7 and the Rpb1 and Rpb6 subunits.[32,33] The stalk is located near the RNA exit channel and the Rpb1-CTD. Indeed, two studies showed that the nascent RNA exits making contacts with the Rpb7 subunit [34,35] Then, this location suggests that the stalk might play a role in the recruitment of factors important for RNA biogenesis [9] and/or CTD modification.[36]

Sub1 is a polypeptide of 292 amino acids that shows strong similarity to PC4 (127 amino acids) over a 64-residue region (amino acids 41–105) that includes a ssDNA binding domain (DBD) and sequences essential for co-activator function.[37,38] Although Sub1 is highly related to PC4, yeast Sub1 is much larger. Specifically, Sub1 has an extra carboxy-terminal (CT) region of approximately 190 amino acids with no functions assigned up to date, but suggesting that Sub1 might have functional differences due to this additional region. Like PC4, Sub1 has the capacity to tightly bind melted DNA and ssDNA *in vitro*.[12] The DBD of PC4 and Sub1 are involved in transcriptional activation[12,39,40] and repression,[38,41] and it has been proposed that the CT region is dispensable for the functions of Sub1 related to its DNA binding capacity, such as regulation of *IMD2* transcription and response to DNA damage.[27,41] Regardless, our studies suggest the CT region is necessary for the stability of Sub1 and it could regulate its DNA binding capacity, while interacting with Rpb4/7.[21]

On the other hand, it has been proposed that PC4 phosphorylation negatively impacts its dsDNA binding capacity, thereby promoting its release from promoters.[42] Yeast Sub1 can be also phosphorylated *in vitro*, binding more weakly to DNA than unphosphorylated Sub1.[12] Interestingly, Sub1 has been identified as a phosphoprotein in proteome-wide studies, being three specific amino acids of the CT sequence the target of these phosphorylations.[43] If the phosphorylated form has a role in elongation, this would explain why lower levels of Sub1 associated with coding regions are usually detected.[14,17] Although it is unknown if Sub1 binding capacity is modulated by phosphorylation *in vivo*, our results indicate that it may be regulated, at least, by its extra CT region, where phosphorylated residues are placed.[43]

Sub1 at the initiation stage

Our more recent work presents the first demonstration that Sub1 directly interacts with RNAPII through the Rpb4/7 stalk, and most likely via Rpb7, because Sub1 binds RNAPII in the absence of Rpb4. However, a fully functional Rpb4/7 heterodimer is necessary for Sub1 to stably associate with chromatin after RNAPII recruitment to the PIC.[21] We have proposed a model, where Sub1 is recruited to RNAPII through interaction with Rpb4/7, TFIIB, and DNA.[12-14,16] One hypothesis is that once at the PIC, Sub1 interacts with Rpb4/7 via its CT region to keep associated to RNAPII and chromatin (Fig. 1). This interaction would help to maintain Sub1 associated to gene promoters until the next step in transcription. Supporting this idea, we identified a specific genetic interaction between *sub1ΔCT* and *rpb4Δ*, but not with a mutation altering Sub1 DNA binding, and showed that the association of Sub1ΔCT with gene promoters is significantly reduced in the absence of

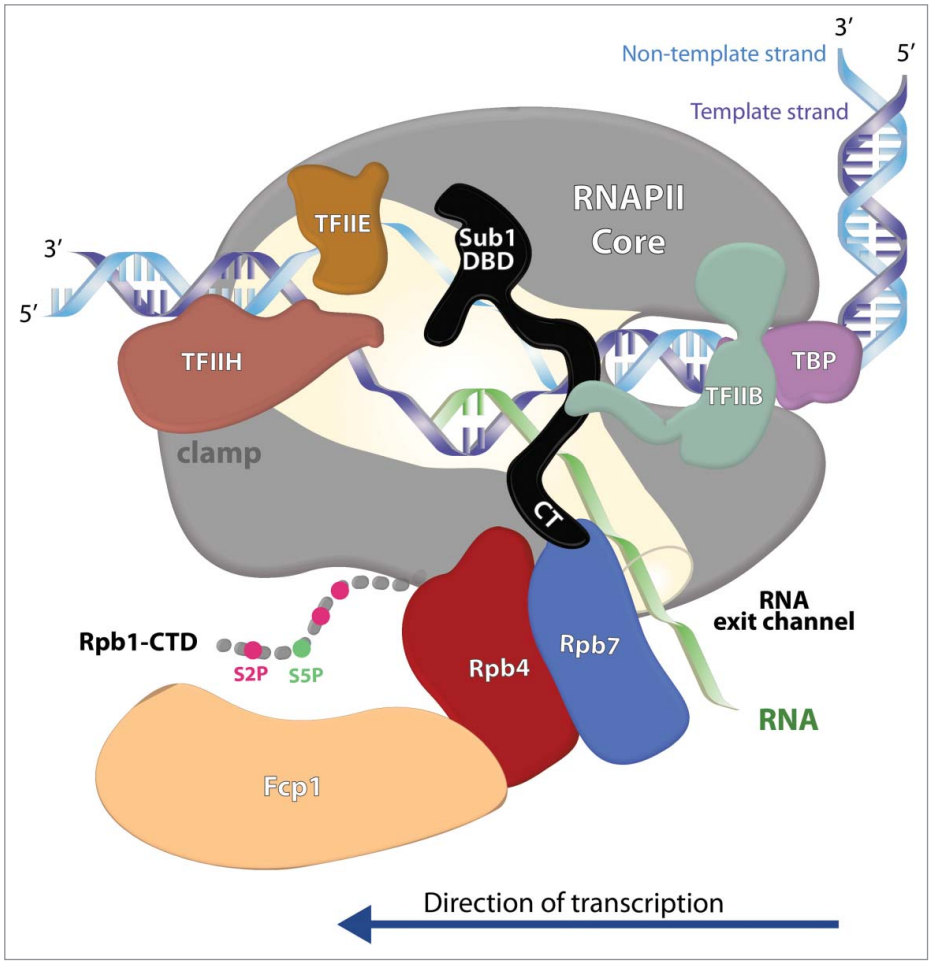

Figure 1. Schematic model showing the hypothetical localization of Sub1 during transcription initiation. Sub1 is bound to the promoter by interacting with upstream DNA at the junction between single- and double-stranded DNA[14] through its DNA Binding Domain (Sub1 DBD) [21]. The proposed localization of Sub1 in this model explains the reported physical and genetic interaction of Sub1 with TFIIB[13,55], as well with TFIIE and TFIIH.[14] The intrinsically-disordered CT region of Sub1 (Sub1 CT) may extend to directly interact with the Rpb4/7.[21] During initiation, Rpb7 contacts with the nascent RNA[34,35]. The genetic interaction between Sub1 and Rpb1 clamp is also illustrated. In addition, the model shows the connections of Fcp1 phosphatase with Rpb4 and Sub1 revealed by our studies[16,21,36]that could occur during the initiation–elongation transition, and consistent with the structural data[56].

RPB4.[21] This is the first evidence for a role of the Sub1-CT region.

With respect to components of the PIC, a direct interaction between Sub1 and TFIIB were described and showed that Sub1 inhibited the formation of the TATA binding protein (TBP)-TFIIB-promoter complexes *in vitro*.[13] Accordingly, TFIIB is required for Sub1 recruitment to the promoters of constitutively transcribed genes,[40] and Sub1 mainly localizes to the promoter region in a manner dependent upon TBP.[14] A model proposed by S. Buratowski and collaborators [14] is that Sub1 is first recruited to the PIC by interactions with transcription factors, likely with TFIIB, and at that point both factors would cooperate in promoter melting. Moreover, they showed that Sub1 is located near the leading edge of the *HIS4* transcription bubble. Hence, in this model, Sub1, upon promoter melting, can interact with the non-template strand or perhaps both strands at the upstream junction between single- and double-stranded DNA. Agreeing with this model, the intrinsically-disordered nature of the Sub1 CT could allow the protein to span the distance between the bubble upstream junction and the RNAPII stalk (Fig. 1). Additionally, genetic data also suggest that Sub1, bound to gene promoters, could help TFIIE and TFIIH to maintain the PIC in a stable, but inactive conformation in the open complex.[14] Comparably, it has been shown that PC4 directly associates with various transcriptional activators, for instance TFIIB and TFIIH, and weakly with TBP. In particular, PC4 participates in the

initiation–elongation transition by binding to melted DNA in collaboration with TFIIE.[44]

Considering more ideas and hypotheses, one possibility is that Sub1 would have effects over the stability of factor interactions established with the clamp domain during initiation and early elongation; for instance cooperating with TFIIE at initiation stage and with Spt5 during elongation. This hypothesis is supported by the fact that *SUB1* deletion causes lethality when combined with the *rpb1-L1397S* mutation localized within the clamp domain,[21,45] and that both TFIIE and Spt5 directly interact with this domain.[46-49] In that sense, it is worthy to mention that during initiation, TFIIE contributes to the formation of the open complex and to the maintenance of the stability of the transcription bubble, thanks to its interaction with the clamp, whose mobility it aids to modulate.[46,47] In Archeae, it has been proposed that the TFIIE homologue TFE could be recruited to the transcription complex via the E/F polymerase subunits (Rpb4/7), and/or TFE could modify the conformation and function of the polymerase via E/F.[6] Furthermore, in these organisms, TFE competes with Spt4/5 for the access to the clamp during the initiation, but after clearance of TFE, Spt4/5, together with E/F, will contribute to the processivity of the polymerase.[6] Sub1 could participate in the replacement of TFIIE by Spt4/5, contributing this way to the initiation–elongation transition.[17]

Sub1 as an elongation factor

In the course of transcription initiation, the clamp controls the entry of dsDNA to the polymerase active center and, throughout elongation it contributes to the separation of the RNA–DNA hybrid at the end of the transcription bubble. For this purpose, the clamp adopts two distinct structural configurations: open, where dsDNA accesses to the active center, and closed, where ssDNA is seated in the active center and facilitates the processivity of the polymerase.[50] Curiously, Rpb7 associates with the polymerase through the interaction with the clamp, restricting its configuration to the closed state.[32] In *S. cerevisiae*, Spt5 extensively interacts with the stalk, and with three other domains formed by Rpb1 and Rpb2: the clamp, protrusion, and wall.[48,49] These interactions may fasten the transcribing DNA onto the central cleft of the RNAPII, as the clamp is in a closed configuration. Thus, Spt5 has been proposed to be crucial for holding the RNAPII complex in a closed conformation that is highly competent for transcription elongation, favoring processivity.[48] In this situation, Sub1 would be recruited to the chromatin in a Rpb4/7 dependent manner, and associate with both complexes, Spt4/5 and Rpb4/7.[17,21]

During transcription initiation, it has been proposed that changes in Sub1, such as phosphorylation[12,42] could reduce its DNA-binding capacity and facilitate promoter clearance.[12-14] During the transition to elongation, Sub1, most likely via its CT, would remain attached to RNAPII through Rpb7,[21] thus joining the elongation complex to influence transcription elongation.[17] This is likely achieved by stabilizing Spt5-Rpb1 association with DNA, because in the absence of Sub1, Spt5-Rpb1 interaction decreases.[17] Accordingly, as mentioned above, *sub1Δ* is synthetically lethal with *rpb1-L1397S*, [21] and also enhances growth phenotypes of the *spt5–194* mutant that alters Spt5 binding to the clamp. In contrast, *SUB1* overexpression suppresses these phenotypes.[17] Thus, we proposed that Sub1 plays a role in the stabilization of the clamp domain, and it is essential when the function of the clamp is altered, as in the *rpb1-L1397S* and *spt5–194* mutants.[17,21] In fact, deletion of *SUB1* has no effect on Spt5 association with chromatin,[17] while *RPB4* deletion affects both Sub1[21] and Spt5 occupancy (unpublished data). Hence, Sub1 interactions with Rpb4/7 and Spt4/5 may concurrently promote closed complex conformation and thus facilitating processivity and transcription elongation (Fig. 2).

A controversial point: Initiation versus elongation

So far, we have focused on the idea that the biological meaning of *rpb1-L1397S sub1Δ* synthetic lethality is directly linked to participation of Sub1 in elongation. It is instead possible this genetic interaction could be explained as a direct consequence of Sub1 role in initiation. In fact, Sub1 is involved in TSS selection,[15] and its association with coding region significantly decreased with respect to promoters. Besides, in *rpb1-L1397S* cells, *IMD2*, and *URA2* are upregulated, which is a hallmark of a defect on TSS selection.[45] However, several facts support that Sub1s role in elongation is linked to the clamp function: (1) the clamp domain is essential for RNAPII processivity, and genome wide association of RNAPII with coding regions is reduced in *rpb1-L1397S*

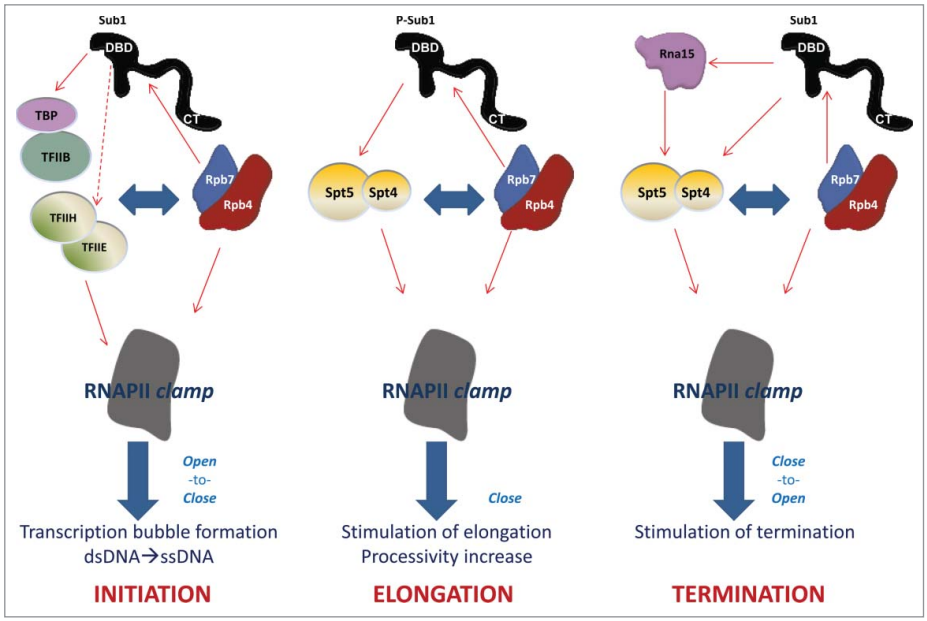

Figure 2. A working model: Sub1 function is linked to the stalk and clamp domains, assisting transcription and processing factors during the whole transcription cycle. See the text for detail. Continuous-line arrows indicate physical interaction/co-purification while discontinuous lines indicate genetic interaction.

cells.[45] (2) Sub1 promotes elongation rate and splicing, in association with Spt5,[15,16] which in turn interacts with the clamp.[48,49] Indeed, Sub1 effect on splicing was corroborated in a nice study that also showed that Sub1 is involved TSS selection.[15] In this study, two groups of RNAPII mutants were identified: one that preferentially initiates upstream and exhibits an increased rate of transcription and other initiating downstream and transcribing slowly. They showed that *sub1Δ* exacerbated slow RNAPII alleles and partially suppressed fast RNAPII alleles. They revealed that fast RNAPII mutations resulted in upstream transcription start and diminished splicing, whereas slow mutations or *SUB1* deletion gave rise to downstream transcription start and enhanced splicing. More interestingly, they showed that only deletion of *SUB1*, and not the *sua7–3* mutation, that also exhibits defects on TSS selection, correlates with splicing defects, as do slow and fast RNAPII mutants. Thus, the catalytic rate of RNAPII has effects on TSS selection. Therefore, Sub1 effects on TSS and elongation could be also due to its role influencing RNAPII rate,[17] which is closed related to the clamp domain function. Consistently, both, the speed of the polymerase and Sub1, impact on the selection of the TSS, splicing, and transcription elongation[15,17]. Furthermore, we have also shown that, though in *sub1Δ* the *IMD2* gene is upregulated, there is also a decrease in *de novo* synthesis of *IMD2* transcript due to defects on elongation [17] and this may be the case for *rpb1-L1397S*. In fact, this mutant is sensitive to 6AU.[45] In any case, we cannot discard the possibility that the lethality of the *sub1Δ rpb1-L1397S* double mutant is a consequence of defects on transcription start site selection.

Sub1 participates in transcription termination

Rpb4 contributes to co-transcriptional recruitment of 3′-end processing factors,[9] and in Archea, E/F stimulates transcription termination dependent on U-rich sequences.[6] The location of Rpb4/7 near the CTD [32] and the fact that Rpb7 contact the emerging RNA [34,35] let us and others[9,36] to propose that the stalk is in a perfect position to stabilize interactions with 3′-end processing factors, the CTD and the polyadenylation site sequences within the nascent transcript. Again, S. Buratowski studies[9] suggested that such stabilization could be mediated by a direct interaction of Rpb4/7 with Rna15, a component of the cleavage factor I (CFI), involved in termination and polyadenylation, with the RNA and with an intermediary factor. Could be this intermediary factor Sub1? Accordingly, on one hand, Sub1 interacts with Rna15, and this interaction is evolutionary conserved in human cells. Strong evidences suggested that Sub1 could exert an anti-terminator function, avoiding premature termination of pre-mRNAs while inhibiting Rna15 function.[19]

Furthermore, Sub1 directly interacts with Rpb7,[51] which in turns contacts the nascent RNA.[34,35] Hence, Sub1 is properly placed to influence pre-mRNA processing, while interacting with the stalk. On the other hand, Rpb4 and Spt4/5 are required for Rna15 recruitment.[9,19,20,52] Because in the absence of Sub1, the Spt5-RNAPII interaction is unstable, it might be that the recruitment and function of the CFI complex would be altered. Then, we can visualize a model for Sub1 role during transcription termination that could imply a new change in the conformation of Sub1 and its localization within the transcription complex or even Sub1 exit. Thus, for instance, its dephosphorylation, coincident with RNAPII dephosphorylation by Fcp1, could relieve the inhibition over Rna15, allowing termination.[19] Simultaneously, this dephosphorylation could promote Sub1 exit from the complex, destabilizing Spt4/5-RNAPII interaction and bring about transcription termination.[52] Hence, Sub1 would not only contribute to the initiation–elongation transition, but also to the elongation–termination transition, participating in transcription regulation from PIC formation to transcription termination.

New insights to understand how Sub1 modulates Rpb1-CTD phosphorylation

The location of Rpb4/7 within the RNAPII complex near Rpb1-CTD hinted that it may play a role in the modulation of its modifications.[32] Indeed, we earlier demonstrated that Rpb4/7, as well as Sub1, have a role in regulating RNAPII CTD phosphorylation levels.[18,36] Rpb4 modulates the functionality of Fcp1,[36] and Sub1 associates with Fcp1, and is required to maintain proper Fcp1 protein levels.[16] Interestingly, we know now that a full length CTD is a requisite for Sub1 association with RNAPII and with chromatin.[21] Altogether, our data provide significant new insight into the relationship among Sub1, Fcp1, Rpb4/7, and the modulation of Rpb1-CTD phosphorylation at least during elongation and termination, which crucially regulates the biogenesis of mRNAs, and RNAPII recycling.[3,4]

A model for Sub1 role during the whole transcription cycle

The data accumulated over the years about Sub1 indicate that Sub1 binds DNA through its rminal DBD, while is stably maintained in the RNAPII-DNA complex by binding to Rpb4/7 via its CT region (Fig. 1). In addition, Sub1 interaction with the stalk and polymerase clamp domains may help to explain how it may influence TSS selection[15] and transcription elongation rate.[17] Similarly, Sub1 association with Rpb4/7 and Spt4/5 offers an insight into Sub1 role during transcription termination; and its localization within RNAPII helps to understand how it may influence Rpb1-CTD phosphorylation. Consistent with all that data, a working model for Sub1 function, linked to Rpb4/7 and the configuration of the clamp (Fig. 2), could be the following: During initiation, Sub1 is recruited to promoters by TFIIB and Rpb4/7, where it helps to maintain the PIC in a stable but inactive conformation (open), for instance collaborating with TFIIE and TFIIH.[14,18] Here, Sub1 binds DNA through its N-terminal DBD, while is stably maintained in the RNAPII-DNA complex by binding to Rpb4/7 via its CT region (Fig. 1). In this situation, Sub1 could be near the emerging RNA, which make contacts with Rpb7.[34,35] After PIC activation, the transcription complex undergoes a conformational change that allows TFIIE exit, and its replacement by Spt4/5, which binds to the clamp and Rpb4/7, assuring the polymerase closed conformation and promoting processivity. At this point, RNAPII is being phosphorylated and transcription starts.[3,4] Then, during elongation Sub1 would interact with Spt4/5 (maybe both as phosphoproteins[42,43,53]), stabilizing Spt5-Rpb1 association with DNA. This could help to decrease the time that this complex is paused immediately after the initiation–elongation transition and will positively influence the transcription elongation rate.[17] Moreover, extensive and specific phosphorylation events targeting RNAPII,[3,4] Spt5,[53] and Sub1 (P-Sub1, Fig. 2) [12,42,43,54] could take place simultaneously and ultimately regulate the initiation–elongation transition and, therefore, transcription elongation. Finally, during termination, Sub1 and Spt4/5 would promote, together with Rpb4/7 the activity of termination and 3′-end processing factors, such as CFI (for instance, Rna15),[9,16,20,52] as well as clamp opening and transcription termination. One possibility is that Sub1 dephosphorylation and/or Sub1-associated factors would trigger its dissociation from Spt4/5 and Rna15, favoring transcription termination and 3′-end processing.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

I thank all people from my lab that contribute to the knowledge of Sub1 functions, and especially to M. Garavís. I also thank C. Fernández-Tornero for its support over the last years; and I would like to express my gratitude to S. Buratowski for reading this manuscript, for his comments, and for his always invaluable help.

Funding

O.C. acknowledges the Spanish Ministry of Economy and Competitiveness (MINECO; [BFU2013–48374-P]) for funding.

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