

# Sub1 associates with Spt5 and influences RNA polymerase II transcription elongation rate

Alicia García, Alejandro Collin, and Olga Calvo

Instituto de Biología Funcional y Genómica, Consejo Superior de Investigaciones Científicas/Universidad de Salamanca, 37007 Salamanca, Spain

**ABSTRACT** The transcriptional coactivator Sub1 has been implicated in several steps of mRNA metabolism in yeast, such as the activation of transcription, termination, and 3'-end formation. In addition, Sub1 globally regulates RNA polymerase II phosphorylation, and most recently it has been shown that it is a functional component of the preinitiation complex. Here we present evidence that Sub1 plays a significant role in transcription elongation by RNA polymerase II (RNAPII). We show that *SUB1* genetically interacts with the gene encoding the elongation factor Spt5, that Sub1 influences Spt5 phosphorylation of the carboxy-terminal domain of RNAPII largest subunit by the kinase Bur1, and that both Sub1 and Spt5 copurify in the same complex, likely during early transcription elongation. Indeed, our data indicate that Sub1 influences Spt5-Rpb1 interaction. In addition, biochemical and molecular data show that Sub1 influences transcription elongation of constitutive and inducible genes and associates with coding regions in a transcription-dependent manner. Taken together, our results indicate that Sub1 associates with Spt5 and influences Spt5-Rpb1 complex levels and consequently transcription elongation rate.

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## INTRODUCTION

Transcription by RNA polymerase II (RNAPII) can be divided into the following steps: RNAPII recruitment to gene promoters and the assembly of the preinitiation complex (PIC) and promoter clearance, initiation, elongation, and termination. For many years, the coordinated recruitment of the transcriptional machinery to form the PIC has been considered to be the principal step for the regulation of transcription. However, recent data show that the elongation phase is also highly regulated in eukaryotic cells and strictly coordinated with other nuclear processes, such as remodeling, DNA repair, recombination, and replication. In fact, in addition to the basal elongation factors, a number of factors involved in mRNA

processing, chromatin modification, remodeling, and mRNA export are also recruited during transcription elongation (reviewed in Shilatifard *et al.*, 2003; Saunders *et al.*, 2006; Selth *et al.*, 2010). In *Saccharomyces cerevisiae*, these factors include the basal elongation factors TFIIS and the Spt4/Spt5 complex, the chromatin assembly factor Spt16/Pob3 (known as FACT in higher eukaryotes), and the polymerase-associated factor (PAF) complex (Shilatifard *et al.*, 2003; Saunders *et al.*, 2006; Selth *et al.*, 2010). Spt5 (NusG in bacteria) is the only RNA polymerase-associated factor that is conserved in all living organisms (Vassylyev *et al.*, 2002; Korkhin *et al.*, 2009; Grohmann and Werner, 2011). It contains a conserved NGN domain that stimulates transcription elongation and RNAP processivity (Burova *et al.*, 1995; Chen *et al.*, 2009). In the case of Eukarya and Archaea, this domain binds to the RNAP and Spt4 to form the Spt4/5 elongation complex (Guo *et al.*, 2008; Hirtreiter *et al.*, 2010; Klein *et al.*, 2011; Martinez-Rucobo *et al.*, 2011).

Transcription elongation is regulated not only by elongation factors but also by the phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNAPII. Phosphorylation of the CTD by CTD-modifying enzymes occurs predominantly on the Ser-2 and Ser-5 residues (Sims *et al.*, 2004; Saunders *et al.*, 2006). The level of Ser-5 phosphorylation peaks early in the transcription cycle due to the action of Kin28 and remains constant or decreases as RNAPII progresses to the 3' end of the gene (Komarnitsky *et al.*, 2000;

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Address correspondence to: Olga Calvo ([ocalvo@usal.es](mailto:ocalvo@usal.es)).

Abbreviations used: 6AU, 6-azauracil; ChIP, chromatin immunoprecipitation; CTD, carboxy-terminal domain; GLAM, gene-length-dependent mRNA accumulation; MPA, mycophenolic acid; PIC, preinitiation complex; RNAPII, RNA polymerase II; Sub1-HA, Sub1-hemagglutinin; TAP, tandem affinity purification; TRO, transcriptional run on; TSS, transcription start site.

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Rodriguez *et al.*, 2000; Schroeder *et al.*, 2000). In contrast, Ser-2 phosphorylation is the predominant modification in the body and toward the 3' end of the gene and occurs concurrently with productive elongation (Komarnitsky *et al.*, 2000; Buratowski, 2009). Ctk1 is the principal kinase responsible for Ser-2 phosphorylation, which is important for the recruitment of the histone methyltransferase Set2 and the association of the polyadenylation and termination factors with RNAPII (Cho *et al.*, 2001; Ahn *et al.*, 2004). In addition to Ctk1, the Bur1/Bur2 kinase complex phosphorylates Ser-2 when RNAPII is near the promoter and stimulates Ser-2 phosphorylation by Ctk1 during elongation (Qiu *et al.*, 2009). Bur1 also phosphorylates the histone modifier Rad6/Bre1 (Wood *et al.*, 2005) and the carboxyl-terminal domain of the elongation factor Spt5, which stimulates the recruitment of the PAF1 elongation complex (Liu *et al.*, 2009; Zhou *et al.*, 2009). In addition, we recently showed that the transcriptional coactivator Sub1 globally regulates RNAPII-CTD phosphorylation during the entire transcription cycle through the action of all CTD kinases (Garcia *et al.*, 2010), suggesting that it might also have a role in transcription elongation.

Sub1 has been implicated in several steps of mRNA metabolism, including initiation, transcription termination, and 3'-end processing (Henry *et al.*, 1996; Knaus *et al.*, 1996; Calvo and Manley, 2001; He *et al.*, 2003). It was originally described as a transcriptional stimulatory protein that is homologous to the human positive coactivator PC4 (Ge and Roeder, 1994; Kaiser *et al.*, 1995; Malik *et al.*, 1998). Sub1 genetically and physically interacts with TFIIB (Henry *et al.*, 1996; Knaus *et al.*, 1996), and most recently it has been reported that Sub1 is a PIC component (Sikorski *et al.*, 2011). In addition, Sub1 contributes to the activation of osmoresponse genes during osmotic shock (Rosonina *et al.*, 2009) and to the regulation of *IMD2* gene expression (Koyama *et al.*, 2008). Moreover, it has been suggested that Sub1 might have a role in transcription elongation based on the following observations: 1) Sub1 function is linked to CTD elongation kinases (Calvo and Manley, 2005; Garcia *et al.*, 2010); 2) *SUB1* genetically interacts with *FCP1*, which encodes the Ser-2 CTD phosphatase Fcp1 (Calvo and Manley, 2005); 3) cells lacking Sub1 display decreased accumulation of Fcp1, altered RNAPII phosphorylation, and decreased cross-linking of RNAPII to transcribed genes (Calvo and Manley, 2005).

Here we present evidence that Sub1 plays a role in transcription elongation. We show that *SUB1* genetically interacts with *SPT5*. Sub1 regulates Spt5 phosphorylation by the CTD kinase Bur1, and both Sub1 and Spt5 copurify in the same complex, likely during early transcription elongation. Indeed, our data indicate that Sub1 influences Spt5-Rpb1 levels. We also show that Sub1 promotes transcription elongation of constitutive and inducible genes and that it associates with coding regions in a transcription-dependent manner. Taken together, our results indicate that Sub1 associates with Spt5 and influences Spt5-Rpb1 complex levels and consequently transcription elongation rates.

## RESULTS

### Sub1 influences the efficiency of transcription elongation of long transcripts

We previously presented evidence suggesting that Sub1 could indirectly influence transcription elongation via its effects on the association of RNAPII with chromatin during active transcription and on the phosphorylation of the RNAPII CTD (Calvo and Manley, 2005; Garcia *et al.*, 2010). Therefore we first investigated whether Sub1 indeed is able to influence transcription elongation, using several different strategies: we studied elongation efficiency by the gene-length-dependent mRNA accumulation (GLAM) assay, *IMD2* gene

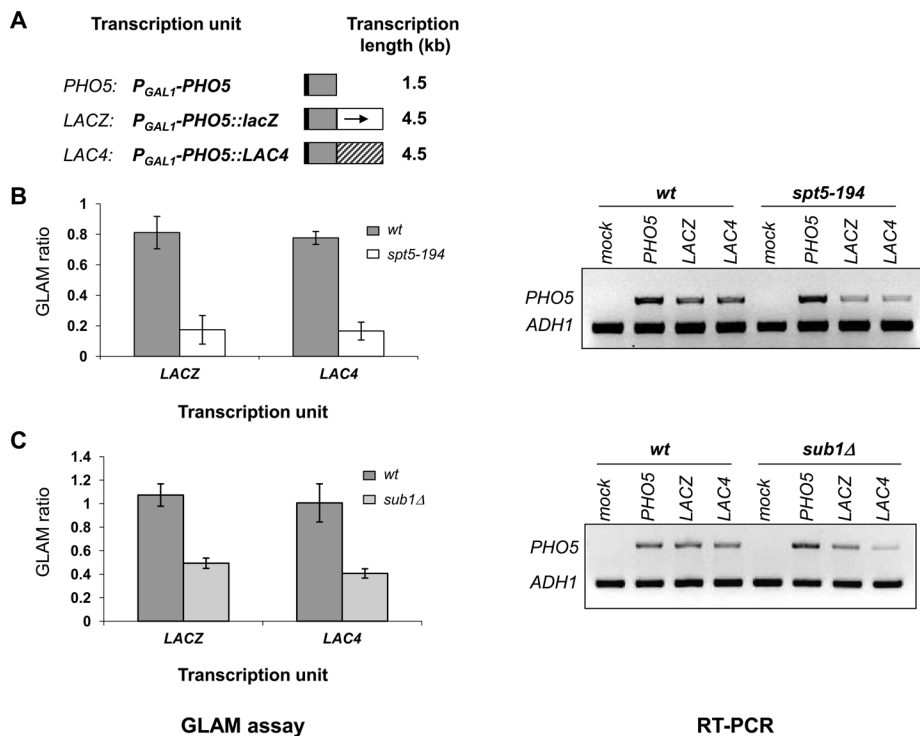
expression by real-time quantitative PCR (RT-qPCR), RNAPII association with constitutive and regulated genes by chromatin immunoprecipitation (ChIP), and levels of active elongation-competent polymerase by transcriptional run-on (TRO) assay.

The GLAM assay specifically detects defects in elongation and is used to measure the efficiency of gene-length-dependent mRNA accumulation (Morillo-Huesca *et al.*, 2006). The wild-type (*wt*) and *sub1Δ* strains were transformed with three plasmids containing transcription units of different lengths, with the same *PHO5* coding sequences under control of the *GAL1* promoter and variable 3' untranslated sequences (Figure 1A). The ratios of acid phosphatase activity for the long transcripts versus the short transcripts (GLAM ratios) were calculated for each strain (Figure 1C). We also analyzed the *spt5-194* mutant, as a positive control for elongation defects, and the corresponding *wt* strain. As shown in Figure 1B (left), the GLAM ratio for *spt5-194* was dramatically reduced compared with the *wt* ratio, consistent with its function as an elongation factor. Similar values have been reported for other elongation factor mutants, such as *spt4Δ*, and for PAF1 complex mutants (Morillo-Huesca *et al.*, 2006; Gaillard *et al.*, 2009). Of interest, the GLAM ratio for *sub1Δ* cells was also significantly reduced (Figure 1C, left) but to a lesser extent than in *spt5-194* cells (Figure 1B, left). The gene-length-dependent defect observed with *spt5-194* and *sub1Δ* cells was further confirmed by RT-PCR assays performed with cDNA generated from *spt5-194*, *sub1Δ*, and the two corresponding *wt* strains bearing the different transcription units or an empty plasmid as a mock control (Figure 1, B and C, right). Our results showed that *PHO5* expression decreased in both *spt5-194* and *sub1Δ* cells when expressed as long transcription units containing either the *lacZ* or *LAC4* 3' untranslated sequences but was not affected when *PHO5* was transcribed from the short transcription unit.

### Sub1 promotes transcription elongation and associates with coding regions

The *IMD2* gene encodes a rate-limiting enzyme in the de novo synthesis of GTP (IMP dehydrogenase 2), and its expression is increased by treatment with the GTP-depleting drugs mycophenolic acid (MPA) and 6-azauracil (6AU; Shaw and Reines, 2000; Escobar-Henriques *et al.*, 2001). Although their mechanism is not well understood (Jenks *et al.*, 2008; Kuehner and Brow, 2008), these compounds have been widely used to identify proteins involved in transcription elongation (Shaw and Reines, 2000; Desmoucelles *et al.*, 2002; Riles *et al.*, 2004). In fact, mutations in several of the genes encoding components of the transcriptional elongation machinery confer sensitivity to 6AU in *S. cerevisiae* due to the resulting inability to induce *IMD2* transcription (Shaw and Reines, 2000; Riles *et al.*, 2004). *IMD2* transcription is regulated by the selection of different start sites. Upstream start sites lead to short, noncoding transcripts that are prematurely terminated and degraded in noninducing conditions, whereas a downstream start is selected upon nucleotide limitation, leading to *IMD2* induction (Jenks *et al.*, 2008; Kuehner and Brow, 2008).

It has been proposed that Sub1 represses *IMD2* gene expression upon binding to the promoter in noninduced conditions (Koyama *et al.*, 2008), in which only upstream short, noncoding transcripts are synthesized and *IMD2* is not transcribed. In fact, deletion of *SUB1* increases the expression of *IMD2* in normal growth conditions, whereas its expression is avoided in *wt* cells (Koyama *et al.*, 2008). However, Sub1 effects on *IMD2* expression may be explained in part by a mechanism involving transcription start site selection (TSS), consistent with recent data showing that *SUB1* deletion alters TSS selection (N. Krogan, personal communication). Thus, in the absence of



**FIGURE 1:** Deletion of *SUB1* leads to altered transcription elongation efficiency of long transcripts. (A) Schematic representation of the three transcription units used in the GLAM assays. (B) The wt and *spt5-194* strains were transformed with the plasmid bearing the three transcription units shown in A. GLAM ratios were calculated as the relative levels of acidic phosphatase activity expressed by the indicated transcription unit ( $GAL$ -PHO5 LACZ or  $GAL$ -PHO5 LAC4) with respect to the acid phosphatase activity from the shortest transcription unit ( $GAL$ -PHO5). Left, a representation of the GLAM ratios. Right, the RT-PCR results of PHO5 expression from the three transcription units and from the mock strain bearing an empty plasmid. ADH1 was used as loading control. PCR products were run on an ethidium bromide-stained gel. (C) GLAM ratios and RT-PCR results for the wt and *sub1Δ* strains.

Sub1 in noninduced conditions, transcription from the downstream start site may be preferred, resulting in constitutive *IMD2* expression. This explains why *sub1Δ* cells are resistant to MPA or 6AU treatment in solid media (Koyama et al., 2008; Garcia et al., 2010). However, we observed that *sub1Δ* cells have reduced growth in liquid media containing 6AU and that the *SUB1* deletion conferred high sensitivity to 6AU when combined with two *bur1* mutations (Garcia et al., 2010). These data suggest that Sub1 could also have a role during transcription elongation. Indeed, recently it was shown that Rpb1 mutations in the trigger loop cause sensitivity or resistance to MPA, which correlates with a shift to upstream or downstream *IMD2* start sites and with increased or reduced elongation rates, respectively (Kaplan et al., 2012).

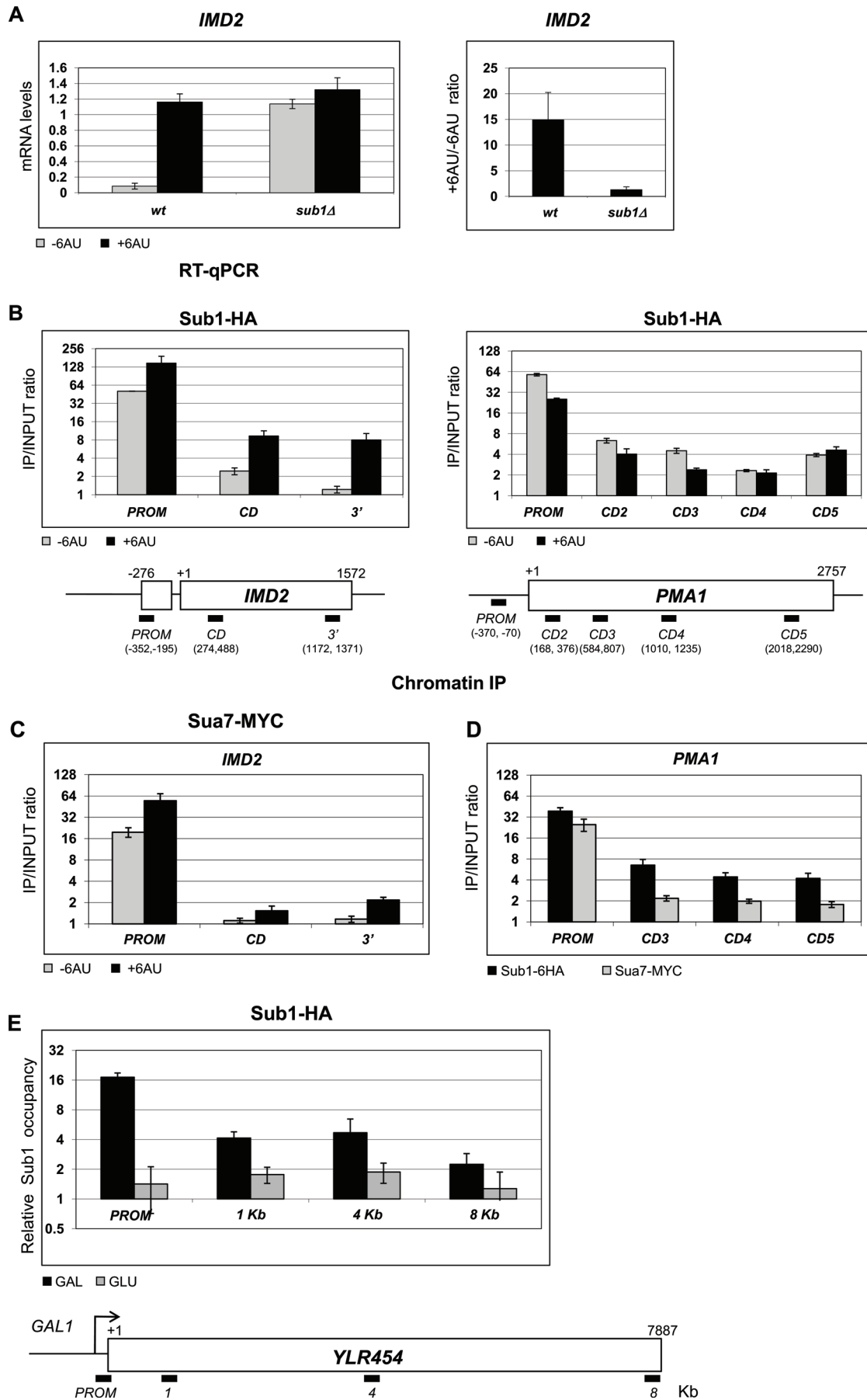
Therefore we first decided to analyze *IMD2* expression in wt and *sub1Δ* cells in the presence or absence of 6AU. As shown in Figure 2A (left), *IMD2* expression increased by more than 10-fold in *sub1Δ* cells compared with wt cells in normal growth conditions. When cells were treated with 6AU, *IMD2* mRNA levels increased significantly in wt cells as expected but did not further increase in *sub1Δ* cells (Figure 2A, left). This is more evident when we calculated the +6AU/-6AU ratios (Figure 2A, right). Then, this result indicates that Sub1 effect on *IMD2* expression is epistatic to 6AU activation. In addition, the lack of further activation of *IMD2* by 6AU in *sub1Δ* cells is in agreement with the fact that these cells are sensitive to 6AU when grown in liquid media and that the *SUB1* deletion enhanced the 6AU sensitivity of *bur1* mutants (Garcia et al., 2010). However,

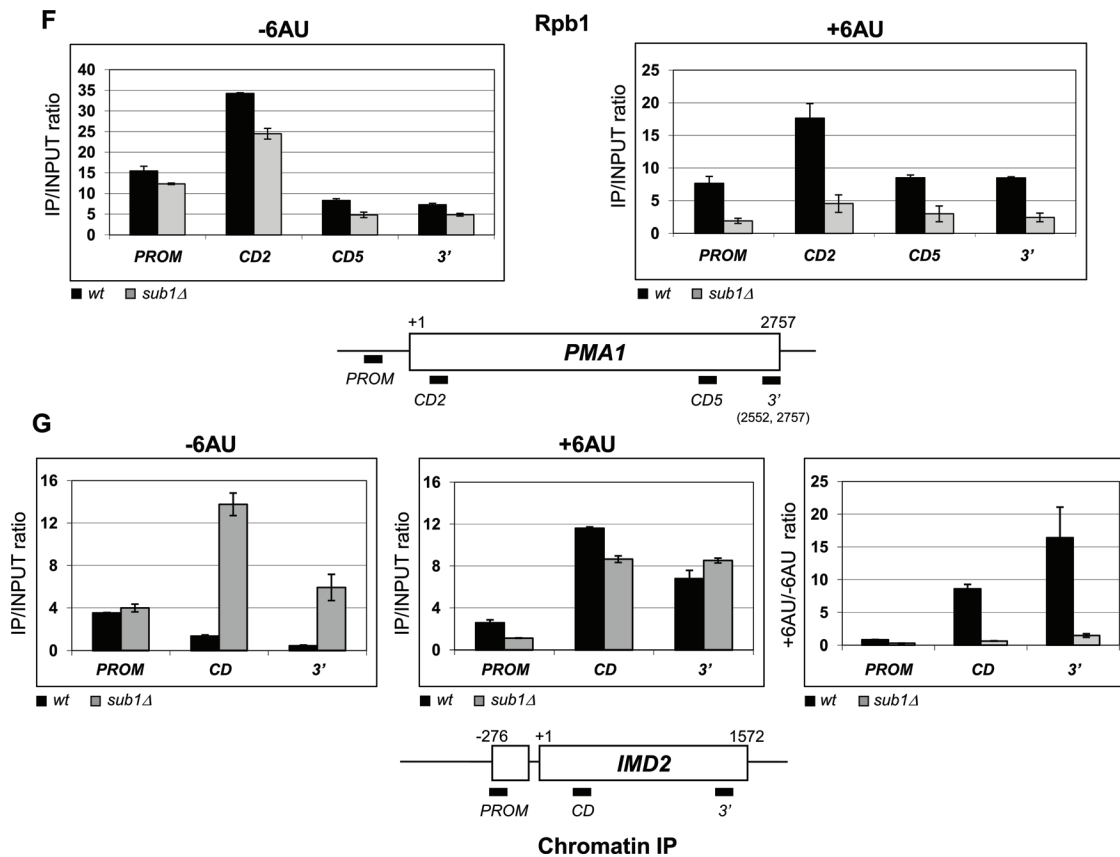
we cannot rule out that this effect on *bur1* cells can be gene specific because we did not analyze *IMD2* expression levels in the *bur1* and *bur1sub1Δ* cells. In any case, our data suggest that full expression of the *IMD2* gene after 6AU induction may be influenced by Sub1 function, and in addition to its involvement in TSS selection, when bound to the promoter in noninduced conditions and when short transcripts from the *IMD2* promoter are being transcribed, Sub1 might also have a role farther downstream during *IMD2* elongation after induction.

To investigate further whether Sub1 participates in transcription elongation, we analyzed the association of Sub1-hemagglutinin (HA) with the *IMD2* and *PMA1* promoters and coding regions in the presence or absence of 6AU. As shown in Figure 2B, and as previously reported, Sub1-HA associated with the *PMA1* and *IMD2* promoters under normal growth conditions (Calvo and Manley, 2005; Koyama et al., 2008). The association of Sub1-HA with the *IMD2* coding region was near the lower limit of detection in the absence of 6AU (Figure 2B, left), and it was almost undetectable in the 3'-end region. Conversely, Sub1-HA was found to be associated with the constitutively transcribed *PMA1* gene from the promoter to the 3'-end regions (Figure 2, B, right, and D; Calvo and Manley, 2005; Sikorski et al., 2011). Curiously, we observed that after 6AU treatment, Sub1-HA association with the *IMD2* promoter increased (Figure 2B, left; note that the graphs are presented on a logarithmic scale), whereas its association with the *PMA1* promoter decreased (Figure 2B, right). In addition, we detected an increase in the association of Sub1-HA with *IMD2* coding and the 3'-end regions of ~4 and 6.5 times, respectively, above the background levels in the noninduced conditions (Figure 2B, left) and a decrease in the association with the *PMA1* 5'-coding regions (Figure 2B, right).

We also analyzed the association of TFIIB (Sua7) with chromatin as a negative control (Figure 2C) because TFIIB is a well-characterized PIC component with no role in transcription elongation and close genetic and functional connections with Sub1 (Knaus et al., 1996; Wu et al., 1999; Rosonina et al., 2009). For this purpose, we used a TFIIB-MYC strain for ChIP assays. As expected, TFIIB only associates efficiently with the *IMD2* promoter region regardless of 6AU treatment, although a slight increase in TFIIB association above background was observed at the 3'-end after induction, possibly due to its role in gene looping (Singh and Hampsey, 2007). In the case of the *PMA1* gene, both Sub1 and TFIIB associate with the promoter, but only Sub1 shows an association that is significantly above background with coding regions (Figure 2D). These results provide strong evidence that Sub1 plays a role in *IMD2* transcription elongation under induction conditions and may also have a role during transcription elongation of the constitutive *PMA1* gene.

We next analyzed Sub1 association with the long transcription unit of the *YLR454* gene (8 kb), which is expressed under the control of the *GAL1* promoter (Mason and Struhl, 2005). The purpose of this experiment was to provide evidence that the signal over the





**FIGURE 2:** Sub1 promotes transcription elongation, associates with coding regions, and influences Rpb1 association to chromatin during transcription elongation. (A) RT-qPCR analysis of the *IMD2* gene. Total RNA was isolated from wt and *sub1Δ* cells grown in SC-URA with or without 50 μg/ml 6AU. cDNA synthesis was performed and coupled with qPCR. *IMD2* expression values were normalized to the 18S rRNA values and graphed. The relative levels of the *IMD2* mRNA in *sub1Δ* vs. wt cells are shown. (B) ChIP analysis of Sub1-HA was performed in the wt strain using an anti-HA antibody. Cross-linking to the *IMD2* (left) and *PMA1* (right) promoter and coding regions in the presence or absence of 100 μg/ml of 6AU was analyzed by qPCR. Quantifications of the results are shown in the graph, where the numbers on the y-axis represent the ratio of the values obtained from specific primer products to the negative control (intergenic region of chromosome VII) and normalized to the input controls. A schematic representation of the *IMD2* and *PMA1* genes is shown below the corresponding graph; the PCR primers pairs corresponding to the promoter and coding regions at the specified positions are indicated. In the case of the *IMD2* gene, the upstream white box represents the promoter transcribed region leading to short, noncoding transcripts (Jenks et al., 2008). (C) ChIP assay to analyze the Sua7-MYC association with the *IMD2* gene as in B. (D) ChIP analysis of the Sub1-HA and Sua7-MYC association with the *PMA1* gene. (E) Sub1-HA occupancy during galactose induction and after 4 min of glucose treatment. A schematic representation of the *GAL1-YLR454* gene; the PCR primers pairs corresponding to the promoter and coding regions at the specified positions are indicated. (F) Rpb1 occupancy at the promoter and coding regions of the *PMA1* gene was assayed by ChIP in wt and *sub1Δ* cells in the presence or absence of 100 μg/ml of 6AU with the 8WG16 antibody. Real-time qPCR quantifications were graphed as described. (G) Rpb1 occupancy at the promoter (left) and coding (middle) regions of the *IMD2* gene assayed as in F. Right, the +6AU/-6AU ratio. Rpb1 cross-linking to promoter and coding regions was independently quantified in wt and *sub1Δ* cells, and the +6AU/-6AU ratio was then calculated and graphed.

genes is meaningful and not due to a decrease in the strong promoter signal over distance as a consequence of the average chromatin shearing (~300 base pairs). In addition, by analysis of the Sub1 association with a long gene, the coding region signal can be separated from a promoter signal due to any tandem-oriented downstream gene near the 3'-end region. As shown in Figure 2E, Sub1-HA associates with coding regions during galactose induction and disassociates after glucose-mediated transcriptional repression. The Sub1 association with chromatin remains significant as far as 4 kb downstream from the promoter, whereas its association with the most 3'-end is slightly above background. It is worth noting that most yeast genes are ~1.5–2 kb long, and genes as

long as *YLR454* are rarely found in *S. cerevisiae*. Nonetheless, this result provides further evidence that Sub1 associates with at least the coding regions of average size and even longer genes (e.g., *PMA1*) in *S. cerevisiae* and further supports the hypothesis that Sub1 may have a role during transcription elongation.

We also investigated whether Sub1 has a role in transcription elongation by analyzing Rpb1 occupancy from the promoter to 3'-end regions of the *IMD2* and *PMA1* genes in both wt and *sub1Δ* cells in the presence or absence of 6AU. We (Calvo and Manley, 2005; Garcia et al., 2010) and others (Rosonina et al., 2009; Sikorski et al., 2011) have already reported that there is decreased association of RNAPII with the chromatin of constitutively transcribed genes

in the promoter and coding regions in cells lacking Sub1. Here we extended our study to the *IMD2* gene, regulated by guanine nucleotide levels. In agreement with our previous data, we observed a reduction in the association of Rpb1 with the *PMA1* gene in *sub1Δ* cells compared with *wt* cells when grown under normal conditions, and this reduction was further enhanced with 6AU treatment (Figure 2F). Rpb1 association with the coding and 3'-end regions of the *IMD2* gene was undetectable in *wt* cells, as expected, in accordance with *IMD2* repression in normal growth conditions (Figure 2G, left). However, after 6AU treatment, Rpb1 was efficiently recruited to *IMD2* coding regions. The opposite was observed when we analyzed Rpb1 association with the *IMD2* gene in *sub1Δ* cells (Figure 2G, middle). That is, an increase of Rpb1 association with the coding regions of *IMD2* in noninduced conditions and a reduction after induction was observed. However, this reduction is not further enhanced at the 3'-end regions. In any case, the Rpb1 ChIP data completely paralleled the *IMD2* expression levels (Figure 2A) because there was a decrease in the association of Rpb1 with the *IMD2* gene along with a reduction of *IMD2* mRNA after 6AU treatment in *sub1Δ* cells compared with *wt* cells. In fact, when we calculated the +6AU/−6AU ratio of Rpb1 association (Figure 2G, right), we clearly observed a dramatic reduction along the entire gene in *sub1Δ* cells compared with *wt* cells.

In summary, our results indicate that the reduction in nucleotide levels after 6AU treatment causes transcription elongation to become more dependent on the presence of Sub1. This is supported by the fact that Sub1-HA associates with the *IMD2* coding regions during transcription elongation and the fact that Rpb1 association to chromatin is reduced in *sub1Δ* cells during transcription elongation after induction. Taken together, our data support the hypothesis that Sub1 influences transcription elongation, although they do not definitively indicate that Sub1 plays a direct role in that process. In fact, as *SUB1* deletion affects *IMD2* expression, and thus nucleotide levels could be lower in *sub1Δ* cells, suggesting the possibility that the effect of Sub1 on transcription elongation could be indirect through *IMD2*.

### Sub1 influences RNAPII transcription elongation rate

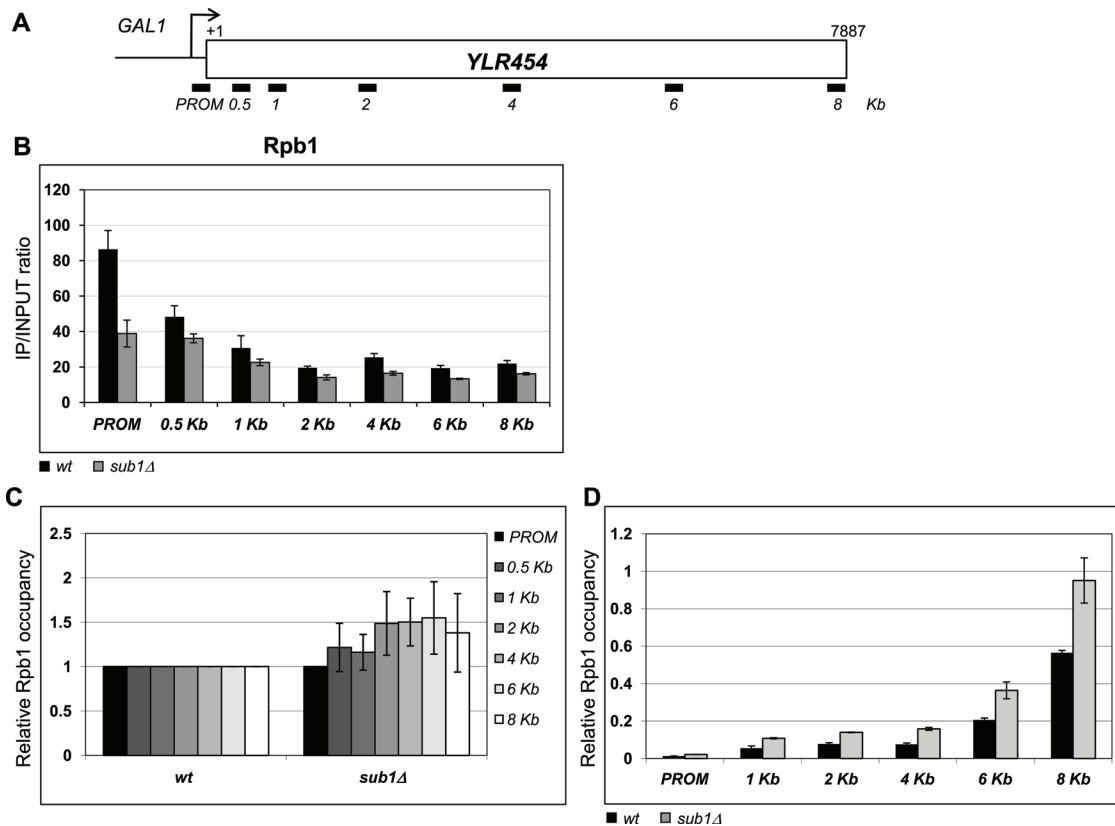
Mason and Struhl (2005) developed an assay to measure two distinct aspects of the elongation process: the rate of transcription and the processivity of RNAPII. They showed that for RNAPII processivity, mutations on the well-known transcription elongation factors fall into three categories: 1) mutations that reduce RNAPII processivity; 2) mutations that reduce processivity only in the presence of 6AU; and 3) mutations that do not affect processivity. Transcription factors with roles in transcription elongation have subsequently been described (Chanarat *et al.*, 2011; Gonzalez-Aguilera *et al.*, 2011; Kruk *et al.*, 2011). For example, the Ccr4–Not complex belongs to a new category because mutations on the subunits of Crc4–Not cause increased RNAPII processivity and reduced elongation rate (Kruk *et al.*, 2011). Furthermore, a mutation on the Spt5 elongation factor (*spt5-242*) affects the transcription elongation rate without affecting processivity (Quan and Hartzog, 2010). We used the same assay as Mason and Struhl (2005) to measure the level of RNAPII association with various positions within the 8 kb of the *GAL1-YLR454* fusion gene, inducible by galactose in *wt* and *sub1Δ* cells (Figure 3A). Our data showed that, as in the case of the *PMA1* gene, RNAPII levels are decreased from the promoter to the 3' end in *sub1Δ* cells when compared with *wt* cells (Figure 3B). However, the decrease of Rpb1 density in the 3'-end regions is significantly less pronounced than at the promoter. This finding suggests that the absence of Sub1 does not cause a processivity defect. Next, we analyzed the relative Rpb1

occupancy along the *GAL1-YLR454* gene as previously described (Mason and Struhl, 2005) to study RNAPII processivity (Figure 3C). Surprisingly, deletion of *SUB1* resulted in either no effect or a slight increase of Rpb1 association downstream of the promoter and along the gene. We then analyzed whether the decrease of Rpb1 density from promoter to 3'-end regions (Figure 3B) was due to the slowed progression of RNAPII across the gene. We analyzed the relative Rpb1 association to chromatin after repressing transcription of the *GAL1-YLR454* gene with glucose for 4 min (Mason and Struhl, 2005; Quan and Hartzog, 2010). All regions examined in the *sub1Δ* mutant showed significantly higher RNAPII occupancy than in *wt* cells (Figure 3D). This result is similar to what was described for the *spt5-242* mutation, which affects the elongation rate without affecting processivity (Quan and Hartzog, 2010). These results support the hypothesis that elongation of RNAPII across the gene is impaired in *sub1Δ*, resulting in more RNAPII association within the body of the gene after the last wave of transcription.

ChIP assays measure the levels of polymerases associated with chromatin during active transcription (Mason and Struhl, 2005). However, ChIP does not specifically distinguish the active, elongation-competent form of the polymerase, for instance, from arrested or paused polymerase (Hirayoshi and Lis, 1999; Rodriguez-Gil *et al.*, 2010). However, the TRO assay enables measurement of the density of actively transcribing RNA polymerases by labeling nascent pre-mRNA (Birise *et al.*, 1998; Hirayoshi and Lis, 1999; Rodriguez-Gil *et al.*, 2010). We therefore used TRO to study the distribution of transcriptionally competent polymerases along the *GAL1-YLR454* gene in *wt* and *sub1Δ* cells. We designed 300–base pair probes distributed along the gene (Figure 4A) and performed TRO. A representative slot blot filter and quantification of three independent experiments are shown in Figure 4, B and C, respectively. We observed that active RNAPII is significantly decreased in *sub1Δ* cells when compared with *wt* cells and that this decrease is stronger in the 5' region of the *YLR454* gene (Figure 4B). This result agrees with the ChIP experiments (Figure 3B). However, when we analyzed the relative levels of active RNAPII along the gene with respect to the 5' region (ATG; Figure 4C), we observed a significant reduction of active polymerase in *sub1Δ* cells. This result indicates that the higher occupancy of Rpb1 along the *GAL1-YLR454* gene observed by ChIP in the absence of Sub1 (Figure 3D) corresponds to inactive, arrested polymerases that most likely cannot efficiently resume transcription. Taken together, the data presented here show that Sub1 influences transcription elongation.

### Sub1 is genetically linked to the Spt4/Spt5 elongation complex

We just showed that *SUB1* deletion causes transcription elongation rate defects similar to those displayed by the *spt5-242* mutant (Quan and Hartzog, 2010). In addition, our previous work showed a functional connection between Sub1 and the CTD-elongation kinase Bur1 (Calvo and Manley, 2005; Garcia *et al.*, 2010). We then decided to investigate whether Sub1 is genetically and functionally linked to the essential elongation factor Spt5, which is also phosphorylated by Bur1 (Liu *et al.*, 2009; Zhou *et al.*, 2009). Spt5 associates with Spt4, and the Spt4/5 complex is required for efficient elongation by RNAPII (Hartzog *et al.*, 1998; Wada *et al.*, 1998). We first performed genetic studies using two *spt5* mutants—*spt5-194* and *spt5-242*—which impair RNAPII transcription elongation: *spt5-194* grows slowly at 37°C and has very moderate sensitivity to 6AU, whereas *spt5-242* is cold sensitive and not sensitive to 6AU (Winston *et al.*, 1984; Swanson *et al.*, 1991; Swanson and Winston, 1992; Hartzog *et al.*, 1996, 1998). In contrast, *sub1Δ* cells grow normally and are not



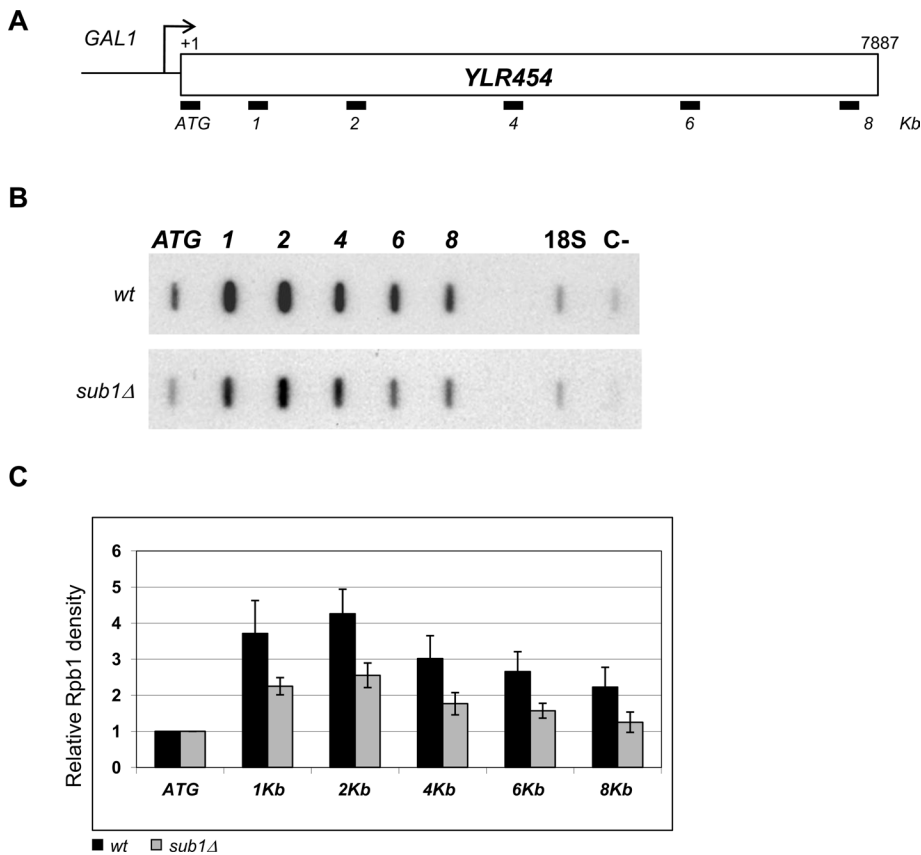
**FIGURE 3:** Deletion of *SUB1* leads to transcription elongation rate defects. (A) A schematic representation of the *GAL1-YLR454* gene. PCR primer pairs corresponding to the promoter and coding region at the specified positions are indicated. (B) Total Rpb1 occupancy at the promoter and coding regions of the *GAL1-YLR454* gene was assayed by ChIP, using 8WG16 antibody in *wt* and *sub1Δ* galactose-induced cells. Quantifications of qPCR were graphed as described in Figure 2. (C) Measurement of RNAPII processivity. Relative Rpb1 occupancy at the *GAL1-YLR454* gene in *wt* and *sub1Δ* cells at the indicated positions after inducing conditions. The qPCR values were first normalized to the values at the promoter (P) and then with respect to the *wt* cells values, taken as 1, for each probe as previously described (Mason and Struhl, 2005). (D) Measurement of the elongation rate as described in Mason and Struhl (2005). Relative Rpb1 occupancy of galactose-induced cells in *wt* and *sub1Δ* cells along the *GAL1-YLR454* gene after 4 min of glucose treatment (glucose/galactose ratio). Increased association of RNAPII with the coding region in the *sub1Δ* cells results in reduced polymerase dissociation after the last wave of transcription, leading to an elongation rate defect.

sensitive to 6AU in solid media (Figure 5, A and C; Garcia *et al.*, 2010). However, *spt5-194 sub1Δ* and *spt5-242 sub1Δ* double mutants both have increased temperature-sensitive growth defects at 37 and 28°C, respectively, and are highly sensitive to 6AU (Figure 5A). Overexpression of *SUB1* suppresses the growth defects of the *spt5-194* mutant (Figure 5B), although not entirely. In fact, overexpression of *SUB1* in *wt* cells causes a slight growth defect, as already reported (Calvo and Manley, 2001). Subsequently, we also tested whether *Sub1* is genetically linked to *Spt4*. As shown in Figure 5C, combining the *SUB1* deletion with the temperature-sensitive mutant *spt4Δ* results in a very strong synthetic growth defect and extreme 6AU sensitivity. Therefore *Sub1* is genetically linked to both *Spt4* and *Spt5*, suggesting that *Sub1* might be functionally linked to the *Spt4/Spt5* elongation complex.

#### Deletion of *SUB1* enhances *Spt5* phosphorylation by *Bur1* but not its recruitment to chromatin

We next tested whether *Sub1* affects *Spt5* phosphorylation by *Bur1*, because it has been shown that *Bur1* phosphorylates the carboxy-terminal domain of *Spt5*, thereby stimulating the recruitment of PAF (Liu *et al.*, 2009) and histone-modifying proteins (Zhou *et al.*, 2009) to the elongation complex. HA-*Bur1*-containing complexes were

immunoprecipitated (IPed) from *wt* and *sub1Δ* whole-cell extracts prepared from cells expressing HA-*Bur1* via the HA epitope tag. IP products were subjected to an *in vitro* IP kinase assay, with or without the addition of ATP (Garcia *et al.*, 2010), using a recombinant hexahistidine (6HIS)-*Spt5* protein (rSpt5; Liu *et al.*, 2009) as the substrate. Immunoblotting with the phosphospecific anti-*Spt5*-P antibody (Liu *et al.*, 2009; Figure 6A, top) and an antibody that recognizes the N-terminal region of *Spt5* (Figure 6A, bottom) showed that *Bur1* activity was higher in *sub1Δ* than in *wt* cells. As a control, equivalent IP of *Bur1*-HA was confirmed by Western blot using an anti-HA antibody (Figure 6A, right). To extend these results, we performed an *in vitro* kinase assay as a positive control for *Bur1*-HA kinase activity. We tested the ability of *Bur1*-HA IPed from *wt* and *sub1Δ* strains and *Bur1*-23-HA (with reduced kinase activity) IPed from *bur1-23* cells (Keogh *et al.*, 2003) to phosphorylate the glutathione *S*-transferase (GST)-CTD fusion protein on Ser-5. As shown in Figure 6B, *Bur1*-HA IPed from *wt* and *sub1Δ* cell extracts was fully active because it efficiently phosphorylated GST-CTD as determined by immunoblotting. In addition, *Bur1* activity was greater in *sub1Δ* cell extracts than in *wt* cells, as previously reported (Garcia *et al.*, 2010), and there was also a significant reduction in the phosphorylation of GST-CTD in the *bur1-23* cell extracts, which is in agreement with previous work (Keogh



**FIGURE 4:** Active elongation-competent RNAPII is decreased in *sub1Δ* cells. (A) Diagram of the *GAL1-YLR454* gene. Locations of the probes used in the TRO analysis are indicated. (B) TRO of *wt* and *sub1Δ* cells growing in galactose-containing medium. (C) Quantitation of blot hybridizations. Hybridization signals were quantitated by PhosphorImager analysis. The bacteria DNA background signal (C-) was subtracted from each probe, and, after normalization for 18S rRNA signal, the results were normalized to the ATG probe, which was fixed at 1.

*et al.*, 2003). Taken together, these results show that Sub1 negatively influences Bur1 kinase activity toward the CTDs of both Spt5 and RNAPII (Figure 6, A and B; Garcia *et al.*, 2010).

In light of the forgoing results, we next tested the extent of Bur1-HA-specific kinase activity on the CTD of Spt5 in our assays using extracts from *wt* and *sub1Δ* strains expressing another kinase, such as Ctk1-HA (Garcia *et al.*, 2010). In addition, we also tested the activity of the Bur1-23-HA mutant kinase with rSpt5. As shown in Figure 6C, only wild-type Bur1-HA (from *wt* and *sub1Δ* cell extracts) was able to phosphorylate the Spt5 CTD efficiently, which is consistent with previous work (Zhou *et al.*, 2009), and Spt5 phosphorylation was greater in the absence of Sub1.

We next determined whether Sub1 also affects Spt5 recruitment to gene promoters. Genome-wide occupancy profiles for yeast transcription factors showed that elongation factors are recruited to the transcriptional machinery during a 5' transition that occurs ~150 nucleotides downstream of the transcription start site (Mayer *et al.*, 2010). We therefore analyzed the recruitment of Spt5 to the 5' coding region of several constitutively transcribed genes (*ADH1*, *PYK1*, *PMA1*, and *PDR5*) between +150 to +400 nucleotides downstream of the transcription start site in the presence or absence of Sub1. For this purpose, we performed ChIP coupled with qPCR using *wt* and *sub1Δ* cells expressing Spt5-FLAG. As shown in Figure 6D, the *SUB1* deletion did not affect the association of Spt5 with the 5' coding region of the genes tested. We obtained the same result when we analyzed the association of Spt5 to the middle and 3'-end

of the tested genes (unpublished data). Taken together, our data support the idea that Sub1 associates with the transcription complex and modulates the phosphorylation of the CTDs of Rpb1 (Garcia *et al.*, 2010) and Spt5.

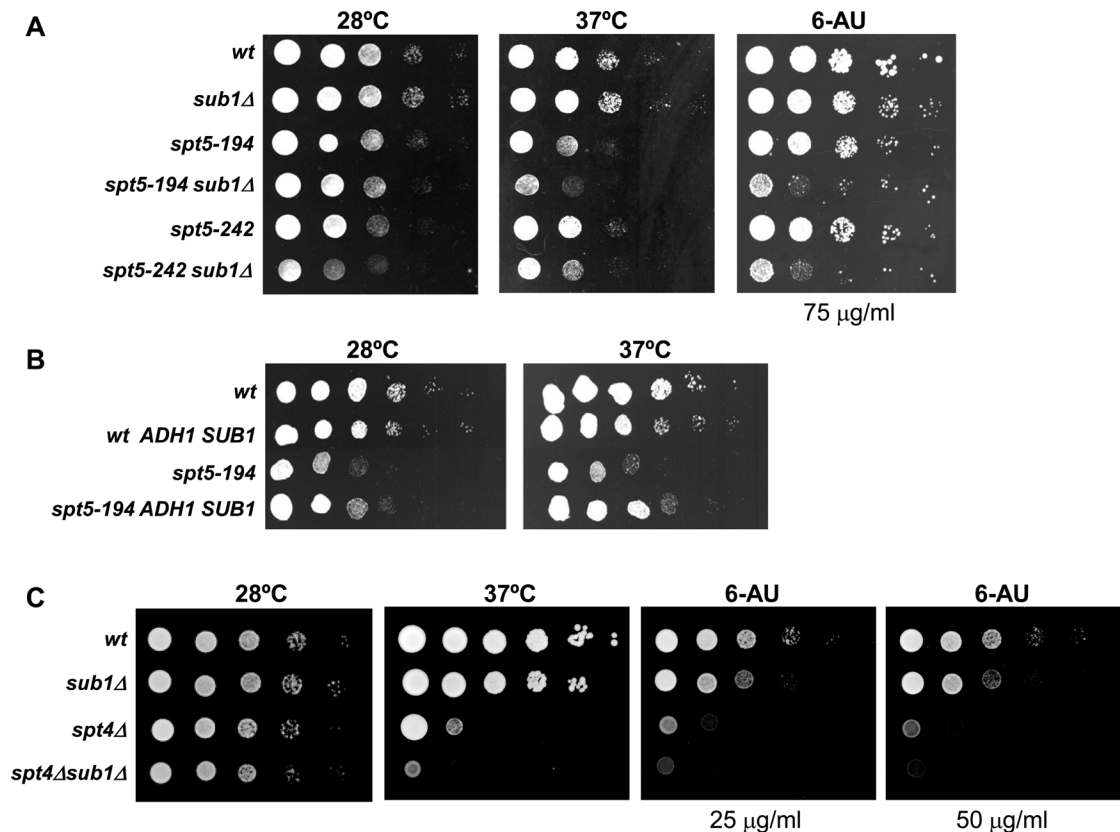
The exact mechanisms by which Sub1 influences Bur1 kinase activity remain unknown. One possibility is that the effects of Sub1 on Bur1 activity may be indirect, through Kin28, because *SUB1* deletion increases Kin28 recruitment and activity (Garcia *et al.*, 2010) and Kin28 promotes Bur1 kinase recruitment and Spt5 phosphorylation (Qiu *et al.*, 2009, 2012). Another possibility is through replication protein RPA, because both Sub1 and Bur1 have been genetically and functionally linked to RPA. The Bur1/2 complex interacts with RPA and maintains genome stability during replication stress (Clausing *et al.*, 2010). In contrast, the Rfa1 subunit of RPA seems to compete with Sub1 for chromatin binding to promoter regions (Sikorski *et al.*, 2011). Indeed, Rfa1 is able to associate with promoter regions during active transcription only in the absence of Sub1. The increased Rfa1 association in *sub1Δ* cells could then enhance Bur1 recruitment (because they both directly interact) and therefore kinase activity. However, Bur1 recruitment is not altered when Sub1 is lacking (Garcia *et al.*, 2010). Nevertheless, we cannot rule out that Sub1 may indirectly influence Bur1 kinase activity during transcription elongation through Rfa1.

### ***SUB1* deletion suppresses the splicing defect of a *spt5-194* mutation**

It has been shown that the transcription elongation rate influences splicing (de la Mata *et al.*, 2003; Howe *et al.*, 2003; Shukla and Oberdoerffer, 2012). Thus RNAPII mutants that slow transcription or cells treated with MPA and 6AU display increased exon inclusion (Howe *et al.*, 2003). Because Sub1 influences the transcription elongation rate, we decided to test whether *SUB1* deletion also affects splicing.

Spt5 has been implicated in pre-mRNA processing because unspliced pre-mRNAs accumulate in *spt5* mutants (Lindstrom *et al.*, 2003; Xiao *et al.*, 2005). Therefore we analyzed splicing of the *RPL26* mRNA in the *wt*, *sub1Δ*, *spt5-194*, and *spt5-194 sub1Δ* strains. We isolated RNA from all four strains and performed RT-PCR to analyze the levels of the unspliced and mature *RPL26A* mRNA (Figure 6E; Lindstrom *et al.*, 2003). We also analyzed the expression of *PYK1*, which does not contain introns. As shown in Figure 6E, the levels of mature and unspliced *RPL26* pre-mRNA were similar in *wt* and *sub1Δ* cells. However, there was a large increase in the level of unspliced *RPL26* pre-mRNA in the *spt5-194* mutant, which is consistent with previous work (Lindstrom *et al.*, 2003). Of interest, when the *SUB1* deletion was combined with the *spt5-194* mutation, there was a significant reduction in the accumulation of unspliced *RPL26*. This result is consistent with a reduction of the transcription elongation rate in *sub1Δ* cells (Figure 3) and in agreement with the fact that elongation rates influences splicing (de la Mata *et al.*, 2003; Howe





**FIGURE 5:** Sub1 is genetically linked to Spt5. Genetic interaction between *SUB1*, *SPT5*, and *SPT4*. (A) *SUB1* deletion enhances the growth defects of *spt5-194* and *spt5-242* and causes 6AU sensitivity to them, and (B) overexpression of *SUB1* suppresses *spt5-194* growth phenotypes. To overexpress *SUB1*, its open reading frame was cloned under the control of the *ADH1* promoter (*ADH1SUB1*). (C) *SUB1* deletion enhances the growth thermosensitivity and 6AU sensitivity of *spt4Δ* mutant. Yeast strains with the indicated genotypes were spotted onto SC-URA and SC-URA media with 75, 50, or 25  $\mu\text{g/ml}$  of 6AU and grown at 28 or 37°C for 2–3 d.

*et al.*, 2003). Similarly, N. Krogan and colleagues (personal communication) observed that slow RNAPII mutations or *SUB1* deletion enhance splicing.

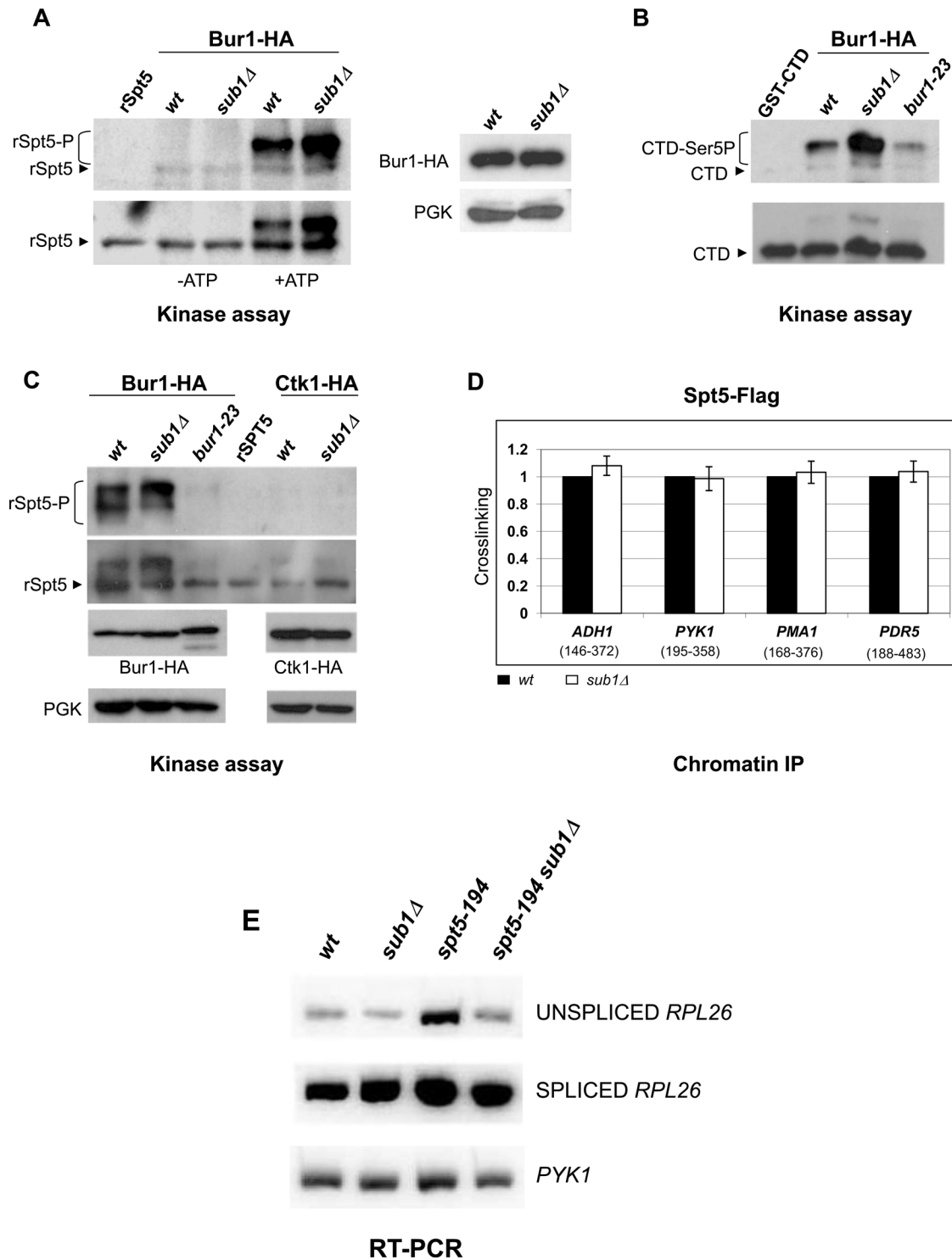
### Sub1 and Spt5 copurify early during transcription elongation

Lindstrom *et al.* (2003) affinity purified Spt5-FLAG from yeast extracts and used mass spectrometry to identify a large number of proteins involved in transcription and pre-mRNA processing, including Sub1. Nevertheless, the copurification of these two proteins was not further confirmed. Therefore we performed a tandem affinity purification (TAP) assay to investigate whether Spt5 copurified with Sub1-TAP and subsequently confirmed our results by coIP. We first purified Sub1-TAP from yeast extracts (Rodriguez-Navarro *et al.*, 2004), and we used an isogenic strain lacking the TAP tag (mock) as a control for nonspecific purification. Purified samples from both strains (Sub1-TAP and mock) were analyzed with a polyclonal anti-Spt5 antibody (Figure 7A). In accordance with previous work (Lindstrom *et al.*, 2003), Spt5 associated with Sub1-TAP, indicating that Sub1 and Spt5 might be part of the same complex. We also confirmed the copurification by IP experiments. We performed an anti-HA IP assay using whole-cell extracts from the Sub1-HA and the double-tagged Sub1-HA/Spt5-FLAG strains. We used the Spt5-FLAG strain as a negative control for the anti-HA IP. As expected, Sub1 was efficiently IPed from the Sub1-HA and Sub1-HA/Spt5-FLAG cell extracts (anti-HA immunodetection in Figure 7B, bottom,

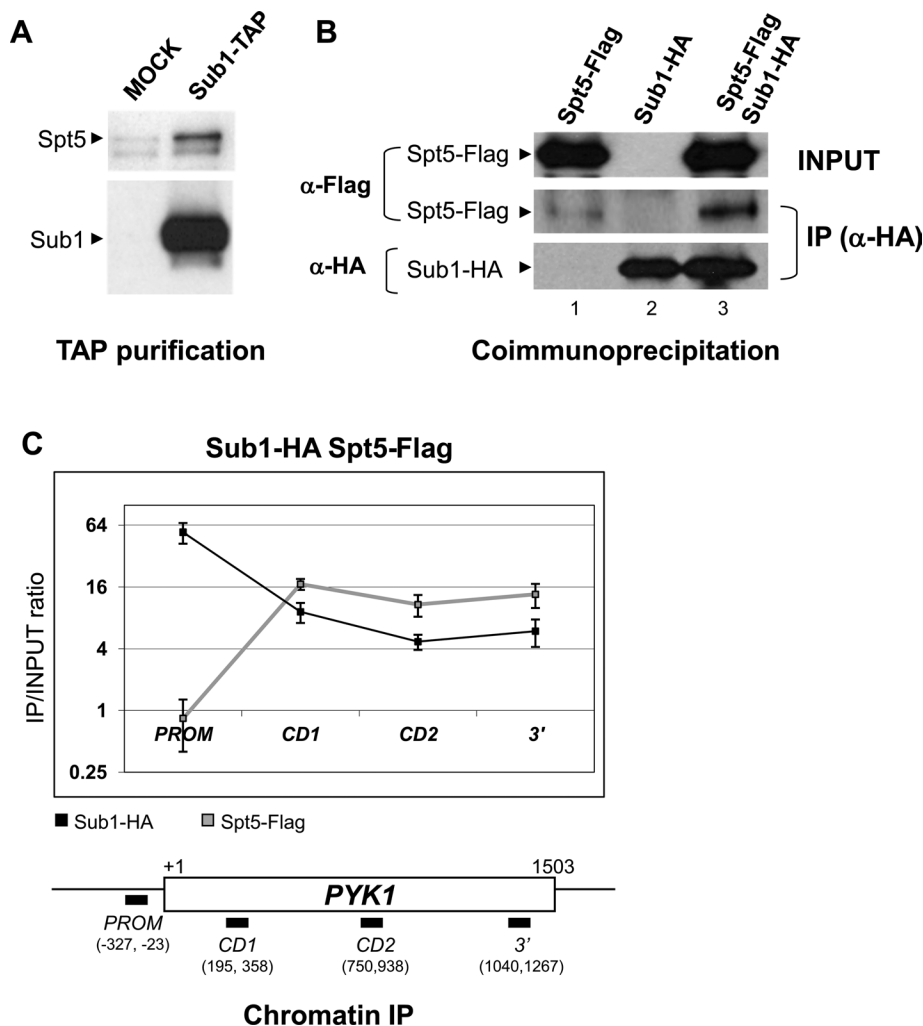
lanes 2 and 3, respectively). We immunoblotted the same membrane with anti-FLAG antibody and only a fraction of Spt5-FLAG colPed with Sub1-HA, as shown in Figure 7B (middle, lane 3). Then, it is possible that Sub1 associates with Spt5 in a transient manner. Consistent with this hypothesis, when we analyzed the association of Spt5-FLAG and Sub1-HA with the chromatin of several actively transcribed genes (*PYK1* in Figure 7C and *ADH1*, *PGK1*, *PMA1* and *YEF3* in Supplemental Figure S1) we observed an increase in the association of Sub1-HA with the promoter regions, which rapidly declined downstream of the promoters but was maintained at a lower level throughout the 3' end of the genes (Figure 7C; Nedeá *et al.*, 2003; Rosonina *et al.*, 2009; Sikorski *et al.*, 2011). In contrast, the association of Spt5-FLAG with the middle and 3' regions of the open reading frames increased (Figure 7C and Supplemental Figure S1; see Mayer *et al.*, 2010). Thus there is a small region, ~150–400 nucleotides downstream of the transcription start site (Figure 7C), where Spt5 and Sub1 might directly or indirectly interact as part of the same complex during early elongation. Alternatively, Sub1 might be less efficiently detected by ChIP in coding regions due to conformational changes occurring in the elongation apparatus that weaken the ability of Sub1 to bind to DNA but permit interaction with the Spt4/Spt5 complex (see *Discussion*).

### Deletion of *SUB1* affects Spt5–Rpb1 interaction

Several lines of evidence suggested that Spt4/5 association with Rpb1 could be altered in *sub1Δ*. First, there is genetic interaction of



**FIGURE 6:** Deletion of *SUB1* enhances Spt5 phosphorylation by Bur1 but not its recruitment to the 5' regions of genes. (A) In vitro kinase assay. WCEs were prepared from wt and *sub1Δ* strains expressing HA-tagged Bur1. The HA-tagged kinase complexes were IPed with 12CA5-protein A beads, and kinase activity was assayed using recombinant 6HIS-Spt5 as substrate. SDS-PAGE and immunoblot analysis were performed to analyze Spt5 phosphorylation using the following antibodies: anti-P Spt5 (Liu et al., 2009), anti-Spt5, 12CA5 (anti-HA for Bur1-HA), and anti-PGK (for total protein level control). (B) WCEs were prepared from the Bur1-HA (wt and *bur1-23* mutant) and Bur1-HA *sub1Δ* strains. In vitro kinase assays were conducted as described but using recombinant GST-CTD as substrate, and CTD Ser-5 phosphorylation was analyzed with the CTD4H8 (anti-CTD Ser5P) and 8WG16 (anti-CTD) antibodies. (C) WCEs were prepared from the wt and *sub1Δ* strains expressing HA-tagged Bur1 and Ctk1 and from *bur1-23*-HA mutant cells. In vitro kinase assays were performed to analyze Spt5 phosphorylation as described. (D) ChIP analyses were performed in the wt and *sub1Δ* strains expressing Spt5-FLAG. Spt5 binding to the 5' region (the nucleotide position relative to the start codon is shown between brackets) of four constitutively expressed genes—*ADH1*, *PYK1*, *PMA1*, and *PDR5*—was examined by qPCR,



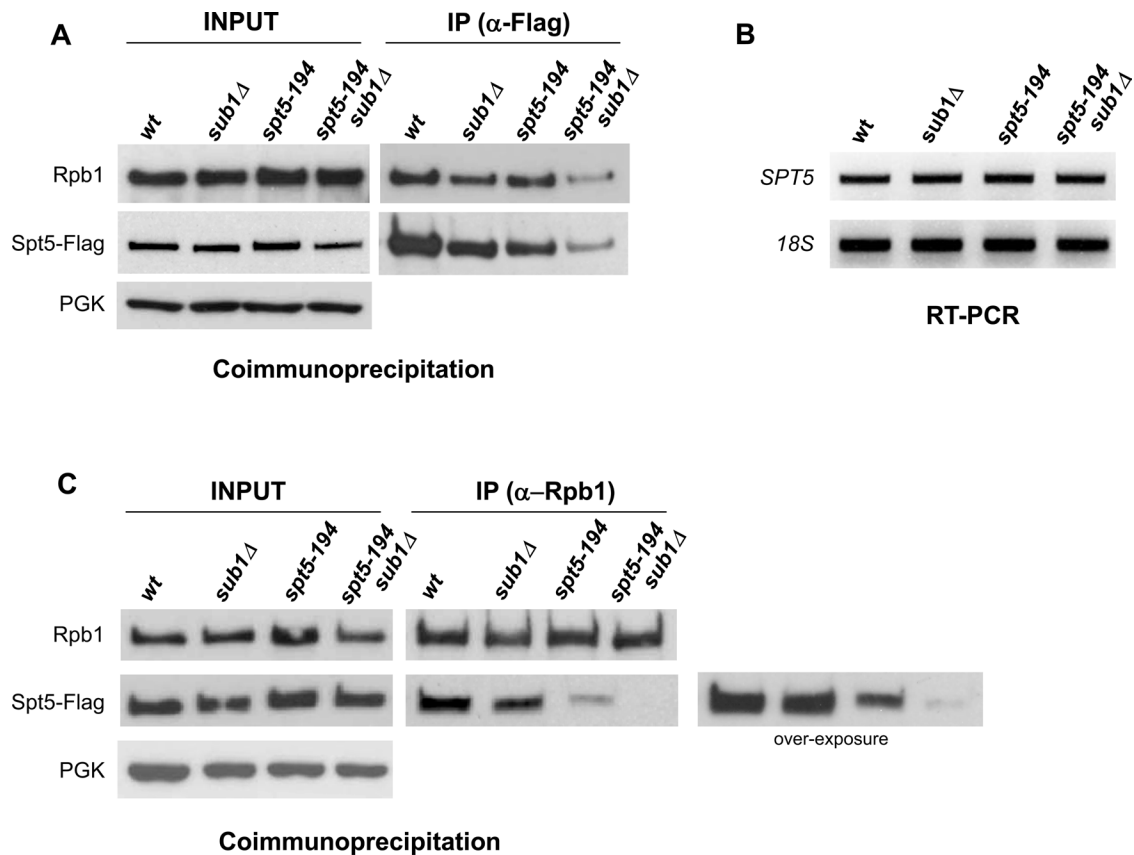
**FIGURE 7:** Sub1 and Spt5 copurify during early transcription elongation. (A) Spt5 copurifies with Sub1-TAP. Tandem affinity purification was performed with a yeast strain expressing Sub1-TAP, and purified extracts were analyzed by Western blot using a polyclonal anti-Spt5 antibody. An isogenic strain lacking the TAP tag was used as a control for nonspecific purification (mock). (B) Spt5-FLAG coimmunoprecipitates with Sub1-HA. WCEs were prepared from the strains Spt5-FLAG, Sub1-HA, and Sub1-HA/Spt5-FLAG, and anti-HA IPs were carried out. After extensive washing, inputs and IPs were analyzed by Western blotting. The upper and middle membranes were immunoblotted with anti-FLAG and the lower membrane with anti-HA. (C) ChIP analysis of Spt5 and Sub1 was conducted in the double-tagged Spt5-FLAG/Sub1-HA strain using anti-FLAG and anti-HA antibodies, respectively. Spt5-FLAG and Sub1-HA cross-linking to the promoter, coding, and 3' regions of the *PYK1* gene was analyzed by qPCR, and the quantifications of the results are graphed as described in Figure 2. A schematic representation of the *PYK1* gene is shown; the numbers indicate the nucleotide position relative to the start codon (+1), and the bars above the genes represent the PCR products analyzed by ChIP.

*sub1Δ* with *spt4Δ*, and with *spt5-194* and *spt5-242* mutations located in the NGN domain (Figure 5). The *spt5-194* mutation disrupts Spt4–Spt5 interaction (Guo *et al.*, 2008), and *spt5-242*

mutation causes elongation rate defects (Quan and Hartzog, 2010). Second, the NGN domain is required to interact with Rpb1 (Klein *et al.*, 2011; Martinez-Rucobo *et al.*, 2011) and overexpression of Sub1 partially suppresses *spt5-194* growth defects. Third, the levels of active elongating polymerase are reduced in *sub1Δ* cells (Figure 4, B and C). Therefore we decided to investigate the interaction of Spt5 with Rpb1 in *wt*, *sub1Δ*, *spt5-194*, and *spt5-194 sub1Δ* cells expressing Spt5-FLAG proteins. We IPed Spt5-FLAG from whole-cell extracts and analyzed the Rpb1 levels by Western blot analysis. As shown in Figure 8A (inputs), the levels of Spt5 protein were unaffected in *sub1Δ* and *spt5-194* cells when compared with *wt* cells but were slightly decreased in *spt5-194 sub1Δ* double-mutant cells. This was not due to an effect of *SUB1* deletion on *SPT5* transcription, as revealed by the RT-PCR experiments (Figure 8B), but it may be an indication that Spt5 is more unstable in the double-mutant cells. When we analyzed the Spt5-FLAG IPs, we observed less Rpb1–Spt5 complex in *sub1Δ* and *spt5-194* cells. Furthermore, this defect is significantly enhanced in the case of the *spt5-194 sub1Δ* double mutant, in which levels of the Rpb1–Spt5 complex were greatly reduced. To corroborate this result, we next IPed Rpb1 and analyzed the levels of colPed Spt5-FLAG (Figure 8C). Similar levels of Rpb1 were IPed from *wt*, *sub1Δ*, *spt5-194*, and *spt5-194sub1Δ* strains. However, slightly less Spt5 associated with Rpb1 in *sub1Δ* compared with *wt* cells, and in the case of *spt5-194* cells, the amount of Spt5 colPed with Rpb1 was significantly reduced. Moreover, this reduction was extraordinarily enhanced in the *spt5-194sub1Δ* cells, in which Spt5 was almost undetectable.

Thus our results suggest that Sub1 might help to maintain appropriate levels of Spt5 protein levels, directly or indirectly increasing its stability, and with special significance in the *spt5-194* background. This is consistent with the essential nature of Spt5, with the requirement of a functional NGN domain for the Spt5–Rpb1 interaction, and with the fact that overexpression of *SUB1* suppresses *spt5-194* growth defects. Taken together, our data indicate that Sub1 influences the

and quantifications were graphed (see *Materials and Methods*). The numbers on the y-axis represent the association of Spt5 with the 5' regions of genes in *sub1Δ* cells relative to *wt* cells, in which cross-linking is considered 1. (E) RT-PCR analysis of the spliced and unspliced forms of the *RPL26* gene. RNA was prepared from *wt*, *sub1Δ*, *spt5-194*, and *spt5-194sub1Δ* cells. Unspliced pre-mRNAs were specifically amplified using one primer from intron sequences and another from the second exon as previously described (Lindstrom *et al.*, 2003). Another pair of primers was used to amplify the spliced *RPL26* mRNA. As a loading control, *PYK1*, which does not contain introns, was also analyzed by RT-PCR. All PCRs were performed using <sup>32</sup>P-dCTP, and the PCR products were run on an 8% polyacrylamide gel.



**FIGURE 8:** Deletion of *SUB1* negatively affects the Spt5-Rpb1 interaction. (A) CoIP of Spt5-Rpb1. WCEs of wt, *sub1* $\Delta$ , *spt5-194*, and *spt5-194sub1* $\Delta$  expressing the corresponding Spt5-FLAG and Spt5-194-FLAG proteins were used to perform anti-FLAG IPs. Western blots of the inputs and IPs were analyzed with anti-Rpb1 (top), anti-FLAG (middle), and anti-PGK (bottom). The CoIP was performed at least three times, and a representative experiment is shown. (B) RT-PCR analysis of the *SPT5* gene. 18S rRNA was used as loading control. PCR products were run on an ethidium bromide-stained gel. (C) CoIP of Spt5-Rpb1 as in A, but the IPs were performed with anti-Rpb1 (8WG16). Western blots of the inputs and IPs were also analyzed using the same antibodies as in A.

interaction of Spt5 with Rpb1, consistent with all our demonstration that Sub1 influences transcription elongation.

## DISCUSSION

Over the past several years, there has been an extraordinary increase in the number of proteins known to influence transcription elongation (Selth *et al.*, 2010). Many of these proteins do not directly affect transcription elongation (Mason and Struhl, 2005), although they possess some of the criteria used to define them as elongation factors. These criteria include the ability to travel with elongating RNAPII and associate in vivo with coding regions in a transcription-dependent manner (e.g., Krogan *et al.*, 2002; Pokholok *et al.*, 2002; Mason and Struhl, 2003; Kruk *et al.*, 2011). One important characteristic of a transcription elongation defect is the reduced association of RNAPII with transcribed regions from 5' to 3' ends, which may be a consequence of a defect in the elongation rate, processivity, or both (Mason and Struhl, 2005; Quan and Hartzog, 2010; Chanarat *et al.*, 2011; Kruk *et al.*, 2011). Here we provided new insight into this process by showing that Sub1, a PIC component, associates with the Spt5 elongation factor and influences transcription elongation rate.

### Sub1 affects transcription elongation efficiency and elongation rate

We presented data showing a reduced elongation efficiency of long transcripts in *sub1* $\Delta$  cells by the GLAM assay. However, we

cannot conclude from this assay alone whether a factor is involved directly in elongation, since mutations in factors involved in pre-mRNA processing also show reduced GLAM ratios (Morillo-Huesca *et al.*, 2006; Gaillard *et al.*, 2009). In addition, ChIP analysis showed that in the *sub1* $\Delta$  mutant RNAPII association to chromatin was reduced, although measurement of in vivo processivity showed that the relative RNAPII association with chromatin is not altered or very slightly increased in *sub1* $\Delta$  cells. On the other hand, we observed a higher density of RNAPII after the last wave of transcription by ChIP in the absence of Sub1, indicating an elongation rate defect, which results in a decrease of active, competent elongating polymerase measured by TRO. Thus it is possible that the polymerase recruited to the promoter in *sub1* $\Delta$  cells is slower than in wild-type cells or does not efficiently resume transcription after transient stalling or arrest. Moreover, the role of Sub1 in the transcription elongation rate is further supported by data demonstrating that *SUB1* deletion suppresses *spt5-194* splicing defects, which is consistent with the fact that elongation rates influence the splicing and selection of alternative splice sites (de la Mata *et al.*, 2003; Howe *et al.*, 2003).

The elongation rate defect has been also described for an Rpb2 mutation (Mason and Struhl, 2005), for Ccr4-Not elongation complex mutants (Kruk *et al.*, 2011), and for the *spt5-242* mutation (Quan and Hartzog, 2010). Therefore, given that the Spt5/Spt4 complex affects RNAPII processivity and elongation rate (Mason

and Struhl, 2005; Quan and Hartzog, 2010) and Sub1 appears to influence levels of an Rpb1–Spt5 complex, this provides an explanation for the defects in the polymerase elongation rates observed in *sub1Δ* cells during transcription elongation. However, we cannot rule out that the Sub1 role in elongation may also derive from a promoter role and/or from a function in gene looping due to its close functional link with TFIIB (Knaus *et al.*, 1996; Wu *et al.*, 1999; Rosonina *et al.*, 2009). Although different from TFIIB, Sub1 associates not only with promoters, but also with coding regions.

### Sub1 is functionally linked to the elongation factor Spt5

Spt5 is present in the elongation complex but is not detected in the PIC. It is recruited to the transcriptional machinery during the initiation–elongation transition (Mayer *et al.*, 2010), and structural studies recently showed that the conserved Spt5 NGN domain locks the nucleic acids in the polymerase cleft and maintains the transcription bubble (Martinez-Rucobo *et al.*, 2011). It was recently reported that Sub1 is a PIC component that localizes at the promoter, where DNA must be unwound for transcription (Sikorski *et al.*, 2011), and we showed that it remains associated with coding regions during active transcription. Because Spt5 and Sub1 bind to open transcription bubbles (Klein *et al.*, 2011; Martinez-Rucobo *et al.*, 2011; Sikorski *et al.*, 2011) and are associated with chromatin during early transcription elongation, and *sub1Δ* cells display reduced levels of RNAPII occupancy in coding regions, it is possible that Sub1 could redundantly help Spt5 associate with RNAPII during the initiation–elongation transition and positively promote transcription elongation. In agreement with this, Spt5 levels associated with Rpb1 are slightly reduced in *sub1Δ* cells, and this defect is significantly enhanced in the double mutant *spt5-194 sub1Δ*. This is consistent with the fact that the *spt5-194* mutation is located in the NGN domain of Spt5, which directly binds to Rpb1 and Spt4 (Klein *et al.*, 2011; Martinez-Rucobo *et al.*, 2011). In addition, the *spt5-194* mutation interrupts Spt4–Spt5 binding (Guo *et al.*, 2008). This could explain the growth defects displayed by the *spt5 sub1Δ* and *spt4Δ sub1Δ* double mutants and the correction of the *spt5-194* growth defects by overexpressing *SUB1*.

Based on structural studies, it has been proposed that the initiation–elongation transition may become irreversible after Spt4/5 binding because Spt4/5 maintains the transcription bubble and prevents RNA release (Martinez-Rucobo *et al.*, 2011). On the other hand, it has been proposed that initiation factors, such as TFIIE, that bind to overlapping sites on the polymerase clamp domain (Chen *et al.*, 2007) will likely avoid premature binding of Spt4/5 during initiation and therefore Spt5 phosphorylation. It is interesting that Sub1 has also been genetically linked to TFIIE, and it has been suggested that Sub1 could help TFIIE and TFIIH to maintain the open complex (Sikorski *et al.*, 2011). It is possible that Sub1 cooperates with TFIIE as a PIC component, where Bur1 and Spt5 entry is avoided, and with Spt5 as an elongation factor to maintain the transcription bubble, depending on Sub1 phosphorylation status. In particular, Sub1 could function with Spt5 to help stabilize the Rpb1–Spt5 elongation complex and increase elongation rate. Furthermore, Sub1 regulates RNAPII and Spt5 phosphorylation, as shown here and previously (Garcia *et al.*, 2010), which is required to promote transcription elongation (Buratowski, 2009; Liu *et al.*, 2009; Zhou *et al.*, 2009). Conversely, the effect of Sub1 on in vitro phosphorylation appears to indicate a negative effect. It is possible that these negative effects are limited in vivo to a specific time at which phosphorylation by Bur1 should be inhibited, for example, during preinitiation (Bur1 is not

detected in the PIC complexes; Liu *et al.*, 2004; Garcia *et al.*, 2010), thus blocking Bur1 kinase activity and Spt5 recruitment and phosphorylation as well.

Our results are consistent with recently proposed models for Sub1 function in which Sub1 is recruited to promoters and helps to maintain the PIC in a stable but inactive conformation, possibly by helping TFIIE (Sikorski *et al.*, 2011) and/or inhibiting Kin28 recruitment and activity (Garcia *et al.*, 2010), required to phosphorylate RNAPII-CTD on Ser-5 and to recruit capping enzymes (Komarnitsky *et al.*, 2000; Buratowski, 2009). During transcription initiation, the phosphorylation of Sub1 would reduce its DNA-binding capacity (Ge *et al.*, 1994; Henry *et al.*, 1996; Jonker *et al.*, 2006), alleviate kinase inhibitory effects, and facilitate promoter clearance (Henry *et al.*, 1996; Knaus *et al.*, 1996; Sikorski *et al.*, 2011) and capping enzymes recruitment (Garcia *et al.*, 2010). During the initiation–elongation transition, initiation factors are exchanged by elongation factors (Pokholok *et al.*, 2002) and Spt4/Spt5 complex is recruited, binding the polymerase (e.g., Martinez-Rucobo *et al.*, 2011) and the capping complex (Pei and Shuman, 2002). Thereafter, Bur1 phosphorylates Spt5 and RNAPII-CTDSer5P on Ser-2 to promote extensive phosphorylation by Ctk1 farther downstream in coding and 3'-end regions (Qiu *et al.*, 2009), the capping complex is released, and productive transcription elongation is triggered (reviewed by Buratowski, 2009). In that context, our data indicate that Sub1 joins the early elongation complex containing Spt4/Spt5 and influences transcription elongation, most likely by 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 positively influences the transcription elongation rate. Moreover, extensive and specific phosphorylation events targeting RNAPII (e.g., Buratowski, 2009), Spt5 (Liu *et al.*, 2009; Zhou *et al.*, 2009), and Sub1 (Ge *et al.*, 1994; Henry *et al.*, 1996; Jonker *et al.*, 2006) could take place simultaneously and ultimately regulate the initiation–elongation transition and therefore transcription elongation.

### Sub1 associates predominantly with promoters as a PIC component but also with coding regions as an elongation factor

If Sub1 associates with the Spt5 elongation factor and has a role during transcription elongation, why is Sub1 found associated predominantly with the promoter and 5' coding regions? One possibility is that conformational changes occur in the elongation apparatus that may weaken the ability of Sub1 to bind to DNA or may interfere with detection by ChIP in the middle and 3' ends of genes. It has been proposed that Sub1 competes with the more abundant single-strand DNA (ssDNA)–binding protein Rfa1, which participates in transcription elongation and was purified with Sub1 in the PIC (Sikorski *et al.*, 2011). In fact, Rfa1 associates with promoters in the absence of Sub1, whereas its association with coding regions is Sub1 independent. Because the in vitro ability of phospho-Sub1/PC4 to bind to DNA is reduced, thus allowing it to escape from promoters (Ge *et al.*, 1994; Henry *et al.*, 1996; Jonker *et al.*, 2006), it is possible that phosphorylated Sub1 will be less strongly associated with coding regions where Rfa1 ssDNA-binding affinity is increased (Sikorski *et al.*, 2011). Alternatively, in coding regions these two proteins could be bound to DNA in a noncompetitive manner. In fact, a *SUB1* and *RFA1* genetic interaction suggests some cooperative function (Sikorski *et al.*, 2011), and both have been genetically and functionally linked to Bur1 function (Clausing *et al.*, 2010; Garcia *et al.*, 2010). Another possibility is that the majority of Sub1 is associated with promoter regions, and only a small pool of Sub1

participates in transcription elongation of constitutively transcribed genes (e.g., *PMA1*), whereas its participation could be very relevant for genes whose transcription directly depend on it (e.g., *IMD2*). For instance, the Ccr4–Not1 complex has been recently described as a bone fide elongation factor, but it is recruited only to several induced genes (Kruk *et al.*, 2011). In any case, there is ample evidence demonstrating that Sub1 is recruited to promoters and associates in a transcription-dependent manner with coding regions as part of the transcription elongation complex, at least during early elongation. This is strongly supported by the fact that Sub1 associates with the Spt4/Spt5 elongation complex and influences transcription elongation rate.

## MATERIALS AND METHODS

### Yeast strains and media

The strains used in this study are listed in Supplemental Table S1. Yeast strain construction and other genetic manipulations were performed following standard procedures (Burke *et al.*, 2000).

### In vitro kinase assays

To analyze GST-CTD and 6HIS-Spt5 phosphorylation, we performed in vitro kinase assays as previously described (Garcia *et al.*, 2010). Cells were grown to an OD<sub>600</sub> of 0.8, collected, washed, and suspended in lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.6, 200 mM KOAc, 10% glycerol, 1 mM EDTA; Keogh *et al.*, 2003) containing protease and phosphatase inhibitors. Yeast whole-cell extracts were prepared by glass bead cell disruption using a FastPrep system. Protein concentrations were determined, and 150 µg were incubated with 15 µl of protein G–Sepharose–coupled 12CA5 (anti-HA) antibody for 2 h at 4°C to immunoprecipitate Bur1-HA. The immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.6, 7.5 mM MgOAc, 100 mM KOAc, 2% glycerol). The beads were resuspended in 25 µl of kinase buffer with 2.5 mM of ATP and incubated with 30 ng of GST-CTD or 100 ng of rSpt5 for 30 min at 30°C. The following antibodies were used: 8WG16 (nonP-CTD; Covance, Berkeley, CA); CTD4H8 (CTD-Ser5P; Millipore, Billerica, MA); anti-Spt5 (yN-20; Santa Cruz Biotechnology, Santa Cruz, CA), and a phospho-specific anti-Spt5-P antibody (a gift from S. Hahn [Fred Hutchinson Cancer Research Center]; Liu *et al.*, 2009). Bur1-HA and Bur1-23-HA are C-terminal tagged; however, Bur1-HA is expressed from a chromosomal copy and Bur1-23-HA from a centromeric plasmid (pRS315), both under the control of their own promoters (a gift from S. Buratowski [Harvard Medical School]; see Supplemental Table S1).

### Chromatin immunoprecipitation

Chromatin purification, immunoprecipitation, RT-qPCR amplification, and data analysis were performed as described previously (Garcia *et al.*, 2010). Briefly, PCR of purified chromatin, following immunoprecipitation, was performed by RT-qPCR with a CFX96 Detection System (Bio-Rad, Hercules, CA), using SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) and following manufacturer's instructions. Four serial 10-fold dilutions of genomic DNA were amplified using the same reaction mixture as the samples to construct the standard curves. All real-time PCRs were performed in triplicate and with at least three independent ChIPs. Quantitative analysis was carried out using the CFX96 Manager Software, version 0.2.1. The values obtained for the IPed PCR products were compared with those of the total input, and the ratio of the values from each PCR product from transcribed genes to a nontranscribed region of CVII was calculated.

Numbers on the y-axis of the graphs are detailed in the corresponding figure legend. ChIP analysis to study elongation rate and processivity was performed as described (Mason and Struhl, 2005). Briefly, to study processivity, yeast cells were grown in rich medium with 2% raffinose as the carbon source at 28°C to an OD<sub>600</sub> of 0.5, followed by galactose induction for 2.5 h. To study the elongation rate, we treated the galactose-induced cells with glucose at a final concentration of 5% for 4 min. ChIP was performed three times with the 8WG16 antibody using chromatin from three independently grown cultures. To represent RNAPII processivity in wild-type and mutant cells, the data are expressed as the ratio of RNAPII occupancy in each strain relative to the promoter position followed by the normalization of each value to the corresponding position in wild-type strain, which was set to 1. To represent the elongation rate, the RNAPII association with chromatin after 4 min of glucose treatment was normalized to RNAPII association under galactose induction conditions at every region tested in the wild-type and *sub1Δ* cells.

### Transcriptional run on

Twenty-five milliliters of culture at an OD<sub>600</sub> of 0.3–4 growing at 30°C and induced with 2% galactose was permeabilized and nascent RNA labeled with [ $\alpha$ -<sup>32</sup>P]UTP for 5 min at 30°C as described (Birse *et al.*, 1998; Calvo and Manley, 2001). After partial hydrolysis, RNA was hybridized directly to filter immobilized YLR454 probes. Hybridization signals were quantitated by PhosphorImager analysis (Personal Molecular Imager; Bio-Rad). Each experiment was performed at least three times.

### RNA isolation and RT-PCR

Total RNA was extracted as previously described (Schmitt *et al.*, 1990), and RT-qPCR was performed using the PrimeScript RT Reagent Kit (Takara Bio), following the manufacturer's instructions. All qPCRs were performed in triplicate with at least three independent cDNA samples.

### 6-Azauracil induction

Cells were grown in synthetic complete (SC) media without uracil to an OD<sub>600</sub> of 0.5. They were then split into two cultures, and 6AU was added to one of the cultures to a final concentration of 50 or 100 µg/ml. Both cultures were incubated for 1 h, collected, washed, and resuspended in the appropriate buffer to isolate RNA. RT-qPCR was performed as described.

### GLAM assay

The GLAM assay was carried out as in Morillo-Huesca *et al.* (2006) with cells grown to mid-log phase in selective synthetic medium lacking uracil with 2% galactose. Acidic phosphatase activity was measured at least twice in cells grown in three to four independently induced cultures for each strain. The mean values and standard deviations are represented in the corresponding figures.

### TAP purification, immunoprecipitation, and Western blot analysis

Purification of Sub1-TAP was performed as described (Rodriguez-Navarro *et al.*, 2004). Sub1-6HA IP was performed as follows: cells expressing Sub1-6HA were grown on rich medium to an OD<sub>600</sub> of 1.0, harvested, washed with water, and resuspended in 250 µl of lysis buffer (10 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a cocktail of protease inhibitors). Cell lysis was achieved at 4°C using a FastPrep System. The clarified whole-cell

extracts (WCEs) were IPed for 2 h at 4°C with anti-HA antibody coupled to protein A–Sepharose. The IPs were extensively washed (lysis buffer with 0.5% NP-40) and subsequently resuspended in SDS–PAGE sample buffer. Western blot analysis was performed using the appropriate antibodies. A similar protocol was followed for Spt5-FLAG IP, although it was performed overnight at 4°C using anti-FLAG antibody.

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