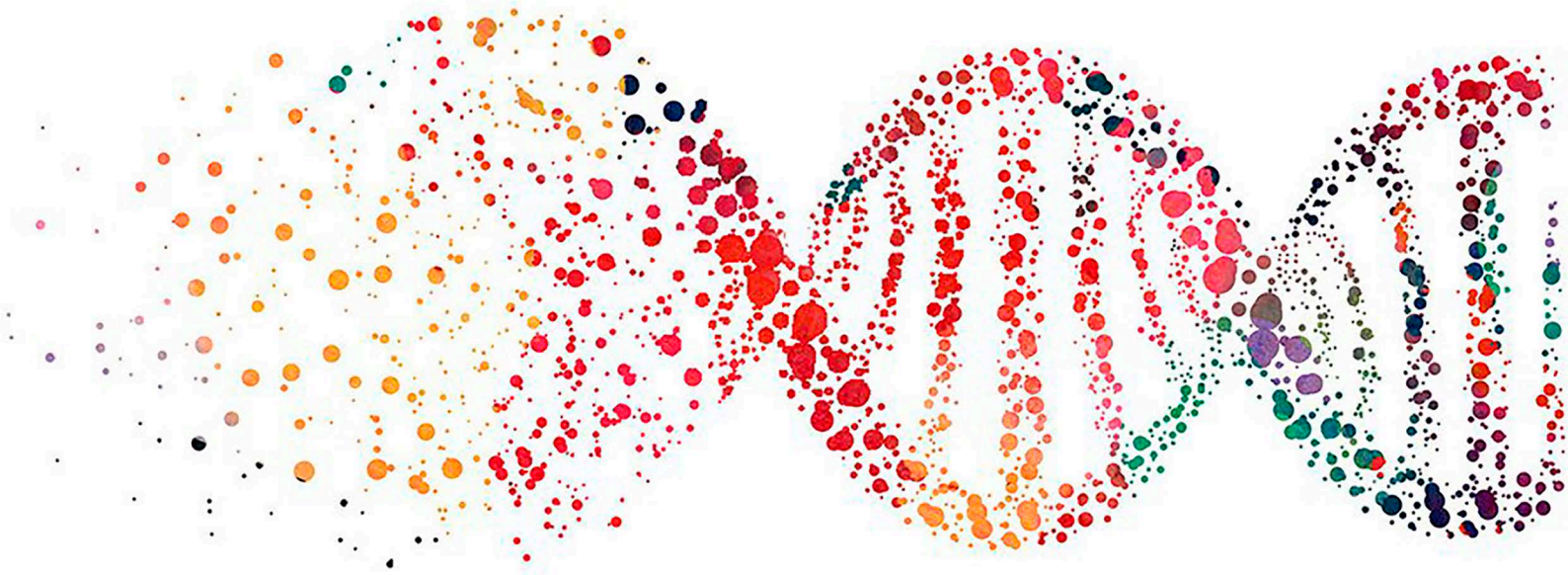
Papel de Rpb4/7 en la fosforilación de la RNA polimerasa II y en la formación de los bucles génicos

Paula Allepuz Fuster

Salamanca 2018



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Que la licenciada Paula Allepuz Fuster ha realizado el trabajo titulado "**Papel de Rpb4/7 en la fosforilación de la RNA polimerasa II y en la formación de los bucles génicos**" bajo mi dirección, en el Instituto de Biología Funcional y Genómica (IBFG), centro mixto de la Universidad de Salamanca y del Consejo Superior de Investigaciones Científicas (CSIC), para optar al grado de Doctora en Biología.

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide el presente certificado.

Salamanca, 18 de Diciembre de 2017

Dra. Olga María Calvo García

El **Dr. Francisco del Rey Iglesias**, Catedrático del Departamento de Microbiología y Genética de la Universidad de Salamanca,

CERTIFICA:

Que la memoria titulada "**Papel de Rpb4/7 en la fosforilación de la RNA polimerasa II y en la formación de los bucles génicos**" presentada por la licenciada Paula Allepuz Fuster para optar al grado de Doctora en Biología por la Universidad de Salamanca, ha sido realizada bajo la dirección de la Dra. Olga María Calvo García, en el Instituto de Biología Funcional y Genómica, centro mixto de la Universidad de Salamanca y del Consejo Superior de Investigaciones Científicas (CSIC).

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide el presente certificado.

Salamanca, a 18 de Diciembre de 2017

Dr. Francisco del Rey Iglesias

A Eliseu

"Perquè hi haurà un dia
que no podrem més
i llavors ho podrem tot"

Vicent Andrés Estellés

AGRADECIMIENTOS

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Introducción

La transcripción es uno de los procesos más regulados de la expresión génica. Dicho proceso es llevado a cabo por las enzimas denominadas RNA polimerasas (RNAP), las cuales se encargan de sintetizar RNA a partir de un DNA molde.

El RNA tiene un papel fundamental en la expresión de los genes en todos los organismos vivos, por ello no es sorprendente que las enzimas encargadas de su síntesis, las RNAPs, se encuentren altamente conservadas en la evolución (Werner, 2008). Mientras que arqueas y bacterias poseen una única RNAP para transcribir todo su genoma, en eucariotas hay varias RNAPs nucleares especializadas en transcribir distintas clases de genes (revisado en Werner and Grohmann, 2011). Así, la RNAPI se encarga principalmente de la transcripción de los RNA ribosomales (rRNAs) 18S, 28S (25S en levaduras) y 5,8S. La RNAPII transcribe todos los genes que codifican para proteínas, RNA mensajeros (mRNAs), algunos RNAs de pequeño tamaño no codificantes (snRNAs y snoRNAs), además de transcribir los CUTs (del inglés "*cryptic unstable transcripts*"), SUTs (del inglés "*stable unannotated transcripts*") y XUTs (del inglés "*Xrn1-sensitive transcripts*") (Housley and Tollervey, 2009; Van Dijk *et al*., 2011; Xu *et al*., 2009). La RNAPIII es la responsable de la síntesis del rRNA 5S, de los RNA de transferencia (tRNAs) y de algunos RNAs pequeños, como son los elementos SINE (Haag and Pikaard, 2011). Adicionalmente, en plantas existen otras dos RNAP nucleares, la RNAPIV y la RNAPV, que sintetizan pequeños RNAs de interferencia (siRNA) encargados de regular la metilación de las secuencias de DNA complementarias, participando en el silenciamiento génico (Haag and Pikaard, 2011).

Todas las RNAPs están relacionadas evolutivamente y presentan propiedades comunes a nivel estructural y funcional (Cramer *et al*, 2002b). La estructura de todas las RNAPs nucleares eucariotas está altamente conservada, así hay subunidades comunes a todas las polimerasas y otras que, aunque son específicas de cada polimerasa, comparten cierto grado de homología entre ellas e, incluso, con subunidades de la RNAP bacteriana (Langer *et al.,* 1995; Werner and Grohman, 2011). Por ejemplo, las dos subunidades mayores de las tres RNAPs eucariotas, donde se encuentran los centros catalíticos de las enzimas, están altamente conservadas, aunque todas ellas tienen particularidades que las hacen diferentes. Así, la subunidad mayor de la RNAPII, Rpb1, posee un dominio exclusivo en su extremo carboxilo terminal, que se denomina CTD, el cual sufre un gran número de modificaciones postraduccionales a lo largo de

todo el ciclo de transcripción. Modificaciones que son de crucial importancia para la regulación de la transcripción en particular y de la expresión génica en general, como veremos más adelante (revisado en Heidemann *et al.*,2012).

1. La RNA polimerasa II: estructura y función

La RNAPII está formada por 12 subunidades denominadas Rpb1-Rpb12, en función de su peso molecular (Figuras I1A), siendo Rpb1 la subunidad mayor y Rpb12 la menor (Cramer *et al*., 2008; Young, 1991). La estructura de la RNAPII se resolvió mediante cristalografía de rayos X, en un primer momento a partir de un cristal que contenía el complejo de 10 subunidades (carente de las subunidades Rpb4 y Rpb7) (Cramer *et al*., 2000; Cramer *et al*., 2001; Gnatt *et al*., 2001) y posteriormente conteniendo las 12 subunidades (Armache *et al*., 2003) (Figura I1A).

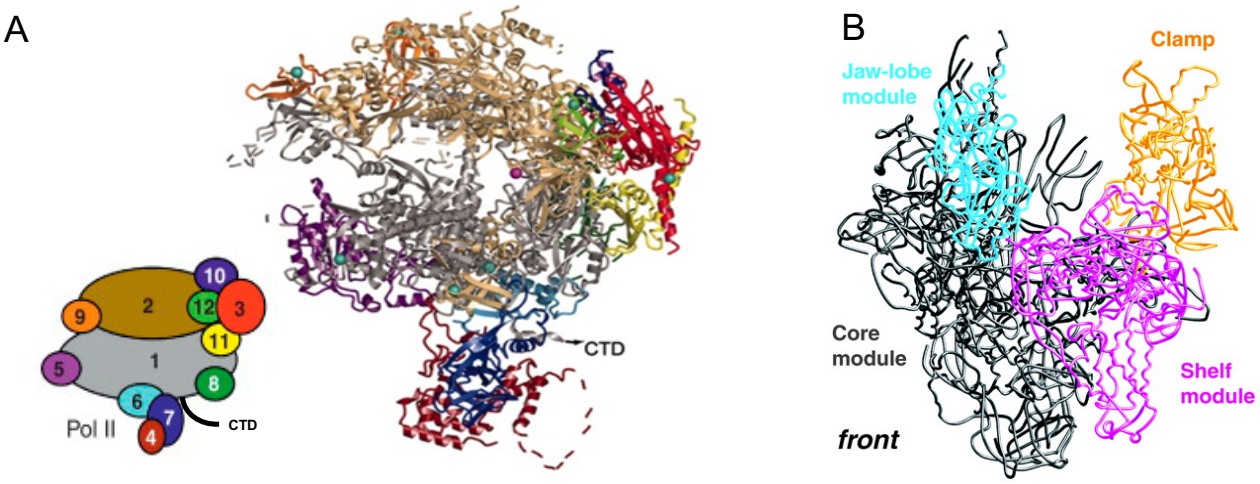

Figura I1. Estructura de la RNA polimerasa II: Rpb1-Rpb12. (A) Representación mediante diagrama de cintas de la estructura completa de la RNAPII. Se representa una vista superior del complejo de la RNAPII. Las 12 subunidades están coloreadas siguiendo el patrón de colores del esquema de bolas que encontramos a la izquierda. Las líneas discontinuas representan zonas de estructura desordenada. Imagen extraída de (Armache et al., 2005). (B) Representación del núcleo de la RNAPII (10 subunidades, sin el heterodímero Rpb4/7). Los módulos *core, jaw-lobe, shelf* y *clamp*, se muestran en negro, azul, rosa y naranja, respectivamente. Imagen extraída de (Cramer *et al*., 2001).

De las 12 subunidades, sólo 2 de ellas no son esenciales para la viabilidad celular, Rpb4 y Rpb9 (Woychik and Young, 1990). Existen 5 subunidades comunes a las tres RNA polimerasas (Rpb5, Rpb6, Rpb8, Rpb10 y Rpb12) y cuatro de ellas presentan homología de secuencia con las correspondientes subunidades de la RNAPI y III (Rpb1, Rpb2, Rpb3 y Rpb11) (Carles *et al*., 1991; Hampsey, 1998; Woychik *et al*., 1990; Young, 1991). Rpb9 es específica de la RNAPII, aunque comparte cierta homología estructural con la región N-terminal de A12.2 de la RNAPI y con el extremo N-terminal

de Rpc11 de la RNAPIII (Chédin *et al.,* 1998). Rpb4 y Rpb7 forman un heterodímero, denominado tallo o *stalk,* que en el caso de *S. cerevisiae* es disociable del resto del complejo (Edwards *et al*., 1991; Cramer *et al*., 2001; Kwapisz *et al*., 2008; Ruprich-Robert and Thuriaux, 2010). Este dominio no se encuentra conservado en eubacterias, y en arqueas y eucariotas se localiza en la periferia de las enzimas, muy cerca del canal de salida del mRNA naciente, así como del CTD de Rpb1 (Werner and Grohmann, 2011) (Figura I1A). De acuerdo con esto, estudios *in vitro* en células humanas sugieren que Rpb7 es capaz de interaccionar directamente con los transcritos nacientes de la RNAPII (Ujvári and Luse, 2006). Aunque estas subunidades no comparten homología de secuencia con ninguna subunidad de la RNAPI y RNAPIII, poseen cierta homología estructural con dos subunidades de la RNAPI (Rpa43/17) y dos de la RNAPIII (Rpc25/17), que también forman un heterodímero (Vannini and Cramer, 2012). Por último, la característica más exclusiva de la RNAPII, como ya hemos mencionado, es el CTD de Rpb1, del que hablaremos en detalle más adelante.

La RNAPII presenta una morfología similar a la de una *pinza de cangrejo*, y las doce subunidades se organizan en cinco módulos estructurales diferentes, que presentan cierta movilidad relativa entre ellos (Figura 1B). Estos módulos son: *core* o "núcleo", *jaw-lobe o* "garra-lóbulo", *shelf* o "plataforma", *clamp* o "grapa" y *stalk o* "tallo" (Cramer *et al*., 2000, 2001; Gnatt *et al*., 2001).

El "núcleo" (*core*) de la enzima está formado por las subunidades Rpb1 y Rpb2, en las que reside el centro activo de la polimerasa, y por Rpb3, Rpb10, Rpb11 y Rpb12, las cuales están implicadas en el proceso de ensamblaje del complejo (Cramer *et al*., 2001). El módulo "garra-lóbulo" (*jaw-lobe*) contiene la garra superior, formado por regiones de Rpb1, Rpb5 y Rpb9, y por el dominio lóbulo de Rpb2. El módulo "plataforma" (*shelf*) contiene la región inferior de la garra (un dominio de Rpb5), el dominio de ensamblaje de Rpb5, Rpb6, las regiones del "pie" o *foot* y la "hendidura" o *cleft* de Rpb1. El módulo denominado "grapa", está constituido por regiones de Rpb1 y Rpb2 (Cramer *et al*., 2001).

El módulo "garra-lóbulo" junto con el módulo *"*plataforma*",* contribuye a la entrada del DNA al centro activo de la enzima, siendo el módulo "grapa" el que, a través de un cambio de conformación posiciona el DNA en el centro activo. Este cambio de conformación produce que el dominio *clamp* o "grapa" pase del estado abierto inactivo,

que permite el acceso del dsDNA al centro activo, a un estado cerrado activo, que posiciona el ssDNA en el centro activo. Esto facilita la separación del hibrido DNA-RNA en el extremo de la burbuja de transcripción, favoreciendo así la procesividad de la enzima durante la elongación (Cramer, 2002, 2004; Cramer *et al*., 2000, 2001; Gnatt *et al.,* 2001).

Por último, el "tallo" o *stalk* forman un subcomplejo de dos subunidades constituido por Rpb4 y Rpb7, que interaccionan entre sí y pueden disociarse del resto del complejo de la RNAPII en *Saccharomyces cerevisiae*. Curiosamente, ambas subunidades se encuentran en la célula en mayor abundancia que el resto del complejo enzimático de la RNAPII, y se han propuesto funciones específicas para las mismas (revisado en Sharma and Kumari, 2013). Dado que el objeto de estudio de este trabajo ha sido descifrar el papel del heterodímero Rpb4/7 en la fosforilación del CTD de Rpb1 de la RNAPII, nos detendremos un poco más en describir las características funcionales de estos dos importantes dominios, el *stalk* y el CTD.

1.1 Rpb4/7: subunidades multifuncionales de la RNAPII

En *Saccharomyces cerevisiae* el heterodímero Rpb4/7 es un complejo muy versátil capaz de actuar como regulador en la mayoría de las etapas de la expresión génica, incluyendo la transcripción, la reparación del DNA acoplada a la transcripción, el transporte del mRNA, su degradación y su traducción (Schulz *et al.,* 2014; Li and Smerdon, 2002; Harel-Sharvit *et al.,* 2010; Lotan *et al.,* 2005; Haimovich *et al.,* 2013; Dahan and Choder, 2013) (Figura I2). Además, también participan en la respuesta a estrés (Choder and Young, 1993; McKune *et al*., 1993; Pillai *et al.,* 2003; Garrido-Godino *et al.,* 2016; revisado en Sharma and Kumari, 2013); y se ha descrito que la tasa de crecimiento de las células en fase estacionaria es dependiente de los niveles de Rpb4 presentes en las mismas (Choder and Young, 1993).

En lo que respecta al ciclo de transcripción, el heterodímero Rpb4/7 además de participar en la iniciación (Edwards *et al.,* 1991) y en la elongación (Sampath *et al*., 2008; Deshpande *et al*., 2014), parece ser que podría tener un papel en la terminación, influyendo en la asociación de ciertos factores de procesamiento del extremo 3' del gen (Runner *et al.,* 2008). Aunque inicialmente se describió que el heterodímero se asociaba a la RNAPII en los promotores y viajaba con la RNAPII a lo largo de toda la

transcripción (Runner *et al*., 2008; Jasiak *et al.,* 2008), existe cierta controversia y en algún estudio se ha mostrado que Rpb4/7 se disocia del complejo a lo largo de la elongación, dependiendo de su interacción con varios factores de elongación (Mosley *et al.,* 2013).

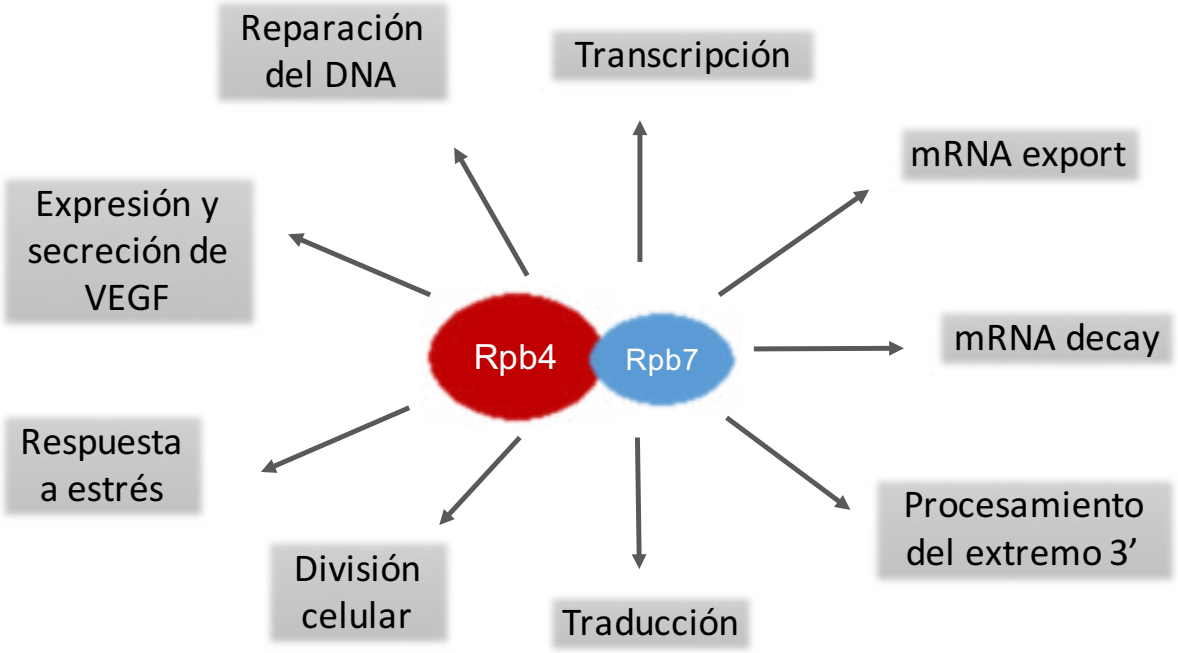

Figura I2. Representación esquemática de los distintos procesos en los que se ha implicado al heterodímero Rpb4/7.

Mientras que Rpb7 es esencial para la viabilidad celular, Rpb4 es dispensable, aunque en su ausencia los niveles de Rpb7 se encuentran muy disminuidos y es difícil detectar su asociació a la RNAPII (Kolodziej *et al*., 1990; Runner *et al*., 2008; Sheffer *et al*., 1999). De hecho, parece ser que una de las funciones de Rpb4 sería la estabilización de la asociación de Rpb7 con el resto de la RNAPII, como lo demuestran los siguientes hechos: Rpb4 es necesario para el crecimiento a altas y bajas temperaturas (Woychik and Young, 1989; Wiychik *et al*., 1991). Así, en células carentes de Rpb4 se produce un completo apagado de la transcripción cuando se las somete a altas temperaturas (Miyao *et al*., 2001). Sin embargo, en estas células la sobreexpresión de *RPB7* rescata su crecimiento lento y la sensibilidad a altas y bajas temperaturas (Sheffer *et al*., 1999). Además, mediante ensayos de co-inmunoprecipitación se ha observado la existencia de una interacción estable de Rpb7 con la RNAPII cuando esta subunidad se sobreexpresa en células carentes de *RPB4*

(Sharma and Sadhale, 1999; Sheffer *et al*., 1999).

Por otra parte, alteraciones en los niveles de ambas subunidades afecta de modo diferente a la expresión génica, resultando en fenotipos celulares distintos. Estas evidencias sugieren que tanto Rpb4 como Rpb7 no solo tienen una función en el contexto del heterodímero Rpb4/7, sino que también desarrollan funciones independientes (revisado en Sharma and Kumari, 2013). De hecho, Rpb4 favorece la esporulación y suprime la formación de hifas mientras que Rpb7 sería a la inversa (Singh *et al.,* 2007). Además, Rpb4 es el responsable de la transcripción de genes de respuesta a estrés térmico (Miyao *et al.,* 2001; Pillai *et al.,* 2003).

En 2010 el heterodímero Rpb4/7 se postuló como coordinador del acoplamiento entre la transcripción dependiente de la RNAPII, la traducción y la degradación del mRNA (Harel-Sharvit *et al*., 2010), adquiriendo un papel relevante en lo que se empezó a conocer como la recirculariación de la expresión del mRNA. Se cree que Rpb4/7 interacciona con el mRNA naciente de una manera dependiente de su interacción con la RNAPII (Goter-Baron *et al*., 2008). Finalizada la transcripción del mRNA, el complejo mRNA-Rpb4/7 interacciona directamente con eIF3 (factor de iniciación de la traducción en eucariotas), estimulando el inicio de la traducción en el citoplasma (Harel-Sharvit *et al*., 2010). De igual manera, Rpb4/7 estimula el acortamiento de la cola poli(A) de los mRNAs, promoviendo la degradación de éstos (Lotan *et al.,* 2007, 2005). Una rápida iniciación de la producción de proteínas, seguida de una degradación eficaz del mRNA es necesaria para un control eficiente de la expresión génica, acelerándose ambos procesos mediante el marcaje de los mRNAs con el heterodímero Rpb4/7 (Figura I3). Así, parece ser que los mRNAs a los que se asocia Rpb4/7 presentan una mayor capacidad para el reclutamiento de la maquinaria de degradación en el citoplasma. De hecho, se cree que el ritmo de degradación de los diferentes mRNAs está en proporción al número de copias de cada mRNA que lleve unido Rpb4/7 (Shalem *et al*., 2011). Así, por ejemplo, el mutante de deleción de Rpb4 presenta una tasa de síntesis de mRNA muy reducida, pero esta disminución es compensaba por una disminución similar en la degradación del mRNA (Schulz *et al.,* 2014) mediante el proceso conocido como *buffering* del mRNA, que conecta transcripción y degradación (Sun *et al.,* 2012; Sun *et al.,* 2013; Miller *et al.,* 2011). Por último, se ha propuesto, que tras la degradación del

mRNA en el citoplasma, el dímero Rpb4/7 vuelve al núcleo donde puede participar en una nueva ronda de transcripción (Figura I3) (Selitrennik *et al*., 2006).

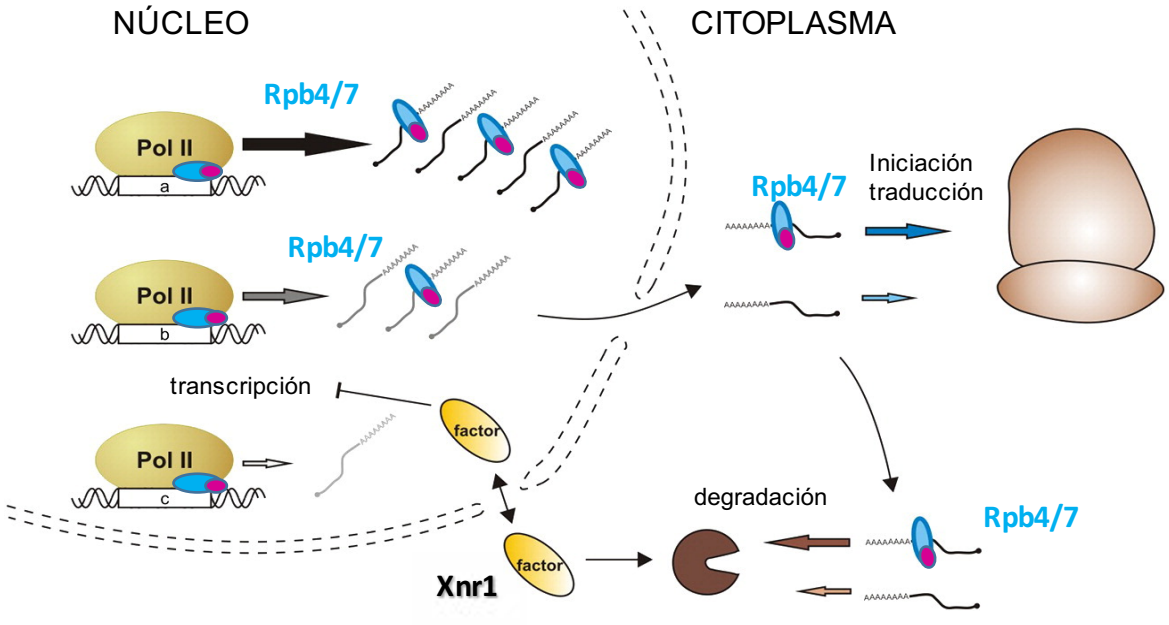

Figura I3. La transcripción y la degradación del mRNA se encuentran acopladas. Una vez se produce la transcripción por la RNAPII, el mRNA es marcado con el heterodímero Rpb4/7 (representado por la elipse azul y rosa) de un modo dependiente de la transcripción. La presencia de Rpb4/7 en el mRNA estimula la traducción de los mismos (flechas azules) y a su vez su degradación (flechas marrones). Xnr1 actuaría como factor de control entre la transcripción y la degradación de los mRNAs (Haimovich *et al*., 2013). Imagen modificada de (Turowski *et al.,* 2013).

2. El ciclo de transcripción

La maquinaria transcripcional de la RNAPII es la más compleja de todas e implica factores de transcripción, co-reguladores, activadores y represores específicos, los cuales aseguran una correcta transcripción (Hahn, 2004).

La transcripción por la RNAPII está regulada a varios niveles: durante el ensamblaje de la RNAPII en la cromatina, antes (pre-iniciación) y durante el inicio de la transcripción, durante la elongación y el procesamiento del mRNA y en la terminación (Shandilya and Roberts, 2012). Durante mucho tiempo se pensó que la pre-iniciación/iniciación de la transcripción era el paso más regulado y limitante, sin embargo, en los últimos años un gran número de trabajos han demostrado que tanto la elongación como la terminación de la transcripción son procesos alta y finalmente regulados (revisado en Kwak and Lis, 2013 y Porrua and Libri, 2015). Además, existe un acoplamiento entre la transcripción y el procesamiento de los transcritos nacientes

mediado por el CTD, lo que supone un incremento en la eficiencia del procesamiento y garantiza que solamente los mensajeros que han sido procesados correctamente se transporten al núcleo.

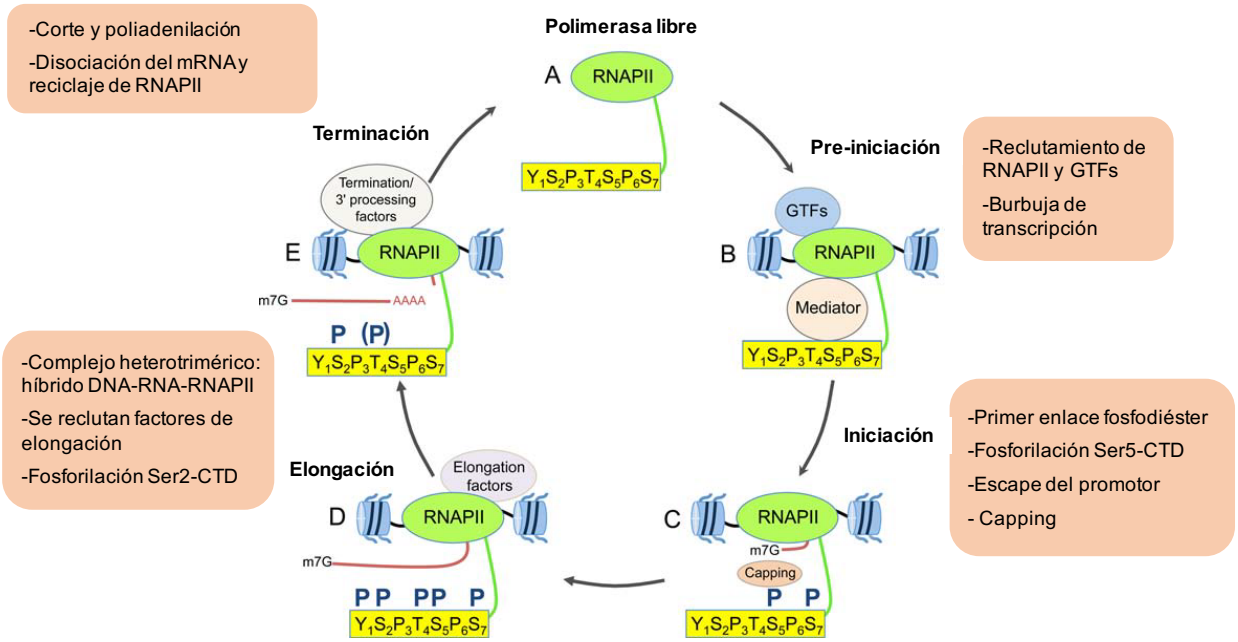

Figura I4. Ciclo de transcripción de la RNAPII. Esquema en el que se muestran las distintas etapas del ciclo de transcripción (pre-iniciación, iniciación, elongación y terminación), con las modificaciones más importantes de la RNAPII, así como los distintos factores que se asocian a esta a lo largo del ciclo. En los recuadros anaranjados se detallan puntos clave de las distintas etapas. Imagen modificada de (Dirck and Geyer, 2013).

2.1. Pre-iniciación/Iniciación: formación del PIC en el promotor

Numerosas rutas de señalización que controlan procesos como son la identidad celular, el crecimiento, la diferenciación y el desarrollo, hacen diana en última instancia en la maquinaria transcripcional durante el inicio de la transcripción que, en el caso de los genes que codifican para proteínas, consisten básicamente en la RNAPII y los factores generales de la transcripción (GTFs: TFIIA, TFIIB, TFIID, TFIIE, TFIIF y TFIIH). Estos están normalmente asistidos y/o regulados por un gran número de proteínas de diversa índole. Así, para dar inicio a la transcripción, un conjunto enorme de proteínas, más de un centenar en humanos, se organizan frecuentemente en grandes complejos multiproteicos que incluyen a los GTFs, la RNAPII y numerosos y diversos complejos que actúan como co-activadores, co-represores, modificadores y remodeladores de cromatina (Gupta *et al*., 2016). Además, existe un gran número de activadores y represores regulando la pre-iniciación, entre los que se encuentra un complejo

multiproteico crucial, denominado Mediador, y que se ha identificado como un regulador global de la expresión génica (Eychenne *et al*., 2017).

La RNAPII es incapaz de unirse por sí sola al promotor, por lo que necesita de otras proteínas capaces de reconocer los elementos que le permitan posicionarse y formar el complejo de pre-iniciación (PIC) e iniciar una ronda de transcripción (Sikorski and Buratowski, 2009; Thomas and Chiang, 2006). Las proteínas que forman el PIC reconocen las secuencias promotoras, reclutando y posicionando a la RNAPII. Además, son necesarias para la formación y estabilización de la burbuja de transcripción, selección del sitio de inicio de la transcripción (TSS) y para facilitar el escape del promotor (revisado en Thomas and Chiang, 2006).

Normalmente, la formación del PIC comienza con el reclutamiento del factor TFIID a los promotores, a través de la caja TATA u otros elementos consenso, uniéndose al DNA y produciendo una serie de cambios que facilitan la unión de otros factores generales de la transcripción (GTFs) y más tarde de la RNAPII. Es importante recalcar que casi todos los estudios bioquímicos y estructurales han estudiado la pre-iniciación en promotores que contienen caja TATA. Aunque los promotores con caja TATA comprenden solamente el 20-30% de los promotores eucarióticos (Ohler *et al.,* 2002; Yang *et al.,* 2007; Rhee and Pugh, 2012), normalmente son los que se encuentran finamente regulados. Por el contrario, los promotores que carecen de cajas TATA dirigen la transcripción de los genes de expresión constitutiva y tienen TSSs heterogéneos (Grungerg and Hahn, 2013).

El ensamblaje de los GTF puede realizarse de forma secuencial o bien asociarse pre-ensamblados en el complejo conocido como holoenzima (revisado en Shandilya and Roberts, 2012; Thomas and Chiang, 2006).

2.2. Iniciación

La formación de un PIC completo en el promotor del gen no garantiza una transcripción productiva *in vivo*. Un PIC bien formado, como hemos dicho, es un complejo estable (complejo cerrado) y posicionado en el DNA, pero inactivo e incompetente para la transcripción. La RNAPII, con ayuda del factor TFIIB, escanea la cadena de DNA molde en busca del TSS correcto. Es en este momento cuando pasa de un complejo cerrado a un complejo abierto de iniciación, lo que implica el acceso del

dsDNA del promotor al centro activo de la polimerasa a través del dominio *cleft,* aproximadamente unos 20 pb aguas abajo de la secuencia TATA (Giardina and Lis, 1993; Cramer *et al*., 2001; Kostrewa *et al*., 2009; Wang *et al*., 1992). Este paso se produce gracias a la actividad helicasa y ATPasa del factor TFIIH (Dvir *et al*., 1997; Holstage *et al*., 1997; Wang *et al*., 1992). En este punto, el dominio estructural *clamp* presenta una conformación cerrada, debido a la presencia del heterodímero Rpb4/7, favoreciendo la formación del primer enlace fosfodiester entre los primeros NTPs (Armache *et al.,* 2003; Bushnell and Kornberg, 2003). La transición de 2-3 nts de RNA a 4-5 nts de RNA es un paso crucial en la superación de la iniciación abortiva. En el caso de producirse una iniciación abortiva, estos pequeños transcritos son liberados y rápidamente degradados (Liu *et al*., 2011).

En la mayoría de los eucariotas, después de haber sintetizado 20-100 bases, la RNAPII puede pausarse (*Promoter proximal pause*) y entonces rompe sus contactos con la secuencia promotora, se disocia de algunos de los componentes del PIC, y establece una unión más fuerte y estable con el RNA naciente. Este paso da lugar a un complejo de elongación completamente funcional en un proceso denominado “escape o salida del promotor” (Westover *et al*., 2004). En levaduras, cuando el transcrito alcanza una longitud de 8-9 nts es lo suficientemente largo como para establecer contactos fuera del canal de salida del RNA, determinando la separación del RNA-DNA molde y produciendo un cambio conformacional en la RNAPII, que pasará a contener un híbrido RNA-DNA estable en su centro activo (Westover *et al.,* 2004). Además, la liberación del factor TFIIB produce el colapso de la burbuja de transcripción, favoreciéndose así la salida de la polimerasa del promotor (Kostrewa *et al.,* 2009; Pal *et al.,* 2005), e iniciándose la elongación. De hecho, la mayoría de los componentes del PIC unidos al promotor se mantienen en su lugar y sólo TFIIB, TFIIF y la RNAPII necesitan ser reclutados durante la reiniciación, incrementado sustancialmente la tasa de transcripción y las rondas subsiguientes de transcripción (Hahn, 2004; Yudkovsky *et al*., 2000).

En todo este proceso inicial de la transcripción, el domino carboxilo terminal (CTD) de Rpb1 se encuentra defosforilado (Myers *et al*., 1998). La fosforilación del CTD en unos residuos serina específicos, de los que hablaremos más adelante, favorece el

escape de la RNAPII del promotor, comenzando así la elongación (revisado en Calvo and García, 2012).

2.3. Elongación

Es en este momento cuando la RNAPII da paso a la elongación, transcribiendo a lo largo de la secuencia del gen. La elongación podría definirse como la etapa durante la cual la polimerasa atraviesa y lee las regiones codificantes del DNA molde sintetizándose el RNA naciente. Una polimerasa elongante presenta dos cualidades muy importantes e imprescindibles para la producción del transcrito maduro: una alta procesividad y una tasa de elongación constante. Ambas cualidades pueden verse comprometidas a lo largo de la elongación (Nechaev and Adelman, 2011). La procesividad puede definirse como la capacidad de la polimerasa para viajar a lo largo del gen molde sin disociarse, evitando una terminación abrupta de la transcripción. La tasa de elongación puede considerarse como la velocidad a la que la polimerasa viaja a lo largo del gen en transcripción (Mason and Struhl, 2005).

En los últimos años la investigación en esta etapa transcripcional ha sido muy intensa, determinando la elongación como un proceso altamente regulado y ampliamente ligado a los estados de fosforilación del CTD. La regulación ocurre tanto en los primeros estadios de la elongación a través de una pausa de la RNAPII, como al ser liberada para entrar en la fase de elongación productiva. Durante los pasos iniciales de la elongación, la RNAPII puede pausarse y acumularse en zonas cercanas a la región promotora, 30-60 nts aguas abajo del TSS, ejerciendo como control de calidad del proceso de *capping* y de las modificaciones en la RNAPII antes de entrar en una elongación productiva (revisado en Jonkers and Lis, 2015).

A lo largo de la elongación encontramos los primeros eventos del procesamiento del transcrito naciente de modo co-transcripcional. Durante la elongación temprana, cuando el transcrito tiene un tamaño de unos 20 nts (Coppola *et al*., 1983; Chiu *et al*., 2002; Moteki and Price, 2002; Rasmussen and Lis, 1993), se produce el reclutamiento de la maquinaria del *capping* que añade una caperuza de 7-metil-guanosina en el extremo 5’ del mRNA naciente. Dicha caperuza estabiliza el transcrito, lo protege de la degradación, promoviéndose una correcta elongación. Además, el *capping* promueve la eliminación de intrones y el procesamiento del extremo 3’ y facilita el transporte de los

mRNAs al citoplasma y su posterior traducción (revisado en Lewis and Izaurralde, 1997). La regulación y coordinación de la elongación con la maquinaria del *capping* está mediada por las fosforilaciones específicas y secuenciales del CTD de Rpb1 (Fabrega *et al*., 2003; Ghosh *et al*., 2011; Komarnitsky *et al*., 2000; McCracken *et al.,* 1997; Shroeder *et al.,* 2000).

Más adelante en la elongación se recluta el *espliceosoma*, encargado de la eliminación de intrones o *splicing* (Ardehali and Lis, 2009). El *splicing* puede iniciarse de modo co-transcripcional y termina post-transcripcionalmente, debido al desfase entre la velocidad de procesamiento del *espliceosoma* y la velocidad de síntesis de la RNAPII (Beyer and Osheim, 1988). Al igual que en el *capping*, el acoplamiento del *splicing* con la transcripción está mediado por la fosforilación del CTD, aunque esta vez en un residuo diferente (Davis and Manley, 2011; Hargreaves *et al*., 2009). Además, la fosforilación del CTD podría regular el *splicing* alternativo mediante la modulación de la concentración local de factores del *espliceosoma* (De la Mata *et al*., 2003; Muñoz *et al*., 2010).

2.4. Terminación

Después de la síntesis completa del mRNA, la RNAPII y el RNA naciente se disocian del DNA molde, marcando el final del ciclo de transcripción. Se trata de un proceso dinámico, el cual no se encuentra restringido a las secuencias 3' de los genes, sino que puede tener lugar a lo largo de toda la ORF (Kuehner *et al*., 2011). Existen dos rutas bien estudiadas para llevar a cabo la terminación, una dependiente de la poliadenilación para la mayoría de los mRNAs (Proudfood *et al*.,2002; revisado en Shandilya and Roberts, 2012) y otra ruta dependiente de la helicasa Sen1 (Steinmetz and Brow, 1996) para los snRNAs y snoRNAs, cuyos extremos 3' son generados por cortes endonucleolíticos y/o exonucleolítico, y a los que no se añade una cola poli(A) en su forma madura, así como para los transcritos crípticos (CUTs) (Revisado en Berendren, 2013).

En el caso de los mRNAs, previamente a la terminación, se produce el procesamiento del extremo 3' de los mRNAs que consiste en un corte endonucleotídico y la posterior adición de una cola de poliadeninas. Los factores de procesamiento del extremo 3' se asocian a la RNAPII así como a su CTD. En levaduras, el reclutamiento

de esta maquinaria depende de nuevo de un patrón de fosforilación determinado.

2.5. El final como nuevo principio – GENE LOOPING

Una vez que se ha transcrito completamente el gen, la RNAPII encuentra una señal de pausa, a la que le sigue tanto la liberación de la propia polimerasa, como del mRNA recién sintetizado. La disociación de la RNAPII es necesaria, además, para poder iniciar una nueva ronda de transcripción.

Análisis topológicos de la cromatina de *Saccharomyces cerevisiae* han permitido identificar bucles génicos que yuxtaponen las regiones del promotor y del terminador de genes transcritos por la RNAPII (O'Sullivan *et al.* 2004; Ansari and Hampsey, 2005; Singh and Hampsey 2007; El Kaderi *et al.* 2009) (Figura I5). Los bucles génicos, o *gene loops*, son estructuras dinámicas cuya formación es dependiente de la transcripción de la RNAPII, y requiere también del factor de transcripción TFIIB y de componentes de la maquinaria de procesamiento del extremo 3' de los pre-mRNAs (Ansari and Hampsey, 2005; Medler *et al.,* 2011; El Kaderi *et al.,* 2009). El *gene looping* parece ser un proceso general y conservado de la transcripción de la RNAPII, de hecho se también se ha encontrado en mamíferos, mosca y el provirus HIV-1 (Perkins *et al*., 2008; Tan-Wong *et al*., 2008; O'Reilly and Greaves, 2007; Erokhin *et al.,* 2011).

Inmediatamente después de la iniciación de la transcripción, la RNAPII abandona el promotor y el TFIIB se disocia del complejo de iniciación, junto con TFIIF. En este momento, no todos los componentes del PIC se disocian, sino que algunos de ellos permanecerán unidos al promotor (TFIID, TFIIA, TFIIH, TFIIE y Mediator; Yudkovski *et al.,* 2000). Este conjunto de factores forman un "andamio", llamado *scaffold*, el cual facilitará las siguientes rondas de transcripción (Yudkovsky *et al.,* 2000). El modelo actual sugiere que el factor TFIIB se disocia del promotor y posteriormente se asocia de nuevo a la RNAPII en la región terminadora, una vez que se ha producido el corte endonucleolítico y la poliadenilación del mRNA. Este complejo terminador-RNAPII-TFIIB se une de nuevo al *scaffold* formando un bucle génico que facilita la formación de un complejo de reiniciación (Singh and Hampsey, 2007) (Figura I5). De acuerdo con esto, una ronda de transcripción inicial es necesaria para la formación de los bucles, facilitando las siguientes rondas de transcripción (Ansari and Hampsey, 2005). Para la translocación de la RNAPII del terminador al promotor también se requiere de la función

de la CTD fosfatasa Ssu72 (Ansari and Hampsey, 2005), la cual cataliza la defosforilación parcial del CTD y promueve la actuación de otra CTD-fosfatasa, Fcp1 (Bataille *et al.,* 2012). La actividad fosfatasa de ambas genera finalmente la forma hipofosforilada de la RNAPII que se requiere para el ensamblaje del PIC (Zhang *et al.,* 2012). Ssu72 es además un componente del CPF (del inglés "cleavage and polyadenilation factor") implicado en el procesamiento del extremo 3' de los pre-mRNAs y en la terminación de la transcripción (Dichtl *et al.,* 2002; He *et al.,* 2003; Krishnamurthy *et al.,* 2004; Steinmetz and Brow, 2003; Xiang *et al.,* 2010).

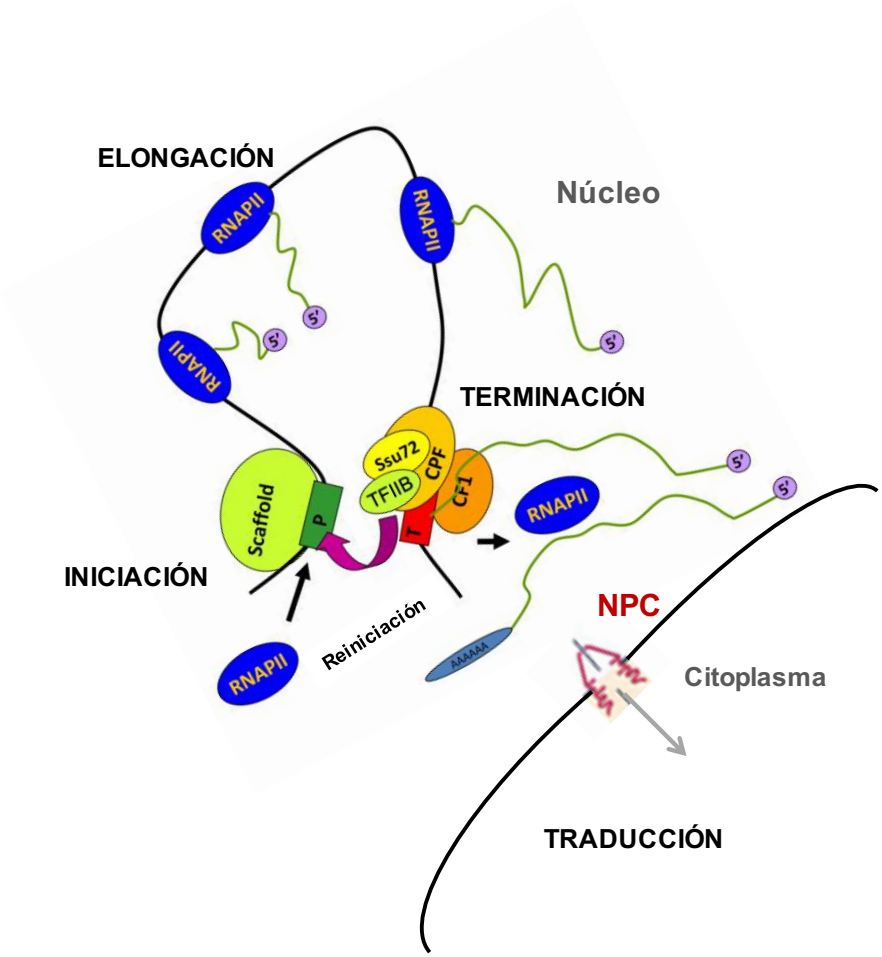

Figura I5. Modelo de la formación de los bucles génicos. La imagen muestra la estructura que presenta el gen en el momento en el que se forma un bucle génico. El rectángulo verde y rojo representan la región promotora y terminadora del gen, respectivamente. CPF corresponde a "*cleavage and polyadenilation factor*" y CFI a "*cleavage factor I*".Imagen modificada de (Hampsey *et al.,* 2011).

Además de promover la reiniciación de la transcripción, se ha demostrado la relevancia fisiológica de la formación de los bucles génicos en varios procesos que regulan la expresión génica:

- **Terminación de la transcripción**

Se ha descrito que un fallo en la formación de los bucles génicos provoca un defecto en la terminación de la transcripción, de manera que la RNAPII continúa la transcripción aguas abajo de la región terminadora, dando lugar al denominado "*readthrough*" (Mukundan and Ansari., 2013). Esto provoca una disminución del reclutamiento de la RNAPII y de los GTFs a las regiones promotoras (Mapendano *et al.,* 2010), así como una disminución en la asociación de los factores de procesamiento del extremo 3' en las regiones terminadoras (Mukundan and Ansari., 2013). Estos resultados apoyan la hipótesis existente de que la RNAPII, una vez formado el bucle génico tras la primera ronda de transcripción, es transferida directamente desde la región terminadora al promotor para reiniciar la transcripción. Hasta el momento, en *S. cerevisae*, todos los mutantes de factores implicados en la formación de los bucles génicos, tienen además alterada la terminación de la transcripción (Mukundan and Ansari., 2013; Mapendano *et al.,* 2010).

- **Regulación transcripcional mediada por intrones**

Parece ser que otra función de los bucles génicos está relacionada con la regulación transcripcional mediada por intrones. En *S. cerevisiae* se ha descrito que genes que contienen intrones, su transcripción se activa de un modo más eficiente. Esto no es dependiente del *splicing*, sino de la configuración que adquiere el bucle génico, cuya configuración es facilitada por el intrón (Moabbi *et al.,* 2012).

- **Direccionalidad transcripcional**

Un gran número de promotores de la RNAPII son bidireccionales en muchas especies, incluyendo humanos (Trinklein *et al*., 2004; Preker *et al*., 2008; Core *et al*., 2008), ratón (Koyanagi *et al*., 2005), *Arabidopsis thaliana* (Wang *et al*., 2009) y *Saccharomyces cerevisiae* (Neil *et al*., 2009; Xu *et al*., 2009). Esto implica que la RNAPII, una vez asociada al promotor de estos genes puede transcribir en ambas direcciones. En este tipo de promotores puede ocurrir que la transcripción en una de las direcciones conduzca a la síntesis de RNAs no codificantes, los cuales pueden ser tóxicos para la célula si se acumulan (Neil *et al*., 2009; Preker *et al*., 2008; Seila *et al*., 2008). Mediante una terminación temprana de la transcripción, seguida de una rápida

degradación del RNA no codificante (ncRNA), se eliminan los resultados de este tipo de transcripción divergente no deseada (Almada *et al*., 2013; Arigo *et al*., 2006; Schulz *et al*., 2013). Por otra parte, resultados recientes muestran la existencia de un mecanismo específico por el que la formación de bucles génicos dirige la transcripción en la dirección productiva "animando" a la RNAPII a transcribir en el sentido codificante de la secuencia y evitando la transcripción de los ncRNAs (Tan-Wong *et al*., 2012). Así, por ejemplo, mutaciones en Ssu72 afectan a la formación de los bucles génicos, lo cual conlleva a que la RNAPII localizada en promotores bidireccionales transcriba los ncRNAs subyacentes en estas zonas y su consecuente acumulación (Lopez and Henderson, 2015). De igual manera, una mutación del TFIIB (*sua7-1*), que altera la formación de los bucles génicos (Singh and Hampsey., 2007) y provoca defectos en terminación, causa la misma alteración en la direccionalidad de la transcripción (Tan-Wong *et al*., 2012; revisado en Grzechnik *et al*., 2014), indicando de nuevo la importancia de la formación de dichas estructuras para mantener la direccionalidad de la transcripción.

- **Memoria transcripcional**

Se trata del mecanismo por el cual se produce una reactivación rápida de la transcripción, causando una respuesta más rápida a un estimulo, una vez se ha pasado por un ciclo inicial de activación y represión del gen. De este modo, aunque mutaciones que alteran el *gene looping* no afectan la transcripción inicial de promotores inducibles, como *GAL1*, si causan una respuesta más lenta a nuevas rondas de activación (Ansari and Hampsey 2005; Singh and Hampsery, 2007). Así, se ha demostrado que la reactivación transcripcional de los genes inducibles *GAL10* y *HXK1* es más rápida que la primera ronda de activación, y que este efecto se pierde en mutantes con ausencia de bucles génicos. Estos resultados indican que el proceso de *gene looping* es fundamental en la reactivación transcripcional posterior a un estímulo previo, confiriéndole un papel importante en el proceso de memoria transcripcional (Tan-Wong *et al.,* 2009; Lainé *et al.,* 2009). Además, se cree que este proceso está vinculado al poro nuclear, facilitando y agilizando el exporte de los mRNAs al citoplasma (Tan-Wong *et al.,* 2009) (Figura I5).

3. La fosforilación de la RNAPII, un proceso esencial en la regulación de la expresión génica

3.1. El dominio carboxilo terminal (CTD) de Rpb1

A diferencia de la RNAPI y RNAPIII, la subunidad mayor de la RNAPII presenta un dominio carboxilo terminal (CTD) exclusivo de ésta. El CTD es esencial para la viabilidad celular, y truncaciones parciales de este o mutaciones de residuos específicos del mismo llevan a defectos en el crecimiento e incluso a la letalidad (Aristizabal *et al.,* 2013; Zhang *et al.,* 2012; Phatnani and Greenleaf, 2006). El CTD no es necesario para la función catalítica de la enzima *in vitro*, pero sí para el procesamiento co-transcripcional de los mRNAs (revisado en Bentley, 2014; y Halen and Churchman, 2017) y para otros procesos de regulación llevados a cabo durante el ciclo transcripcional, como es la modificación y remodelación de la cromatina (revisado en Srivastava and Ahn, 2015; Tanny, 2014).

Se trata de un dominio repetitivo y desestructurado el cual se extiende desde el centro catalítico de la polimerasa a una zona próxima al canal de salida del RNA naciente (Cramer *et al*., 2001; Figura I6). El CTD está formado por una serie de repeticiones en tándem de un heptapéptido consenso. La secuencia de este heptapéptido es la siguiente: Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 ($Y_1S_2P_3T_4S_5P_6S_7$). Esta secuencia consenso está presente en animales, plantas, levaduras y un gran número de protistas (Corden, 1990; Liu *et al*., 2006; Liu *et al*., 2010). Se cree que la organización de la secuencia consenso del CTD se ha originado a través de amplificaciones de una secuencia repetitiva del DNA y que el número de repeticiones presentes en cada organismo parece estar directamente correlacionada con la complejidad genómica del mismo (Chapman *et al*., 2008). Por ejemplo, el CTD de vertebrados superiores presentan 52 repeticiones (Corden *et al*., 1985; Prelich, 2002; Wintzerith *et al*., 1992); el de *Drosophila* 45 (Allison *et al.,*1988); el de levaduras presenta entre 25-27 (Allison *et al*.,1988); y el de los protozoos presenta unas 15 repeticiones (Corden, 1990; Champman *et al*., 2008). El CTD es un dominio desestructurado que sirve como plataforma para coordinar el reclutamiento de proteínas involucradas en las distintas fases de la transcripción y permite la coordinación de ésta con otros procesos nucleares como la maduración del mRNA o modificaciones de la cromatina (Buratowski, 2009; Egloff and Murphy, 2008; Hirose and Ohkuma, 2007;

Muñoz *et al*., 2010; Phatnani and Greenleaf, 2006; Perales and Bentley, 2009; Smith and Shilatifard, 2010).

A

B

RNAPII-CTD Humano				RNAPII-CTD *Saccharomyces cerevisiae*	
1	YSPTSPA	28	YSPTSPS	1	YSPTSPA
2	YEPRSPGG	29	YSPTSPS	2	YSPTSPS
3	YTPQSPS	30	YSPTSPS	3	YSPTSPS
4	YSPTSPS	31	YSPSSPR	4	YSPTSPS
5	YSPTSPS	32	YTPQSPT	5	YSPTSPS
6	YSPTSPN	33	YTPSSPS	6	YSPTSPS
7	YSPTSPS	34	YSPSSPS	7	YSPTSPS
8	YSPTSPS	35	YSPTSPK	8	YSPTSPS
9	YSPTSPS	36	YTPTSPS	9	YSPTSPS
10	YSPTSPS	37	YSPSSPE	10	YSPTSPS
11	YSPTSPS	38	YTPTSPK	11	YSPTSPS
12	YSPTSPS	39	YSPTSPK	12	YSPTSPS
13	YSPTSPS	40	YSPTSPK	13	YSPTSPS
14	YSPTSPS	41	YSPTSPT	14	YSPTSPS
15	YSPTSPS	42	YSPTTPK	15	YSPTSPS
16	YSPTSPS	43	YSPTSPT	16	YSPTSPA
17	YSPTSPS	44	YSPTSPV	17	YSPTSPS
18	YSPTSPS	45	YTPTSPK	18	YSPTSPS
19	YSPTSPS	46	YSPTSPT	19	YSPTSPS
20	YSPTSPS	47	YSPTSPK	20	YSPTSPS
21	YSPTSPS	48	YSPTSPT	21	YSPTSPN
22	YSPTSPN	49	YSPTSPKGST	22	YSPTSPS
23	YSPTSPN	50	YSPTSPG	23	YSPTSPG
24	YTPTSPS	51	YSPTSPT	24	YSPGSPA
25	YSPTSPS	52	YSLTSPAISPDDSDEEN	25	YSPKQDEQKHNENENSR
26	YSPTSPN				
27	YTPTSPN				

Secuencia consenso	$Y_1S_2P_3T_4S_5P_6S_7$
Principales sitios de fosforilación	$Y_1S_2P_3T_4S_5P_6S_7$
Otros sitios de fosforilación	$Y_1S_2P_3T_4S_5P_6S_7$

Figura I6. Rpb1, la subunidad mayor de la RNAPII, y su dominio carboxilo-terminal. (A) Representación lineal de la estructura de Rpb1. Se indican los distintos dominios y los residuos de aminoácidos correspondientes a los límites entre dominios. Imagen extraída de (Cramer *et al.,* 2001). (B) CTD humano y CTD de *S. cerevisiae*. Imagen modificada de (Calvo and García, 2012).

El CTD sufre modificaciones postraduccionales a lo largo de la transcripción, como fosforilaciones, glicosilaciones, metilaciones, ubiquitinaciones e isomerizaciones (Figura I6). Un CTD no fosforilado se asocia a los procesos iniciales de la transcripción, mientras que un CTD fosforilado predomina durante la elongación (Paynte *et al*.,1989).

3.2. El código del CTD

Estudios iniciales sobre la fosforilación de la RNAPII mostraban dos formas de Rpb1 que podían separarse en geles de SDS-PAGE debido a su diferente movilidad. Estas dos formas se definieron como RNAPIIA y RNAPIIO. La RNAPIIA es una forma hipofosforilada de la polimerasa, mientras que la RNAPIIO es la forma hiperfosforilada (Schwartz and Roeder, 1975). Además, ambas formas se describieron como funcionalmente diferentes: la forma IIA (hipofosforilada) se recluta preferentemente al promotor y está presente en el complejo de pre-iniciación, mientras que la forma IIO (hiperfosforilada) es característica de una polimerasa elongante. Una vez se produce la terminación, la RNAPIIO requiere de su defosforilación para estimular de nuevo su reclutamiento a la zona promotora y reiniciar una nueva ronda transcripcional (Cadena and Dahmus,1987; Lu *et al*., 1991; Zhang and Corden, 1991).

Estudios posteriores mostraron que el modelo basado en dos únicas formas de la RNAPII era demasiado simple. Diferentes patrones de fosforilación de la RNAPII son específicos y característicos de los distintos pasos que ocurren a lo largo de un ciclo transcripcional (Komarnitsky *et al*., 2000). De este modo, la coordinación y la correcta progresión de la RNAPII a través del gen y del ciclo transcripcional será dependiente de los cambios en la fosforilación del CTD. Patrones de fosforilación diferentes en el CTD promueven el paso de iniciación a elongación, favoreciendo el intercambio de los GTF durante el escape del promotor (Pokholok *et al*., 2002), por los factores de elongación y el intercambio de éstos por aquellos específicos de la terminación y el procesamiento del 3’ de los transcritos (Kim *et al*., 2004), así como el reciclaje de la polimerasa (Zhang *et al*., 2012). Además, se ha demostrado que los patrones de fosforilación del CTD relacionan el procesamiento del pre-mRNA y otros procesos nucleares con la transcripción (Hirose and Manley, 2000; McCracken *et al*., 1997).

Se sabe que tanto los residuos serina (Ser2, Ser5 y Ser7) (revisado en Zhang and Corden, 1991; Corden, 2007; Paloncade and Bensaude, 2003), como la tirosina (Tyr1) (Baskaron *et al*., 1997; Mayer *et al*., 2012) y la treonina (Thr4) (Hintermair *et al*., 2012; Hsin *et al*., 2011) pueden ser fosforilados. Además, ambos residuos prolina pueden ser isomerizados por una prolyl isomerasa (Wu *et al*., 2000). La glicosilación de las serinas y la treonina también puede ocurrir (Ranunculo *et al.*, 2012; Egloff and Murphy, 2008), e incluso en algunos organismos ciertos residuos del CTD pueden ser metilados,

acetilados o ubiquitinados en sus repeticiones más degeneradas (Dias *et al.,* 2015; Zhao *et al.,* 2016; Li *et al.,* 2007; Voss *et al.,* 2015).

Las numerosas modificaciones que puede sufrir el CTD, especialmente las fosforilaciones, combinadas con el número de repeticiones que presenta, es capaz de generar un número enorme de posibles estados de fosforilación (Figura I7), que hacen que la fosforilación del CTD y su regulación sea extremadamente compleja. El patrón dinámico de modificaciones post-traduccionales que sufre el CTD, referidas sobre todo a las fosforilaciones en Ser2 y Ser5, se denominó por S. Buratowski como "el código del CTD" (Buratowski, 2003). De manera que, cada patrón de fosforilación determina un código reconocible para los factores específicos que participan a distintos niveles en la biogénesis de los transcritos de la RNAPII y en otros procesos nucleares. De este modo, el CTD actúa como una plataforma para reclutar secuencialmente factores que co-regulan y coordinan distintos procesos de la expresión génica (Buratowski, 2003; Corden, 2007; Egloff and Murphy, 2008).

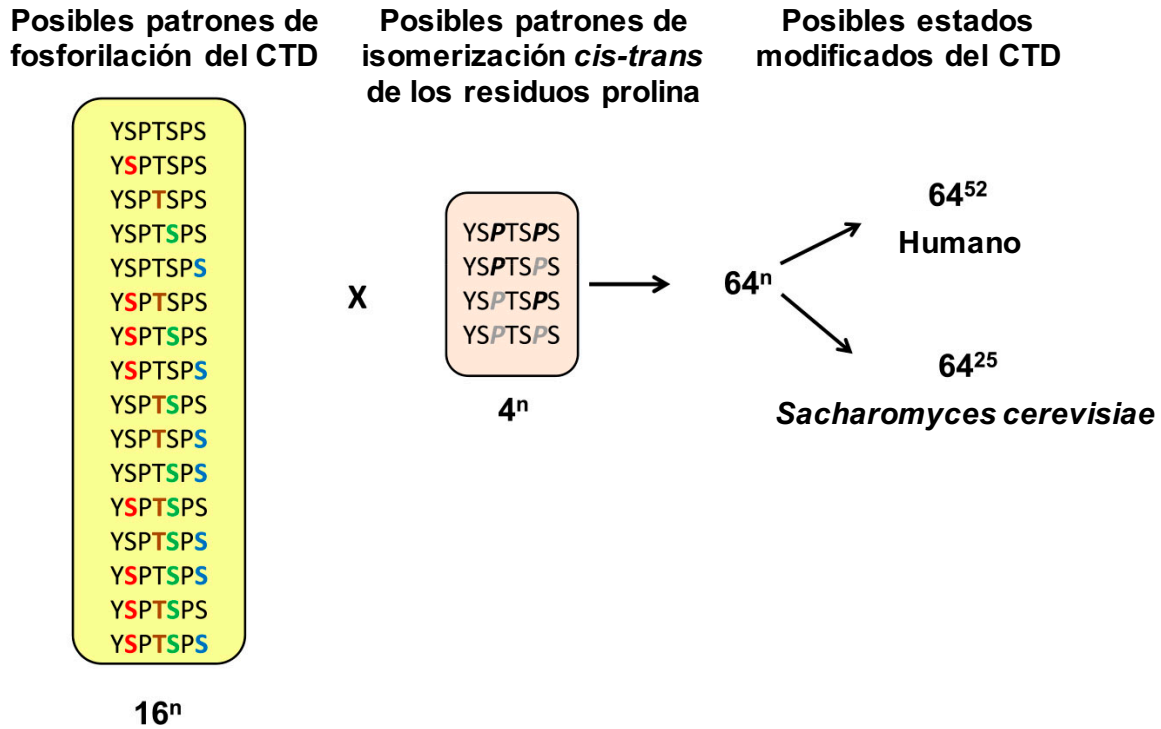

Figura I7. El código del CTD. Posibles estados de fosforilación del CTD humano y de *S.cerevisiae*, resultados de la combinación de la fosforilación e isomerización (*cis-trans*) que pueden sufrir los residuos conservados de la secuencia consenso. Imagen modificada desde (Calvo and García, 2012). n = número de repeticiones de la secuencia consenso.

El código del CTD se genera por la interacción entre quinasas y fosfatasas, que son las que escriben y borran las marcas de fosforilación. Dichas marcas deben ser

reconocidas e interpretadas por factores reguladores, o siguiendo la metáfora, por los lectores o intérpretes del código. La función conjunta y sincronizada de todos estos factores permite la correcta biogénesis de los transcritos de la RNAPII.

3.2.1. *Fosforilación de las serinas: Ser2P, Ser5P y Ser7P*

El abordaje del estudio de la fosforilación del CTD no ha sido fácil debido a las repeticiones del heptapéptido, así como al número de residuos fosforilables de éste. Sin embargo, gracias a la generación de anticuerpos capaces de reconocer las diferentes fosforilaciones del CTD se ha logrado obtener bastante información acerca de las funciones del mismo. Mediante el uso de estos anticuerpos monoclonales en técnicas de inmunoprecipitación de cromatina (ChIP) se observó que diferencias en la fosforilaciones de residuos serina coincidía con el reclutamiento espacio-temporal de diferentes factores transcripcionales (Chapman *et al*., 2007; Egloff and Murphy, 2008; Patturajan *et al*., 1998; Phatnani and Greenleaf, 2006).

Durante un ciclo de transcripción, la RNAPII es reclutada al promotor en su forma hipofosforilada, mientras que los tres residuos serina son fosforilados de manera secuencial y diferencial una vez iniciada la transcripción y hasta que esa finaliza. Se conoce bien que los residuos Ser2 y Ser5, que han sido los más estudiados, son las principales dianas de fosforilación del CTD, aunque la fosforilación en Ser7, la posición más degenerada del heptapeptido, va ganando importancia con estudios más recientes (Heidemann *et al.,* 2012; Hsin and Manley, 2012). De hecho, dos estudios recientes (Schüller *et al.,* 2016; Suh *et al.,* 2016) han demostrado que, tanto en levaduras como en mamíferos, las fosforilaciones de las Ser2 y Ser5 son esencialmente las que generan el código del CTD, y que la fosforilación de la Ser7 tendría funciones más específicas.

El pico de fosforilación de las Ser5 y Ser7 tiene lugar durante el inicio de la transcripción. En las primeras 500 pb, después del sitio de inicio de la transcripción, los niveles de Ser5P ya han descendido prácticamente a la mitad y continuarán descendiendo a lo largo del gen (Bataille *et al.,* 2012; Kim *et al.,* 2010; Mayer *et al.,* 2010), debido a la actividad de dos fosfatasas específicas (Krisnamurthy *et al.,* 2004; Mosley *et al.,* 2009). Por el contrario, los niveles de Ser2P empiezan a incrementarse en el paso iniciación-elongación, presentando su mayor pico en la región 3’ de los

genes (Bataille *et al.,* 2012; Kim *et al.*, 2010; Mayer *et al.,* 2010). Los niveles de Ser7P se mantienen a lo largo del gen, incluso puede presentar una fosforilación de *novo* durante la transcripción (Tietjen *et al.,* 2010). La defosforilación total del CTD ocurre al final de la transcripción, permitiendo el reciclaje de la polimerasa y la reiniciación (Figura I8) (Bataille *et al.,* 2012; Buratowski, 2009; Phatnani and Greenleaf, 2006).

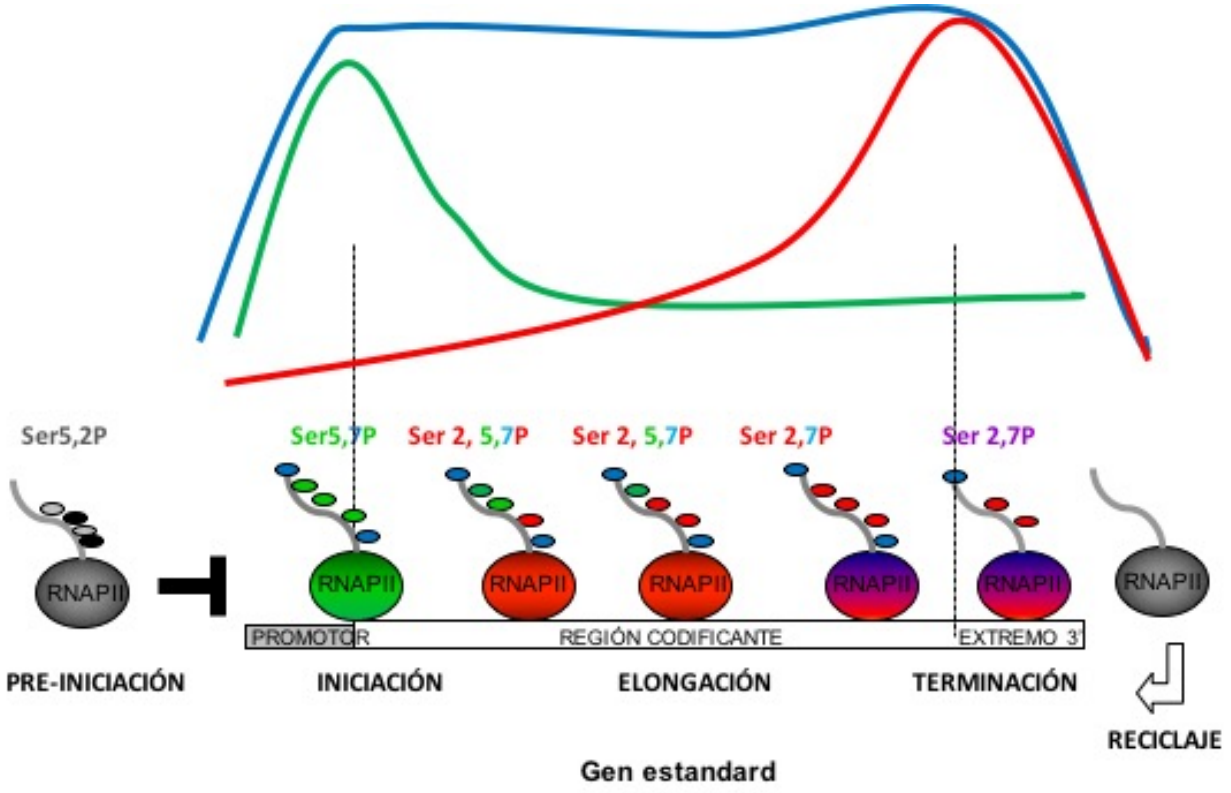

Figura I8. Patrón de fosforilación de las serinas del CTD de la RNAPII en *S.cerevisiae*. Durante la iniciación de la transcripición y el escape del promotor, el CTD de Rpb1 se fosforila en Ser5 (en verde). Al mismo tiempo se fosforila en Ser7 (en azul), estableciéndose una marca doble para los genes transcritos por la RNAPII. Poco después de disociarse del promotor, la marca Ser5P se elimina rápidamente, mientras que van acumulándose los residuos Ser2P (en rojo). En este momento, la fosforilación en Ser7 se mantiene constante. Por último, todas las marcas del CTD se eliminan al final de la transcripción y la RNAPII hipofosforilada (en gris) está lista para ensamblarse de nuevo en el PIC y reiniciar una nueva ronda de transcripción. Los círculos pequeños coloreados representan la fosforilación del CTD en la secuencia consenso. Los círculos grandes indican las distintas formas fosforiladas de la RNAPII a lo largo de la iniciación, elongación y terminación. Imagen modificada a partir de (Calvo and García, 2012).

La fosforilación de las Ser5 por la Kin28 es imprescindible para el reclutamiento de la maquinaria encargada de la adición de la caperuza o *capping* en el extremo 5' (Fabrega *et al*., 2003; Ghosh *et al*., 2011; Komarnitsky *et al*., 2000; McCracken *et al.,* 1997; Shroeder *et al.,* 2000). Por otro lado, la fosforilación de las Ser2 por Ctk1 es la encargada de la coordinación entre la transcripción y el *splicing* (Hargreaves *et al*., 2009; Ahn *et al.,* 2004) y es esencial para la terminación de la transcripción (Lunde *et al.,* 2010). Por su parte, el procesamiento en 3' de los snRNAs se asocia a la

fosforilación de Ser7 tanto en levaduras (Akhtar *et al*, 2009), como en eucariotas superiores (Baillat *et al*., 2005; Egloff and Murphy, 2008; Egloff *et al*., 2007, 2010).

Aunque estudios recientes, como ya hemos comentado, muestran que los residuos más importantes para la generación del código del CTD son las Ser5 y Ser2 (Suh *et al*., 2016; Schüller *et al.,* 2016), la fosforilación de las Ser7 está presente en todos los genes codificantes de proteínas y también en los snRNAs (Akhtar *et al*., 2009; Chapman *et al*., 2007; Egloff *et al*., 2007). Se ha propuesto que la fosforilación de este residuo facilita la elongación, la terminación y suprime la transcripción críptica (Tietjen *et al.,* 2010; Glover-Cutter *et al.,* 2009).

3.2.2. *Tyr1 y Thr4: otros residuos fosforilables*

Estudios recientes han mostrado que los residuos Tyr1 y Thr4 también se fosforilan a lo largo de la transcripción y contribuyen al código del CTD anteriormente propuesto (Heidemann and Eick, 2012).

En levaduras, Thr4P se asocia con las regiones transcritas del gen descendiendo sus niveles de fosforilación en la zona de poliadenilación (Hintermair *et al.,* 2012; Hsin *et al.,* 2011; Mayer *et al.,* 2012), mientras que en células humanas se localiza en las regiones 3' (Mayer *el al.,* 2012). Varios estudios muestran que los residuos de Thr4 no son esenciales para la viabilidad de *Saccharomyces cerevisiae* (Stiller *et al*., 2000) y *Schizosaccharomyces pombe* (Schwer and Shuman, 2011), pero su remplazamiento por valina en pollos (Hsin *et al*., 2011) o por alanina en humanos es letal (Hintermain *et al*., 2012). Estudios recientes sugieren que el residuo Thr4 en levaduras, funciona específicamente en la terminación de la transcripción y en el *splicing* post-transcripcional (Harlen *et al.,* 2016).

Mientras que el remplazamiento del residuo Thr4 es viable en levaduras, no ocurre lo mismo con la Tyr1, siendo letal su eliminación (Schwer and Shuman, 2011), lo que indican una función esencial de este residuo del CTD. Los niveles de Tyr1P se correlacionan con los niveles de Ser2P, son bajos en la zona del promotor incrementándose una vez pasado el TSS. Sin embargo, los niveles de Tyr1P decrecen rápidamente antes del sitio de poli(A), sugiriendo un rol en el reclutamiento de los factores de procesamiento del 3' y de terminación (Schreieck *et al*., 2014; Mayer *et al*., 2012). De hecho, Tyr1P inhibe la unión de los factores de terminación en las zonas

centrales del gen durante la transcripción *in vitro* (Mayer *et al.*, 2012). Recientemente, se ha sugerido que la fosforilación de la Tyr1 ha adquirido funciones esenciales y muy especializadas en eucariotas superiores, siendo importante para la transcripción de promotores antisentido y potenciadores de la transcripción, así como para la estabilidad de la RNAPII (Descostes *et al.,* 2014).

3.3. Enzimas moduladoras de la fosforilación del CTD: CTD-quinasas y CTD-fosfatasas

Las principales enzimas encargadas de modificar el CTD a lo largo del ciclo transcripcional son las CTD-quinasas y las CTD-fosfatasas (Figura I9). Estas enzimas se encuentran altamente conservadas, y aunque se conoce el mecanismo catalítico de las mismas, se desconocen las bases de su especificidad.

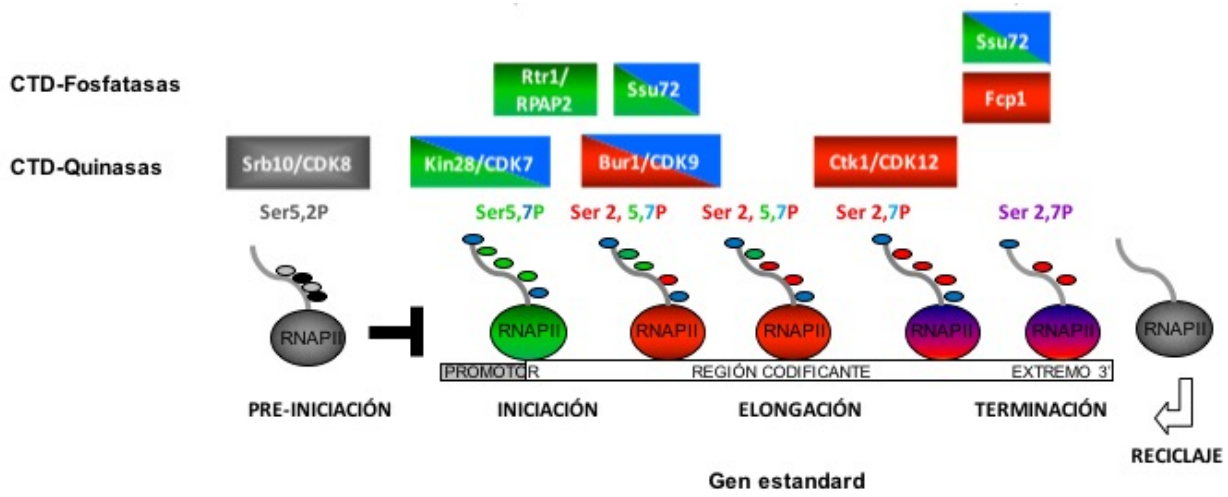

Figura I9. CTD-quinasas y CTD-fosfatasas. Representación de los niveles de fosforilación de las distintos residuos serina a lo largo de un ciclo transcripcional. Se indican en rectángulos las distintas CTD-quinasas y CTD-fosfatasas de *S.cerevisiae* y sus homólogos en humanos. Los distintos colores representados en los rectángulos indican los residuos fosforilados o desfosforilados por parte de las quinasas o fosfatasas respectivamente.

3.3.1. *Escribiendo el código del CTD: CTD-quinasas*

El CTD se fosforila por miembros de la familia de quinasas dependientes de ciclinas (CDK), las cuales consisten en una subunidad catalítica quinasa y una subunidad con función ciclina reguladora. En mamíferos, las mejor conocidas son Cdk7/CycH, Cdk8/CycC, Cdk9/CycT y Cdk12/CycK, las cuales se encuentran evolutivamente conservadas en *Saccharomyces cerevisiae*: Kin28/Ccl1, Srb10/Srb11, Bur1/Bur2, y Ctk1/Ctk2 , respectivamente.

Existen dos CTD-quinasas importantes con funciones durante la pre-iniciación e

iniciación de la transcripción: Cdk8/CycC y Srb10/Srb11 y Cdk7/CycH y Kin28/Ccl1-Tfb3.

Los complejos **Cdk8/CycC** y **Srb10/Srb11**, forman parte del módulo CDK del Mediador (Liao *et al.,* 1995), en mamíferos y levaduras, respectivamente. Ambos complejos pueden fosforilar las Ser2 y Ser5 *in vitro* (Akoulitchev *et al.,* 2000; Larschan and Winston, 2005; Liu *et al*., 2004; Ramanathan *et al*., 2001), aunque su relevancia *in vivo* sigue sin definirse. Varios estudios indican que Srb10/Srb11 podría tener efectos tanto negativos como positivos en la expresión génica *in vivo* (Carlson, 1997; Holstege *et al*., 1998). Srb10 fosforila el CTD de la RNAPII antes de la formación del PIC, regulando negativamente la iniciación de la transcripción (Hengartner *et al*., 1998). Sin embargo, otros trabajos muestran que Srb10 funciona conjuntamente con la Kin28 promoviendo la reiniciación de la RNAPII (Liu *et al*., 2004; Yudkovsky *et al*., 2000). Además, Srb10 fosforila dos subunidades del TFIID en el PIC y algunos factores de transcripción, incrementando su actividad (Galbraith *et al*., 2010; Hirst *et al*., 1999; Vincent *et al*., 2001), o promoviendo su transporte fuera del núcleo y su degradación (Chi *et al*., 2001; Nelson *et al*., 2003). También se ha indicado que Cdk8 promueve la elongación (Donner *et al.,* 2010).

Los complejo **CdK7/CycH** en mamíferos y **Kin28/Ccl1-Tfb3** en levaduras forman parte del factor general de la transcripción TFIIH y son esenciales para la viabilidad celular (Simon *et al*., 1986). Tanto *in vitro* como *in vivo* se ha demostrado que fosforilan Ser5 y Ser7 (Akhtar *et al*., 2009; Cho *et al*., 2001; Glover-Cutter *et al*., 2009; Hengartner *et al*., 1998; Kim *et al*., 2009). Son las primeras quinasas en fosforilar el CTD en el complejo de iniciación, rompiendo la interacción estable entre el CTD y los componentes del PIC y promoviendo el escape del promotor (Akoulitchev *et al*., 2000; Hengartner *et al*., 1998; Jiang *et al*., 1996). Además, la marca de fosforilación en Ser5 producida por Cdk7/Kin28 es esencial para el reclutamiento de la maquinaria del *capping* (Cho *et al*., 1997; Ho and Shuman, 1999; McCracken *et al*., 1997; Rodriguez *et al*., 2000). Además, se necesita para la asociación del complejo de unión al cap (CBC) al transcrito naciente (Wong *et al*., 2007), para el reclutamiento co-transcripcional del factor de elongación Paf1C (Qiu *et al*., 2009), del complejo metil-transferasa de histonas SET1/COMPASS (Ng *et al*., 2003) y del complejo SAGA (Govind *et al*., 2007). Asimismo, la fosforilación del CTD en el residuo Ser5 por Kin28 estimula el

reclutamiento de Bur1/Bur2, promoviendo la fosforilación de Ser2 en zonas cercanas al promotor (Qiu *et al*., 2009).

Avanzando en la transcripción, encontramos tres complejos CTD-quinasas importantes en elongación: Cdk9/CycT y Cdk12/CycK, Bur1/Bur2 y Ctk1/Ctk2-Ctk3

Cdk9 es la subunidad catalítica del factor de elongación P-TEFb (*Positive transcription elongation factor b*), el cual controla la fase de elongación de la transcripción en mamíferos y en *Drosophila* (Saunders *et al*., 2006). Cdk9 es la quinasa principal de Ser2, pero a su vez también contribuye a la fosforilación de Ser5 en la transición iniciación-elongación y a la salida de la pausa de la RNAPII (Ramanathan *et al*., 2001; Wada *et al*., 1998). La actividad de Cdk9 es necesaria para un correcto acoplamiento entre la transcripción y el procesamiento 3' del pre-mRNA (Ni *et al*., 2004). Además, recientemente se ha descrito que Cdk9 es también la quinasa encargada de fosforilar Thr4 (Hsin *et al*., 2011).

Hasta hace relativamente poco tiempo, se pensaba que Cdk9 era la CTD-quinasa encargada de fosforilar las Ser2 en eucariotas superiores, mientras que en levaduras esta función recaía en dos quinasas diferentes: Bur1 y Ctk1. Sin embargo, recientemente se ha demostrado que *Drosophila* presenta un ortólogo para Ctk1, Ctk12, mientras que en humanos existen dos, Cdk12 y Cdk13. Se cree que Cdk12 podría ser la quinasa principal de Ser2 en elongación (Bartkowiak and Greenleaf, 2011; Bartkowiak *et al*., 2010) y más recientemente se ha descrito que dicha quinasa presenta mayor actividad sobre un CTD previamente fosforilado en Ser7 (Bösken *et al.,* 2014). En el caso de Cdk13, estudios recientes la han implicado en el procesamiento del RNA, influyendo en el *splicing* (Liang *et al.,* 2015; Katrin *et al.,* 2016).

Bur1/Bur2 forman un complejo CDK esencial en *S. cerevisiae* que participa en la elongación (Keogh *et al*., 2003; Yao and Prelich, 2002). Bur1 puede fosforilar Ser2, Ser5 y Ser7 (Keogh *et al*., 2003; Lindstrom and Hartzog, 2001; Murray *et al*., 2001; Qiu *et al*., 2009; Tietjen *et al*., 2010). Aunque Bur1 fosforila preferentemente las Ser5 *in vitro* (Keogh *et al*., 2003), estudios posteriores muestran que *in vivo*, Bur1 interacciona con el CTD de la RNAPII y fosforila las Ser2 (Qiu *et al*., 2009; Jones *et al*., 2014). Su actividad es mayor en la elongación temprana de la transcripción. De hecho, la fosforilación de las Ser5 por Kin28 estimula la actividad de Bur1, que a su vez promueve la función de Ctk1 (Qiu *et al*., 2009). Bur1 también fosforila a las Ser7 del CTD en las regiones

codificantes del gen (Tietjen *et al*., 2010). Además la actividad quinasa de Bur1 regula la elongación al fosforilar al factor de elongación Spt5, que posee un extremo carboxilo terminal semejante al CTD (Liu *et al.*, 2009). Por último, Bur1 también regula por fosforilación la actividad de enzimas modificadoras de histonas, como Rad6 (Liu *et al.,* 2009; Zhou *et al.,* 2009).

Ctk1 es la principal CTD-quinasa responsable de la fosforilacion de las Ser2 durante la elongación (Cho *et al*., 2001; Jones *et al*., 2004). Aunque Ctk1 no participa directamente en la elongación (Ahn *et al*., 2004; Mason and Struhl, 2005; Skaar and Greenleaf, 2002), la actividad de esta es necesaria para la asociación de factores de terminación y poliadenilación (Ahn *et al*., 2004), así como de factores modificadores de histonas (Xiao *et al*., 2007). Asimismo, Ctk1 interacciona genética y bioquímicamente con el complejo TREX, el cual acopla la elongación de la transcripción con el exporte del mRNA (Hurt *et al*., 2004; Jimeno *et al*., 2002). Parece ser que también participa en la transcripción de genes de respuesta al daño al DNA (Ostapenko and Solomon, 2003). Por otra parte, Ctk1 promueve la disociación de la polimerasa elongante de factores basales de la transcripción, independientemente de su actividad quinasa (Ahn *et al*., 2009). Por último, se ha implicado a Ctk1 en la transcripción por la RNAPI (Bouchoux *et a*l., 2004; Grenetier *et al*., 2006), y en la fidelidad de la elongación de la traducción en el citoplasma (Hampsey and Kinzy, 2007; Rother and Strasser, 2007).

3.3.2 *Borrando el código de CTD: CTD-fosfatasas*

La defosforilación controlada y dinámica del CTD a lo largo del ciclo transcripcional es imprescindible para la formación de los patrones de fosforilación que regulan y controlan la asociación espacio-temporal de distintos factores de transcripción. Adicionalmente, la completa defosforilación del CTD es necesaria para el reciclaje de la RNAPII.

La defosforilación es llevada a cabo por una serie de fosfatasas del CTD. En levadura se han descrito las CTD-fosfatasas: Ssu72, Fcp1, Rtr1 y Glc7; y en mamíferos encontramos Fcp1, dos fosfatasas pequeñas llamadas SCP1 Y RPAP2 (homóloga a Rtr1). A excepción de Rtr1, las CTD-fosfatasas viajan con el complejo de transcripción durante todo el ciclo. Al igual que las CTD- quinasas, las fosfatasas presentan diferencias en su actividad en las distintas etapas de la transcripción. Por último, se ha

demostrado que Cdc14, una fosfatasa de ciclo celular, es capaz de defosforilar el CTD de la RNAPII específicamente durante la mitosis (revisado por Jeronimo *et al.,* 2013).

Rtr1/RPAP2

A medida que progresa la transcripción, mientras que los niveles de Ser5P van disminuyendo una vez se ha iniciado el ciclo de transcripción, los niveles de Ser2P se van incrementando. Esto significa que los residuos Ser5P deben ser defosforilados activamente. En levaduras, Rtr1 ha sido descrita como la CTD-fosfatasa encargada de la transición Ser5P-Ser2P en la zona 5' de los genes transcritos. De este modo, la deleción de Rtr1 produce una acumulación de Ser5P en extractos celulares, incrementando los niveles de Ser5P a lo largo de las regiones codificantes del gen (Gibney *et al*., 2008; Mosley *et al*., 2009). Sin embargo, como veremos más adelante, también esta función ha sido descrita para la fosfatasa Ssu72. Por otra parte, en levaduras, Rtr1 ha sido propuesta como la fosfatasa encargada de defosforilar el residuo Tyr1P (Hsu *et al*., 2014).

La depleción de la CTD-fosfatasa humana RPAP2 provoca también un incremento en los niveles de Ser5P en el cuerpo de los genes, y afecta a la expresión de los snRNA. El modelo que se propone indica que la presencia de fosforilación en Ser7 reclutaría a RPAP2, produciendo una cascada de eventos críticos para la expresión correcta de estos genes (Egloff *et al*., 2007, 2012; Ni *et al*., 2011).

Inicialmente, se afirmó que Rtr1 no presentaba un sitio activo para la actividad fosfatasa en *Kluiveromyces lactis,* ni se pudo demostrar su actividad fosfatasa, sugiriendo para esta enzima una función más bien reguladora en la defosforilación del CTD (Xiang *et al.,* 2012). Estudios recientes muestran la estructura cristalográfica de la proteína Rtr1 de *Saccharomyces cerevisiae* en la que se ha identificado el sitio activo, perdido en la estructura de *K. lactis* (Irani *et al.,* 2016). Al parecer, el sitio activo en *K. lactis* requiere de un dominio desestructurado en la proteína Rtr1 *in vitro*, explicando porque no fue detectado en trabajos previos (Xiang *et al.,* 2012).

Ssu72

Ssu72 es la CTD-fosfatasa encargada de defosforilar Ser5P y Ser7P (Bataille *et al*., 2012; Krishnamurthy *et al*., 2004; Zhang *et al*., 2012). Inicialmente, Ssu72 se

identificó como un supresor de una mutación en el factor de transcripción TFIIB, *sua7-1*, la cual causaba un cambio en la selección del sitio de inicio por parte de la RNAPII (Sun and Hampsey, 1996; Wu *et al*., 1999). Ssu72 se encuentra presente en el complejo de la RNAPII a lo largo del ciclo de transcripción (Bataille *et al.,* 2012). De hecho, Ssu72 presenta un papel en elongación, así como en el procesamiento en 3' de los pre-mRNAs, ya que forma parte del complejo de corte y poliadenilacion (CPF) (Dichtl *et al*., 2002; He *et al*., 2003; Krishnamurthy *et al*., 2004; Steinmetz and Brow, 2003; Xiang *et al*., 2010).

Mediante análisis a escala genómica se ha observado que la distribución de Ssu72 a lo largo del gen presenta dos picos muy claros: uno en la región 5' de los genes, y otro pico más acusado en la zona de corte y poliadenilación en 3' o inmediatamente después de ésta (Zhang *et al*., 2012). De acuerdo con estos resultados, se ha descrito a Ssu72 como la fosfatasa encargada de la transición Ser5P-Ser2P en el paso de iniciación a elongación (Rosado-Lugo and Hampsey, 2014), lo que podría indicar que tanto Ssu72 como Rtr1 son necesarias para la defosforilación de las Ser5P en la región 5', actuando de un modo no redundante (revisado en Jeronimo *et al.,* 2016). Además, y en sintonía con su localización en la región 3' del gen, Ssu72 se encuentra involucrado en el proceso de reciclado de la RNAPII mediante la formación de bucles génicos (*gene looping*), favoreciendo la completa defosforilación de la RNAPII y facilitando su reciclaje (Ansari and Hampsey, 2005; O'Sullivan *et al*., 2004; Singh and Hampsey, 2007). De hecho, ciertas mutaciones (*ssu72-C15S*) que eliminan la actividad catalítica de Ssu72, provocan una acumulación de fosforilación en Ser7P que impide la reiniciación de la transcripción conduciendo a la muerte celular (Zhang *et al*., 2012).

Se ha identificado un homólogo de Ssu72 en mamíferos. Al igual que en levaduras, Ssu72 de mamíferos se asocia con el factor TFIIB y con la maquinaria de corte y poliadenilación (St-Pierre *et al.,* 2005). Tiene actividad CTD-fosfatasa y presenta una función en el acoplamiento de la transcripción con el procesamiento del extremo 3' de lo pre-mRNAs (St-Pierre *et al*., 2005; Xiang *et al*., 2010).

Fcp1/SCP1

Fcp1 fue la primera CTD-fosfatasa descrita, creyendo que era la encargada de reciclar la forma hiperfosforilada, RNAPIIO, a la hipofosforilada, RNAPIIA (Chambers

and Dahmus, 1994). Se trata de una proteína altamente conservada en eucariotas, y en levaduras es esencial para la viabilidad celular (Archambault *et al*., 1997; Chambers and Dahmus, 1994; Chambers and Kane, 1996; Kimura and Ishihama, 2004, Kobor *et al*., 2009). Fcp1 defosforila directamente la RNAPII, y estudios *in vitro* muestran que su actividad se ve estimulada por TFIIF e inhibida por TFIIB (Archambault *et al*., 1997; Cho *et al*., 2001; Kobor *et al*., 1999). Fcp1 es la CTD-fosfatasa encargada de defosforilar los residuos Ser2P, por lo que se la conoce como la fosfatasa de elongación (Cho *et al*., 2001; Hausmann and Shuman, 2002). En eucariotas superiores, además, están presentes unas CTD-fosfatasa pequeñas (SCPs) que comparten homología con el dominio fosfatasa de Fcp1, pero que presentan especificidad por residuos Ser5P (Yeo *et al*., 2003). Asimismo, ha sido descrito que Fcp1 interacciona directamente con la subunidad Rpb4 de la RNAPII en varios organismos (Kimura *et al.,* 2002; Tombácz *et al.,* 2009). En el caso de *S. cerevisiae*, se ha sugerido que Fcp1 podría estar interaccionando con la RNAPII a través del heterodímero Rpb4/7 (Kamenski *et al*., 2004).

En células donde la actividad de Fcp1 se ve comprometida, la transcripción se encuentra disminuida y se produce una acumulación de RNAPII hiperfosforilada, especialmente en la región codificante de los genes (Kobor *et al*., 1999). Fcp1, además, estimula la elongación *in vitro* de un modo independiente a su actividad fosfatasa, lo que sugiere que se asocia a la polimerasa elongante (Mandal *et al*., 2002). Estudios a escala genómica indican que Fcp1 se asocia a los genes desde el promotor a la región 3', siendo su asociación mayor en la zona de corte y poliadenilación (Cho *et al*., 2001). Esta asociación ocurre después de la disociación de las quinasas Bur1 y Ctk1, y está facilitada por la actividad de Ssu72, lo que permite la completa defosforilación de los residuos Ser2P por parte de Fcp1 (Zhang *et al*., 2012). De hecho, se ha propuesto la existencia de una cooperación entre Fcp1 y Ssu72 en la región 3' de los genes. La defosforilación de Ser5P y Ser7P dependiente de Ssu72 estaría acoplada a la de Ser2P por Fcp1 (Bataille *et al*., 2012). Así, Fcp1 contribuye al reciclaje de la RNAPII (Zhang *et al*., 2012) y es responsable de la defosforilación de la polimerasa una vez se ha disociado del DNA (Kong *et al*., 2005).

Por último, estudios recientes sugieren que Fcp1 es la fosfatasa responsable de la defosforilación de Thr4 en mamíferos (Hsin *et al*., 2014).

Glc7

Al igual que Ssu72, Glc7 es una fosfatasa que forma parte del complejo de corte y poliadenilación (CPF) y es esencial para la viabilidad celular (Krishnamurthy *et al*., 2004; Meinhart *et al*., 2003; Nedea *et al*., 2003). Glc7 es la fosfatasa responsable de la actividad fosfatasa sobre Ser2P y Tyr1P del CPF *in vitro* (Schreieck *et al*., 2014). La depleción de Glc7 *in vivo* produce una acumulación de Tyr1P y un incremento en la ocupación de la RNAPII aguas abajo del gen, indicando problemas en terminación (Schreieck *et al*., 2014). De hecho, recientemente, se ha descrito que el residuo Tyr1 se encuentra mayoritariamente fosforilado al final del ciclo de transcripción y se cree que tiene una función muy importante en la terminación (Mayer *et al*., 2012). Además, Glc7 es esencial para la terminación de los snoRNA (Nedea *et al*., 2008) y para el transporte de los mRNA (Gilbert and Guthrie, 2004).

Cdc14

Cdc14 es una fosfatasa esencial del ciclo celular que regula eventos clave en la mitosis tardía. Está presente en levaduras y tiene ortólogos conservados en eucariotas superiores (Ste Lu *et al.,* 2007; Lu and Zhou, 2007). *In vitro*, es capaz de defosforilar tanto residuos Ser2P como Ser5P (Guillamot *et al*., 2011; Clemente-Blanco *et al*., 2011). Se cree que Cdc14 contribuye a la represión de la transcripción mediante la defosforilación del CTD en regiones repetitivas del genoma de levadura pudiendo contribuir a la segregación telomérica (Clemente-Blanco *et al*., 2011).

3.4. Otros factores que modulan la fosforilación del CTD

Más allá de las quinasas y fosfatasas del CTD, existen otras proteínas capaces de modular el estado de fosforilación del CTD. Esto incluye factores que regulan la conformación del CTD o el acceso y/o afinidad de las enzimas modificadoras del CTD. Entre estos están las isopropil-isomerasas y el coactivador transcripcional Sub1.

3.4.1. *Isomerización de las prolinas: hPin1/Ess1*

El CTD contiene en su heptapéptido consenso dos residuos prolina (P_3 y P_6), los cuales pueden adquirir dos posibles conformaciones espaciales, *cis* o *trans* (Wu *et al.,* 2000), lo que indudablemente influye sobre la estados de fosforilación del CTD.

hPin1/Ess1 son prolil-isomerasas, enzimas encargadas de la rotación del enlace peptídico que precede a un residuo prolina (Lu *et al*., 2007; Lu and Zhou, 2007). En este caso, se trata de prolil-isomerasas específicas de substratos fosforilados (Liou *et al*., 2011). En relación al CTD, hPin1/Ess1 actúan sobre los residuos Pro3 y Pro6 adyacentes a los residuos fosforilables Ser2 y Ser5 (Hanes, 2014; Lu *et al*., 2007). La mutación o depleción de hPin1/Ess1 conlleva un defecto global de la transcripción y una acumulación de Ser5P, sugiriendo que la isomerización de las prolinas presenta un función en la señalización de la transcripción (Mayfield *et al*., 2015; Singh *et al*., 2009). Se ha propuesto que Pin1 en mamíferos estimula la hiperfosforilación del CTD, lo que conlleva la represión de la transcripción y del *splicing* (Xu *et al*., 2003; Xu and Manley, 2007). Por su parte, Ess1 afecta a múltiples pasos en la transcripción, como serían la iniciación y la terminación de la transcripción (Ma *et al.,* 2012; Krishnamurthy *et al.,* 2009).

Mediante estudios de preferencia de substrato se ha podido determinar cuál es la conformación preferida por algunas CTD-fosfatasas en levaduras. Mientras que Fcp1 reconoce substratos fosforilados del CTD tanto en conformación *cis* como *trans* de las prolinas, Ssu72 es estrictamente específica de un CTD fosforilado con *cis*-prolinas (Mayfield *et al*., 2015). De este modo, parece ser que Ess1 promueve la defosforilación de la RNAPII favoreciendo la conformación *cis* del CTD, y permitiendo así la actividad de Ssu72 (Bataille *et al.,* 2012; Krishnamurthy *et al*., 2009).

3.4.2. *Factor de transcripción Sub1*

Sub1 es una proteína de unión al DNA que actúa como regulador de la transcripción por la RNAPII. Inicialmente, en *S. cerevisiae*, fue caracterizado como un coactivador de la transcripción debido a su homología con el coactivador humano PC4, por su interacción genética y física con el TFIIB, así como por su capacidad de estimular la transcripción (Henry *et al.,* 1996; Knaus *et al.,* 1996). Aunque inicialmente Sub1 fue relacionada exclusivamente con la iniciación de la transcripción, actualmente se sabe que Sub1 participa en todo el proceso transcripcional (revisado en Calvo, 2017; Garavís and Calvo, 2017), incluyendo la iniciación (Sikorski *et al.,* 2011; Calvo and Manley, 2005), elongación (García *et al.,* 2012; García *et al.,* 2010) y terminación (Calvo and Manley, 2005). Además hace años se demostró que Sub1, así como su homólogo

PC4, tienen una función relacionada con la fosforilación de la RNAPII (García *et al.,* 2010). De hecho se relacionó funcionalmente a Sub1 con la quinasa del CTD Kin28, así como con la fosfatasa Fcp1 (Calvo and Manley, 2005). Posteriormente, se demostró que efectivamente Sub1 es capaz de regular de forma global la fosforilación del CTD de Rpb1 modulando la funcionalidad de todas las CTD quinasas (García *et al.,* 2010), así como el reclutamiento y niveles celulares de la CTD fosfatasa Fcp1 (Calvo and Manley, 2005). Muy posiblemente, Sub1 lleva a cabo esta función a través de su interacción con el heterodímero Rpb4/7, como hemos demostrado recientemente (Garavís *et al.,* 2017). Como ya hemos dicho, Rpb4/7 se encuentra muy cercano al CTD en el contexto de la estructura de la RNAPII y ya se ha mostrado que interacciona con Fcp1 (Kimura *et al.,* 2002; Tombácz *et al.,* 2009; Kamenski *et al.,* 2004), al igual que Sub1 (Garavís *et al.,* 2017). De nuevo, todo esto apoya fuertemente la hipótesis de que Rpb4/7 podría participar en la regulación de la fosforilación de la RNAPII.

Objetivos

1.-Determinar el papel del heterodímero Rpb4/7 en la fosforilación del CTD de la RNAPII.

2.-Determinar el papel de Rpb4 en la formación de los bucles génicos.

Materiales y métodos

La realización de esta tesis doctoral se ha llevado a cabo utilizando como organismo modelo de estudio la levadura *Saccharomyces cerevisiae*. La bacteria *Escherichia coli* se ha utilizado como herramienta de biología molecular en experimentos de clonación y amplificación de plásmidos.

1. Cepas

1.1. Cepas de *Saccharomyces cerevisiae*

Gran parte de las cepas utilizadas, exceptuando algunas cedidas por otros laboratorios, se construyeron en los fondos genéticos W303 (Mat a/α *ade2-1 his 3-11,15 leu2-3,112 trp1-1 ura3-1*) (Thomas and Rothstein, 1989) y BY4741/BY4742 (*Mat a his3Δ1 leu2ΔO met15Δ0 ura3ΔO/ Mat* α *his3Δ1 leu2ΔO lys2Δ0 ura3ΔO*) (Brachmann *et al.,* 1998). Las cepas utilizadas se especifican en la Tabla MyM1 que se presenta al final de la sección.

1.2. Cepas de *Escherichia coli*

Todos los experimentos con la bacteria *E. coli* se realizaron con la cepa DH5α [*F- endA1 gyr96 hsdR17 ΔlacU169 (f80lacZΔM15) recA1 relA1 supE44-thi-1*] (Hanahan, 1983).

2. Medios de Cultivo

2.1. Medios de cultivo para levaduras

-Medio rico YEPD: 1% de extracto de levadura, 2% de bacto-peptona y 2% de glucosa.

-Medio rico YEPD+KAN: 1% de extracto de levadura, 2% de bacto-peptona y 2% de glucosa, suplementado con 200μg/ml de disulfato de G418.

-Medio completo SC: 0,67% de bases nitrogenadas (YNB) sin aminoácidos, 2% de glucosa y 0,2% de *dropout mix* selectivo. El *dropout mix* es un conjunto de suplementos, como aminoácidos, vitaminas y algunas bases nitrogenadas. Su composición variará dependiendo de los suplementos necesarios para la selección

de las auxotrofias correspondientes en cada cepa. Su composición se detalla en la Tabla MyM2. De este modo para la preparación de cada *dropout mix* se añaden todos los componentes excepto el/los suplemento/s de selección para la auxotrofía/s correpondiente/s.

Dropout mix			
Adenina	0,5g	**Leucina**	10,0g
Alanina	2,0g	**Lisina**	2,0g
Arginina	2,0g	**Metionina**	2,0g
Asparagina	2,0g	**Ácido para-aminobenzoico**	2,0g
Ácido aspártico	2,0g	**Fenilalanina**	2,0g
Cisteina	2,0g	**Prolina**	2,0g
Glutamina	2,0g	**Serina**	2,0g
Ácido glutámico	2,0g	**Treonina**	2,0g
Glicina	2,0g	**Triptófano**	2,0g
Histidina	2,0g	**Tirosina**	2,0g
Inositol	2,0g	**Uracilo**	2,0g
Isoleucina	2,0g	**Valina**	2,0g

Tabla MYM2. Aminoácidos utilizados para la preparación del *dropout mix*

-Medio pre-esporulación Pre-SPO: 0,5% de extracto de levadura, 0,6% de bases nitrogenadas (YNB) con aminoácidos, 0,5% de bacto-peptona, 1% de acetato potásico, y 1,02 % de biftalato potásico, pH ajustado a 5,5 con NaOH saturado.

-Medio de esporulación SPO: 1% de acetato potásico, 0,1% de extracto de levadura y 0,05% de glucosa.

Cuando se requirió, los medios se suplementaron con los aminoácidos y/o bases nitrogenadas necesarioas, en función de las auxotrofías de las cepas utilizadas. Las proporciones utilizadas se detallan en la Tabla MyM3.

Aminoácidos y bases nitrogenadas	Concentración Stock	Volumen/litro	Volumen/placa
Histidina-HCL	100mM	3ml	0,10ml
Isoleucina-Valina	50mM	20ml	0,20ml
Leucina	100mM	20ml	0,20ml
Triptófano	40mM	10ml	0,10ml
Uracilo	20mM	10ml	0,25ml

Tabla MYM3. Aminoácidos y bases nitrogenadas más utilizados para la suplementación de medios.

La preparación de los medios se realizó utilizando agua destilada (Mili Rho). En el caso de medios de cultivo sólidos se añadió agar en una concentración final del 2%. Los medios fueron autoclavados y almacenados sin añadir la glucosa para evitar contaminaciones. La fuente de carbono se añade en el momento en el que se va a utilizar el medio.

2.2. Medios de cultivo para bacterias

-Medio rico LB: 0,5% de extracto de levadura, 1% de Triptona y 1% de NaCl.

-Medio pobre 2xYT: 1% de extracto de levadura, 1,6% de Triptona y 0,5% de NaCl

-Medio rico LB+Amp: 0,5% de extracto de levadura, 1% de Triptona y 1% de NaCl, suplementado con ampicilina sódica a una concentración final de 100 μg/ml, añadida tras el autoclavado.

La preparación de los medios se realizó utilizando agua destilada (Mili Rho). En el caso de medios de cultivo sólidos se añadió agar a una concentración final del 2%.

3. Condiciones de crecimiento

Los cultivos de *S. cerevisiae* en medio líquido se incubaron a la temperatura permisiva de 28ºC a una agitación constante de 200 r.p.m. En casos específicos, la incubación se llevo a cabo a 32ºC o 37ºC. Los experimentos se realizaron siempre con cultivos en fase exponencial, en los que el crecimiento se controló midiendo la absorbancia en un espectrofotómetro a 600nm. En medio sólido, las

temperaturas de incubación fueron de 25ºC, 28ºC, 30ºC, 32ºC, 34ºC y 37ºC, según las necesidades del ensayo.

Las bacterias se incubaron a 37ºC, tanto en medio líquido como sólido.

4. Técnicas Genéticas

4.1. Transformación de *Saccharomyces cerevisiae*

Para la transformación de levaduras se siguieron dos protocolos diferentes dependiendo de la eficiencia requerida. Ambos protocolos se basan en la técnica del acetato de litio descrito por (Giets *et al.,* 1995):

-Para las transformaciones más sencillas de levaduras, donde no se requiere un número elevado de transformantes, se siguió el protocolo modificado según (Amberg and Strathern, 2005). Las células competentes se obtuvieron a partir de cultivos creciendo en YEPD hasta una DO600nm de 0,5-1 ($1x10^7$~$2x10^7$ células/ml). Se utilizaron 5 ml de cultivo por transformación y las concentraciones de DNA variaron desde 100 ng hasta 1 μg, según las necesidades de los experimentos. Para la selección de los transformantes, las células se plaquearon en medios SC selectivos. En el caso de la selección para la resistencia a G418 (KAN), las células, tras ser lavadas con H_2O, se incubaron 2 horas en 1ml de YEPD a 28ºC en agitación, previamente a su plaqueo en YEPD con G418 a una concentración final de 200 μg/ml.

-Para la transformaciones donde se requería una mayor eficiencia en el número de transformantes, se utilizó el siguiente protocolo modificado. En este caso, todas las soluciones se prepararon en tampón TE. Las células competentes se obtuvieron a partir de cultivos saturados creciendo en YEPD. Se inoculó 1 ml de cultivo saturado por cada 50 ml de YEPD. Después de 3 horas y media de crecimiento se procedió a la transformación. Se utilizaron 10 ml de cultivo por transformación y las concentraciones de DNA variaron de 500 ng hasta 1μg. En el caso de la selección para la resistencia al G418, se procedió como se ha detallado más arriba.

4.2. Modificación génica en levaduras

Las cepas generadas para la realización de esta tesis se construyeron por métodos genéticos de cruzamientos o por reemplazamiento génico. En el caso de tratarse de un gen no esencial, se utilizó la técnica descrita por (Longtine et al., 1998) basada en el proceso de recombinación homóloga. El reemplazamiento génico permite la eliminación (deleción) de un gen o la adición de un epítopo en un solo paso. Se utilizan varios plásmidos que contienen distintos marcadores de selección (*TRP1*, *HIS5*, kan^r) y/o distintos epítopos (HA, MYC, TAP) (Tabla MyM4, al final de la sección), como molde para una reacción de PCR. En la reacción se utilizaron como cebadores oligonucleótiodos que anillan tanto en el gen diana que se quiere modificar o eliminar, como en el plásmido molde que contiene el marcador de selección. El DNA amplificado se utiliza para transformar las levaduras. Los reemplazamientos se comprobaron por PCR usando cebadores externos a la zona reemplazada y dentro del marcador génico utilizado para tal propósito. En el caso de la adición de epítopos, se utilizó un cebador interno al marcador de selección adyacente al epítopo y otro interno al gen diana.

4.3. Conjugación, esporulación, y análisis de tétradas

Mediante las técnicas de genética convencional descritas en (Amberg and Strathern, 2005) realizamos los experimentos para la obtención de cepas diploides, su posterior esporulación y para la disección de ascas. Las cepas parentales se mezclaron en una placa de medio YEPD sólido y se incubaron a 28ºC entre 5-6 horas. Posteriormente a este periodo de incubación, los zigotos generados se seleccionaron bien por crecimiento en placas de medio SC selectivo para las auxotrofias correspondientes de cada uno de los parentales, o bien por micromanipulación. Para la esporulación, los diploides se crecieron 24h en medio de pre-esporulación líquido (pre-SPO) a 28ºC y posteriormente se incubaron en medio SPO sólido durante 3-4 días. Las ascas con las cuatro ascosporas se diseccionaron usando un micromanipulador (Singer Instruments), para lo cual previamente se digirió la pared del asca con glusulasa (dilución 1:10, PerkinElmer) durante 20 minutos a temperatura ambiente. El análisis de la segregación de los

diferentes caracteres genotípicos se realizó mediante réplica en los medios selectivos correspondientes para los marcadores genéticos de cada cepa parental. Además, siempre que fue posible, también se analizaron las ascosporas bien por PCR o por *Western Blot*.

4.4. Transformación de *Escherichia coli*

Las células competentes de *E. coli* fueron preparadas por el personal técnico del Instituto de Biología Funcional y Genómica, siguiendo el protocolo descrito por (Hanahan *et al.,* 1991), basado en el método químico del calcio/rubidio. Para transformarlas, se mezclan 100 μl de células competentes con 100 pg-10 ng de DNA, y se incuban en hielo durante 20 minutos. A continuación, se someten a un choque térmico de 2 minutos a 37ºC o 1,5 minutos a 42^{o}C, tras lo cual se mantienen durante 2 minutos en hielo. Posteriormente, se añaden 400 ml de LB precalentado y se incuban a 37^{o}C durante 45-60 minutos en agitación suave. Finalmente, las células se recogen por centrifugación y se siembran en LB solido más ampicilina (100 μg/ml).

5. Plásmidos

En la Tabla MyM4 se detallan todos los plásmidos utilizados para la realización de esta Tesis Doctoral.

6. Métodos de manipulación y Análisis de Ácidos Nucleicos

6.1. Aislamiento de DNA y RNA

6.1.1. *Aislamiento de DNA plasmídico de E. coli*

Para la obtención de DNA plasmídico a pequeña escala se utilizó el sistema comercial Nucleospin® Plasmid de Macherey-Nagel, siguiendo las instrucciones del fabricante.

6.1.2. *Aislamiento de RNA total de levadura*

El RNA total de *S. cerevisiae* se obtuvo siguiendo el protocolo descrito por (Schmitt *et al.,* 1990). Las células se crecen hasta alcanzar una absorbancia de 1,0 a DO600nm, tras lo cual se recogen por centrifugación y se lavan con agua

estéril. En este punto, las células pueden ser congeladas a -80ºC para ser utilizadas posteriormente. Todo el proceso de recogida de las células se lleva a cabo en frío. Para la extracción del RNA se resuspenden las células en 300 μl de tampón AE (NaAc 50 mM, EDTA 10 mM, pH 5,2), más 30 μl de SDS 10%. A esta mezcla se añaden posteriormente 1,2 volúmenes de fenol ácido saturado calentado a 65ºC. Durante 5 minutos, la muestra se somete a intervalos de 20 segundos de agitación en el vórtex, seguidos de 20 segundos de incubación a 65ºC. Tras este periodo, se enfría en hielo seco y seguidamente se centrifuga a temperatura ambiente durante 10 minutos a 13.200 r.p.m. Se recupera la fase acuosa y se transfiere a un tubo nuevo y se repite el proceso con el fenol ácido a 65ºC. De nuevo, se recupera la fase acuosa y seguidamente se realiza una nueva extracción con fenol ácido:cloroformo:alcohol isoamílico (25:24:1). Posteriormente, el RNA se precipita con 1/10 del volumen de NaAc 3 M pH 5,2 y 2 volúmenes de etanol 96% (v/v) frío a -20ºC durante toda la noche. A continuación, se centrifugan las muestras durante 20 minutos a 4ºC a 13.200 r.p.m y después se lavan con etanol 70% (v/v). Se vuelve a centrifugar 5 minutos a 13.200 r.p.m, se retira el sobrenadante y el precipitado se seca en el *Speed Vac Concentrator*. Finalmente, el precipitado se resuspende en 50 μl de agua. La concentración de RNA se determina por espectrofotometría en un equipo Nanodrop a una absorbancia de 260 nm y se determina la relación 260/280 para comprobar la pureza de la muestra. La calidad del RNA obtenido se comprobó por electroforesis en geles de agarosa al 1,8% teñidos con bromuro de etidio (EtBr). El agua destilada y todas las soluciones acuosas utilizadas fueron previamente tratadas con DEPC al 0,1% y autoclavadas.

6.2. Electroforesis de ácidos nucleicos

La separación de fragmentos de DNA se realizó mediante electroforesis en geles de agarosa. Dependiendo del tamaño de las moléculas a separar, se usaron geles de concentración de agarosa variable (1,0%-2% p/v) en tampón TAE (Tris-acetato 40 mM, EDTA 1 mM) y conteniendo EtBr (100 μg/ml). A las muestras se les añadió tampón de carga 6X (azul de bromofenol 0,25% (p/v), xilen cianol

0,25% (p/v), glicerol 30% (v/v). Las electroforesis se realizaron a voltaje constante (100-120V) en tampón TAE. Para visualizar el DNA mediante iluminación con luz UV se utilizó el equipo Gel Doc XR (Bio-Rad) equipado con una cámara CCD para la captura de imágenes. Los tamaños de las moléculas se estimaron por comparación con el marcador de peso molecular 1Kb Plus DNA ladder (Invitrogen).

Para la electroforesis de RNA, se prepararon geles de agarosa de una concentración 1,8% en tampón TAE y con EtBr (100 μg/ml), en los que se cargaron 2 μg del RNA extraído. El tampón TAE utilizado fue previamente autoclavado para asegurar la integridad de los RNAs. Las condiciones de electroforesis fueron voltaje constante (90-110V) durante aproximadamente 45 minutos. En este caso, se prestó especial cuidado al lavado de las cubetas de electroforesis, que se trataron previamente con SDS 1% (p/v).

6.3. Reacción en cadena de la polimerasa (PCR) y transcripción reversa (RT)

6.3.1. *PCR*

Para la realización de PCRs para amplificar DNA de forma rutinaria en el laboratorio, se utilizaron dos kits: Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific), para la amplificación con alta fidelidad de módulos de integración utilizados en la construcción de cepas; My Taq™ Red Mix (Bioline) para la amplificación desde DNA genómico purificado, cDNA o incluso directamente desde una pequeña cantidad de biomasa de células (o colonias aisladas) de *S. cerevisiae*, para la comprobación de cepas y otros procedimientos frecuentes. Ambas enzimas se usaron según las recomendaciones del fabricante. En el caso de las PCRs usando como molde directamente la biomasa de la levadura, las colonias se calentaron en un microondas durante 2 minutos a la máxima potencia. Las amplificaciones se realizaron en un termociclador T Personal (Biometra). La longitud de los ciclos de extensión y la temperatura de anillamiento se modificó, dependiendo del tamaño del fragmento a amplificar y de la temperatura de fusión o temperatura de *melting* (Tm) de los oligonucleótidos, respectivamente.

6.3.1.1. PCR cuantitativa (qPCR)

Las qPCRs para el análisis del DNA purificado en los experimentos de inmunoprecipitación de cromatina (ChIP) se realizaron con el equipo CFX96™ Real-time System (Bio-rad).

Para la reacción de PCR se utilizaron los reactivos del paquete comercial SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara) o el paquete SsoFast™ EvaGreen® Supermix (Biorad), siguiendo las instrucciones del fabricante y los requerimientos específicos del termociclador. El fluorocromo intercalante utilizado para la cuantificación del DNA de las muestras varió dependiendo del paquete comercial utilizado, SYBR® Green o EvaGreen®. La reacción tipo constó de 1 ciclo de 30 segundos a 95ºC, 40 ciclos de [5 segundos a 95ºC para la desnaturalización, y 31 segundos a 65ºC para el anillamiento y la extensión], y un ciclo de disociación para comprobar la obtención de un único producto de amplificación en la reacción. La temperatura de anillamiento de 65º se modificó dependiendo de la eficiencia de los *primers* a utilizar. En casos concretos, fue necesario cambiar la reacción tipo a una en tres paso que consta de 1 ciclo de 45 segundos a 95ºC, 40 ciclos de [5 segundos a 95ºC para la desnaturalización, 10 segundos a 56ºC para el anillamiento y la extensión y 31 segundos a 72ºC], y un ciclo de disociación.

Para poder realizar una cuantificación de los resultados es necesario una recta patrón, representando el ciclo umbral de amplificación exponencial (Ct), frente a la cantidad relativa de DNA. La recta se compone de 4 diluciones seriadas de 1/10 a partir de una muestra de DNA. Para las cuantificaciones de los experimentos de ChIP se utilizó el DNA total correspondiente a una muestra INPUT diluida 1/10 como primer punto de la recta. A cada punto de esta recta se le otorga un valor arbitrario a partir de los cuales el equipo estimará la cantidad de DNA presente en las muestras problema. Para el análisis de datos se utilizó el programa Bio-Rad CFX Manager 3.1 (Bio-Rad).

6.3.1.2. Transcripción reversa o retrotranscripción (RT)

Previamente a la síntesis del cDNA mediante RT, 5μg de RNA total se trata con 0,25μl de DNasa I (10 U/μl Roche) en un volumen final de 50 μl a 37ºC durante 30 minutos. La reacción se para añadiendo 2μl de EDTA 0,2M pH 8,0. Una vez tratado con DNasa I, se realizó la síntesis de cDNA con el kit iScript™ Reverse Transcription Supermix (Bio-rad), siguiendo las especificaciones del fabricante a partir de 200ng del RNA tratado (2μl). Tanto para las PCR convencionales como para las qPCRs utilizamos 1μl de una dilución 1/5 del cDNA obtenido.

6.4. Marcaje *in vivo* de RNA naciente (Transcriptional run-on ó TRO)

Los ensayos de *transcriptional run-on* se realizaron según los protocolos descritos previamente (Birse *et al.,* 1998; Hirayoshi and Lis, 1999; Mukundan and Ansari, 2013).

Las células se crecieron en 100ml de medio SC con sulfato amónico como fuente de nitrógeno a 28ºC hasta alcanzar una DO600nm de 0,3. Los cultivos se recogieron por centrifugación, y se crecieron en 100ml de medio SC con serina/treonina como fuente de nitrógeno hasta alcanzar una DO600nm de 0,7. El pellet obtenido a partir de 100 ml de cultivo se lavó con 10ml del tampón TMN frio (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl) y se resuspendió posteriormente en 940μl de agua DEPC fría. Para la permeabilización del sedimento de células se añadió 60 μl de Sarkosyl 10% y se incubó durante 25 minutos en hielo. Una vez permeabilizadas las células se centrifugaron a bajas revoluciones y se utilizaron directamente para realizar el ensayo.

La elongación de los transcritos iniciada *in vivo* se reanudo resuspendiendo las células en 120μl de buffer de reacción 2.5X (50 mM Tris-HCl pH 7.5, 500 mM KCl, 80 mM $MgCl_2$, 5 mM DTT), 45μl de NTPs/RNase inhibitor mix (10 mM de cada CTP, ATP, and GTP y 300 unidades del inhibidor de RNasas (RNase Inhibitor), y 10μl de [α-^{32}P]-UTP (3000 Ci/mmol, 10 μCi/μl). La reacción se incubó a

30ºC durante 2 minutos para permitir la elongación, tras lo cual se paró añadiendo 1ml del buffer TMN. Posteriormente, las células se centrifugaron a baja velocidad para eliminar los nucleótidos no incorporados. El RNA marcado se extrajo utilizando el tampón LETS (Litio, EDTA, Tris-HCl, SDS)-phenol/chloroformo saturado y se precipitó con 10M LiCl.

La hibridación de los RNA marcados a la membrana prehibridada se llevó a cabo como en (Birse *et al.,* 1998). Todas las señales del TRO fueron cuantificadas utilizando el sistema GEL LOGIC 200 (KODAK) y normalizadas respecto al control. En este caso el*18S* rRNA.

7. Oligonucleótidos

En la Tabla MyM5 (al final de la sección) se detallan los oligonucleótidos utilizados para llevar a cabo las PCR cuantitativas de los experimentos de ChIP y RT-PCR.

8. Métodos de Manipulación y Análisis de Proteínas

8.1. Obtención de extractos proteicos y cuantificación de proteínas

En todos los experimentos se trabajó con extractos proteicos en condiciones nativas. Se prepararon, salvo en los casos especificados, en tampón de lisis que contiene HEPES-KOH 20 mM pH7,6, acetato potásico 200 mM, glicerol 10% (v/v) y EDTA 1 mM. La lisis celular se llevó a cabo en tampón de lisis con inhibidores de proteasas (cóctel de inhibidores de proteasas de Sigma (30 μl/ml) o coctel de inhibidores en tableta cOmplete Mini de Roche (media pastilla/10ml), y PMSF (10 mM); e inhibidores de fosfatasas: 10 mM NaF, 20 mM ortovanadato sódico). Para la rotura mecánica se usaron perlas de vidrio de 45 mm y un sistema Fast Prep realizando 5 pulsos de 30 segundos a velocidad 4,5 K, dejando las células 2 minuto en hielo entre pulso y pulso. En el caso de extractos proteicos utilizados para la realización de co-inmunoprecipitaciones y algunas ChIPs, la rotura celular se realizó también por lisis mecánica en nitrógeno líquido usando un sistema Freezer/Mill 6770 (Spex Sample Prep). La rotura se llevó a cabo mediante 4

pulsos de 1 minuto a máxima potencia (15 CPS), entre pulso y pulso se realiza una pausa de 1 minuto de enfriamiento. La cuantificación de la concentración de proteínas en los extractos se estimó a partir de la medida de absorbancia a 280 nm realizada en equipo de espectrofotometría Nanodrop 1000 (Thermo scientific).

8.2. Inmunoprecipitaciones y co-inmunoprecipitaciones de proteínas

Para los ensayos de inmunoprecipitación de proteínas, se incuban inicialmente 25μl de proteína A-sepharosa (Amersham) equilibrada en tampón de lísis con 1-5 μl (aproximadamente 1 ug) del anticuerpo específico durante 1,5-2h en agitación suave a 4ºC. Para garantizar una correcta incubación de la resina con el anticuerpo se añade buffer de lisis hasta un volumen de 500 μl. Posteriormente, se centrifuga durante 1 minuto a 5.000 r.p.m y se descarta el sobrenadante con el exceso de anticuerpo no unido. La resina se lava tres veces con 1 ml de tampón de lisis, incubando durante 5 minutos a 4º en agitación suave. La inmunoprecipitación se realiza con 10mg de extractos proteicos, los cuales se incuban durante toda la noche a 4ºC con la resina. Posteriormente, se llevan a cabo lavados sucesivos de la inmunoprecipitación con tampón de lisis y se procede a su análisis mediante *Western Blot*. En el caso de inmunoprecipitaciones de proteínas etiquetadas con el epítopo TAP se utiliza IgG-sepharosa 6 Fast Flow (GE Healthcare), y se procede como se ha descrito más arriba.

8.3. Fraccionamiento de cromatina y estudio de proteínas asociadas

El fraccionamiento de cromatina y el estudio de las proteínas asociadas a la misma se basa en el protocolo descrito en (Liang and Stillman, 1997) con modificaciones, según se describe en Miron-García *et al*., (2013). Se utilizan células procedentes de cultivos de 50 ml en fase exponencial (D.O600nm ~0,6-0,8), que se lavan con 5 ml de H_2O MiliQ e incuban durante 10 minutos a temperatura ambiente en 3 ml de PIPES-KOH 100 mM pH=9,4, ácida sódica 0,1%. Tras la incubación, se centrifugan las células a 2.000 r.p.m durante 2 minutos a temperatura ambiente, se resuspenden en 2 ml de fosfato potásico 50 mM pH 7,5, con Sorbitol 0,6 M, DTT 10 mM, 4 μl de Zimoliasa 20T (20 mg/ml) (US Biological) y

se incuban a 37^{o}C hasta la formación de esferoplastos. Para comprobar la formación de esferoplastos se mide la absorbancia (dilución 1:200) cada 10 minutos y, cuando esta se reduce a 1/10 de la inicial se detiene la reacción. Los esferoplastos se verifican mediante la visualización en microscopio de contraste de fases. Una vez obtenidos los esferoplastos, 40 μl de esta mezcla se recogen y se utilizan como *INPUT*. El resto de los esferoplastos se recogen por centrifugación suave a 800 r.p.m durante 5 minutos a 4^{o}C, se lavan con 800 µl de tampón HEPES-KOH 50 mM pH=7.,5 conteniendo KCl 100 mM, MgCl2 2.5 mM y sorbitol 0.4 M, se centrifugan a 800 r.p.m durante 5 minutos a 4ºC . Después se resuspenden en 80 µl de tampón EBX (HEPES-KOH 50 mM pH=7,5, con KCl 100 mM, MgCl2 2,5 mM, Triton X-100 0,25%, PMSF 0,5 mM, DTT 0,5 mM, conteniendo cóctel inhibidor de proteasas 1x (cOmplete, Roche) y se incuban en hielo durante 5 minutos, agitando ocasionalmente para conseguir la rotura celular. El extracto, una vez resuspendido, se transfiere a un Eppendorf con 400 μl de tampón EBX-S (compuesto por tampón EBX con sacarosa 30%), en un tubo eppendorf, y se centrifuga a 12.000 r.p.m durante 10 minutos a 4^{o}C. Tras el gradiente de sacarosa, el pellet resultante se resuspende en 80 μl de tampón EBX y un nuevo gradiente de sacarosa se realiza de la misma forma. El pellet se lava dos veces con 400 μl en tampón EBX y se resuspende en tampón Laemli 1,5x (1x: Tris-HCl 10 mM pH=6,8, SDS 2%, azul de bromofenol 0.02%, glicerol 10%, β-mercaptoetanol .0.77M))

Finalmente, se cargan 15μl y se añade tampón Laemli hasta un volumen final de 20 μl. Para las muestras enriquecidas en cromatina, se cargan 5 μl de muestra y se añade tampón Laemli 1,5x hasta un volumen final de 20 μl.

8.4. Análisis por *Western Blot*

El análisis de proteínas por *Western Blot* se ha realizado siempre en condiciones desnaturalizantes, utilizando geles de poliacrilamida con SDS (SDS-PAGE) (Laemmli, 1970). Las muestras se preparan en tampón de lisis y se desnaturalizan en presencia del tampón de carga Laemmli 2x e incubándolas

durante 20 minutos a 65ºC o hirviéndolas 5 minutos a 95ºC, tras lo cual se cargan el gel (SDS-PAGE), cuya concentración varía dependiendo del tamaño de la/s proteína/s objeto/s de estudio. Después, las muestras se someten a electroforesis a voltaje constante (100-120V), usando un tampón de carrera que contiene Tris-base 25 mM, glicina 250 mM y SDS 0,1% (p/v). Tras la electroforesis, las proteínas se transfirieren a un soporte sólido, en nuestro caso, membranas de PVDF (Millipore) por electrotransferencia húmeda a 200mA durante 3 horas en frío. El tampón de transferencia contiene Tris-base 50 mM, glicina 380 mM y metanol 10% (v/v). Finalizada la transferencia, las membranas se lavan en PBS y se bloquean incubándolas con leche desnatada en polvo al 5% (p/v) en tampón PBS (NaCl 137 mM, Na_2HPO_4 10 mM, KH_2PO 10 mM, KCl 2,7 mM, pH 7,4), durante 30 minutos en agitación suave. Posteriormente, se incuban con el anticuerpo primario, que se diluye en una solución con leche en polvo desnatada al 1% (p/v) y Tween-20 al 0,05% (v/v) en PBS. El tiempo de incubación con el anticuerpo primario depende de las características de cada anticuerpo, que puede variar desde 1 hora a temperatura ambiente a toda la noche a 4ºC. Tras la incubación con el anticuerpo primario, las membranas se lavan tres veces con PBS durante 5 minutos y después se incuban con el anticuerpo secundario, que se prepara en la misma solución utilizada para la incubación con el anticuerpo primario. La incubación con el anticuerpo secundario se realiza a temperatura ambiente durante 30 minutos, seguida de tres lavados de 5 minutos con PBS. Finalmente, para se realiza la detección de las proteínas mediante quimioluminiscencia, utilizando el sistema Pierce® ECL Western Blotting Substrate (Thermo scientific) y/o Westar ηC 2.0 (Cyagen) y siguiendo las instrucciones del fabricante. Los anticuerpos se detallan en la Tabla MyM6 (al final de la sección) .

8.5. Inmunoprecipitación de cromatina o ChIP

Los experimentos de inmunoprecipitación de cromatina (ChIP) se realizaron siguiendo el protocolo descrito en (Keogh *et al.*, 2003) y (Calvo and Manley, 2005) con ciertas modificaciones (García *et al.*, 2010, 2012).

8.5.1. *Preparación y fijación del cultivo*

A partir de un preinóculo saturado, se diluye el cultivo en un volumen de 200ml de medio rico YEPD a una D.O600nm de aproximadamente 0,15-0,20. El cultivo se crece a 28ºC hasta alcanzar una D.O600nm de 0,5-0,6. Una vez alcanzada esta densidad óptica se realiza la unión covalente de la proteínas al DNA (fijación o entrecruzamiento). Para ello las células se tratan con formaldehido al 1%, durante 20 minutos a temperatura ambiente en agitación constante y suave. El formaldehido al 37% se diluye previamente al 11% con una solución llamada *diluent* (0,143 M NaCl, 1,43 mM EDTA, 71,43 mM HEPES-KOH y pH 7,5 ajustado con KOH), que posteriormente se añade al cultivo, diluyéndolo 10 veces para que quede aproximadamente al 1%. El entrecruzamiento se detiene añadiendo glicina a una concentración final de 360 mM, durante 5 minutos más. Por último, se centrifuga el cultivo a 5.000 r.p.m durante 5 minutos a 4^{o}C y las células fijadas y precipitadas se lavan tres veces con tampón TBS frío (Tris- HCl 20 mM pH 7,6, y NaCl 150 mM) y una vez con tampón de lisis FA (tampón de lisis: HEPES-KOH 100 mM, NaCl 300 mM, EDTA 2 mM, Tritón x-100 2% (v/v), deoxicolato sódico 0,2% (p/v), SDS 0,1% (p/v). En este punto las células se congelan a -80^{o}C o se procede a la preparación de los extractos y la fragmentación del DNA.

8.5.2. *Preparación del extracto celular y fragmentación del DNA*

Las células se resuspenden en 500 μl de tampón de lisis FA con inhibidores de proteasas (cóctel de inhibidores de Sigma (30μl/ml) y PMSF 10 mM) y fosfatasas (NaF 10 mM, ortovanadato sódico 20 mM). A continuación, se añade un volumen de bolitas de vidrio de 45 mm, similar al volumen de sedimento celular, y se lisan las células en un sistema Fast Prep, realizando 5 pulsos de 30 segundos a velocidad 4,5 K. Entre pulso y pulso las muestras se dejan 2 minuto en hielo. Finalizada la rotura celular, se eliminan las bolitas por centrifugación y se añade 1 ml más de tampón de lisis con inhibidores al lisado celular y se transfieren a tubos de 15 ml para su fragmentación mediante sonicación en un sistema Bioruptor TM (Diagenode). En los casos que fue necesario, debido a la inestabilidad de las proteínas a estudiar, los extractos proteicos se realizaron mediante el sistema

Freezer/Mill 6770 (Spex Sample Prep). La rotura se llevo a cabo mediante 4 pulsos de 1 minuto a máxima potencia (15 CPS), entre pulso y pulso se realiza una pausa de 1 minuto de enfriamiento. En este caso las células se recogen directamente en 1,5 ml de tampón de lisis FA con inhibidores.

El DNA se fragmenta para obtener DNA de un tamaño de 300-500 pb. Para ello, se someten las muestras a 8 ciclos de sonicación (15 choques 30"on/30"off a potencia alta). Entre ciclo y ciclo las muestras se dejan reposar en hielo durante 5 minutos. A continuación, se transfiere la muestra sonicada a tubos de 2ml y se centrifuga a 13.200 r.p.m durante 10 minutos a 4^{o}C, para eliminar los restos celulares. El sobrenadante se transfiere a un tubo nuevo y se le añade N-Lauroilsarcosina sal sódica (Sarcosyl, Sigma-Aldrich) una concentración final de 1% (p/v). Finalmente se estima la concentración de proteínas en el Nanodrop a 280 nm.

8.5.3. *Inmunoprecipitación*

Rutinariamente, se inmunoprecipitan 20 mg de proteínas, usando para ello el anticuerpo específico requerido (aproximadamente 1 μg) en cada experimento concreto, y 25 μl de proteína A-sefarosa (Amersham), equilibradas con tampón de lisis FA y bloqueadas con ssDNA de salmón (2 mg/ml). Previamente se retira 1/10 del volumen a inmunoprecipitar, como muestra control de DNA total (INPUT). Las inmunoprecipitaciones se realizan mediante una incubación inicial de los extractos durante 3-4 horas a 4ºC en agitación suave con el anticuerpo, tras lo cual se le añade la proteína A y el ssDNA y se deja incubando toda la noche a 4ºC. Tras la incubación, se realizan dos lavados sucesivos de la resina con 1.400 μl de tampón de lisis FA, un lavado con 1.400 μl tampón de lisis con NaCl a 500 mM, un lavado con 1.400 μl tampón de LiCl (Tris-HCl 10 mM pH 8,0, LiCl 250 mM, NP-40 0,5% (v/v), deoxicolato sódico 0,5% (p/v), EDTA 1 mM) y un lavado con 1.400 μl de TE. Después de cada lavado, la resina con la cromatina inmunoprecipitada se centrifuga en frío a 5.000 r.p.m durante 1 minuto. Después del último lavado, la resina se resuspende en 100 μl de tampón TE con 20 mg de RNasa A para digerir

el RNA. En este punto, las muestras INPUT también se tratan con RNAsa A, para ello se sube el volumen con TE hasta 100 μl y se añade la endonucleasa. Tanto las muestras INPUT como los inmunoprecipitados(IPs) se incuban durante 30 minutos a 37ºC con la RNAsa A. Posteriormente, las proteínas se eluyen de la resina mediante dos incubaciones sucesivas con 250 μl de tampón de elución ($NaHCO_3$ 0,1 M, SDS 1% (p/v), durante 15 minutos a temperatura ambiente y en agitación. Ambas eluciones se juntan en un mismo tubo. El volumen de las muestras INPUT se ajustó hasta 500 μl añadiendo tampón de elución.

8.5.4. *Procesamiento del INPUT y del PRECIPITADO*

Para revertir la fijación y separar las proteínas del DNA, las muestras eluidas se incuban durante 5-6 horas a 65^{o}C. Posteriormente, se lleva a cabo la digestión de las proteínas de las muestras añadiendo a cada muestra 25 μl de Tris-HCl, 10 μl de EDTA 0,5M pH 8,0 y 40 mg de proteinasa K e incubando a 37^{o}C durante 1 hora. Tras lo cual se extrae el DNA.

Para la extracción del DNA, se fenolizan las muestras con 500 μl de fenol:cloroformo:alcohol isoamílico (25:24:1), utilizando columnas Phase-lock (5-Prime) y centrifugando a temperatura ambiente durante 5 minutos. La fase acuosa que contiene el DNA se recupera a un tubo nuevo y se precipita durante toda la noche a -20^{o}C con 1/10 del volumen de acetato sódico 0,3 M pH 5,2, más 2,5 volúmenes de etanol 96% (v/v) y 1 μl de glicógeno (20 mg/μl, Roche). Posteriormente, las muestras se centrifugan a 13.200 r.p.m durante 30 minutos a 4^{o}C y el precipitado correspondiente se lava con etanol 70% (v/v). El precipitado se seca por centrifugación en vacío y se resuspende en 100 μl de TE. Por último, se realiza la purificación del DNA por columnas de afinidad, utilizando el producto comercial GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare). Las muestras de DNA se eluyen de las columnas con 70 μl del tampón de elución. Para el análisis del mismo mediante qPCR se utiliza 1 μl de una dilución 1/10 de las muestras INPUT y 1 μl directamente del DNA inmunoprecipitado.

8.6. *Chromosome Conformation Capture* (3C)

Los experimentos de 3C se llevaron a cabo siguiendo el protocolo descrito en (Singh *et al*., 2009).

8.6.1. *Preparación y fijación del cultivo*

Las células se crecieron en 50 ml de YEPD o SC con las auxotrofías requeridas hasta una OD600nm de 0,6-0,8, preferiblemente 0,7, a la temperatura indicada. Posteriormente se llevó a cabo la fijación o *crosslinking* de las células mediante un tratamiento con formaldehido al 1%, durante un periodo de 20 minutos en agitación a temperatura ambiente. El entrecruzamiento se detuvo añadiendo 5,4 ml de glicina 1,25 M, e incubándo el cultivo 5 minutos más en agitación. El cultivo fijado se centrifugó durante 10 minutos a 3.000 r.p.m a 4^{o}C. En este punto el pellet de células puede ser congelado directamente a -80ºC para su posterior uso. Cuando continuamos con el proceso las células se lavaron con 10 ml de TBS 1X con 1% Tritonx100 frío.

8.6.2. *Preparación del extracto celular y obtención de la cromatina*

Las células se resuspendieron en 500 μl de tampón de lisis FA 1X y se pasaron a un Eppendorf nuevo frio. Se añadió 5 μl de PMSF 10mM y un volumen de 500 μl de bolitas de vidrio de 45 mm y se lisaron las células en un sistema Fast Prep, realizando 5 pulsos de 30 segundos a velocidad 4,5 K. Las células se dejaron 2 minutos en hielo entre pulso y pulso. Posteriormente, se eliminaron las bolitas por centrifugación y se centrifugaron los extractos a 13.200 r.p.m., durante 20 minutos a 4ºC. Finalmente, El pellet se resuspendió en 500 μl de TRIS 10mM pH=7,5.

8.6.3. *Digestión con enzimas de restricción*

Para la digestión se utilizó un volumen de 80 μl de la cromatina obtenida. La digestión se llevó a cabo con la enzima Hind III (200 U Fermentas) durante 5 horas a 37ºC en agitación (120 r.p.m). Después, para inactivar la digestión, se añadieron 10 μl de SDS 10% y se incubó durante 20 minutos a 65ºC. Posteriormente, se

incorporó 75 μl de 10% Triton x100 a la reacción y se centrifugó a temperatura ambiente durante 10 minutos a 13.200 r.p.m. El precipitado se dejó en reposo durante unos 10 minutos, tras lo cual, y con mucho cuidado, se resuspendió en 100 μl de TRIS 10mM pH=7,5.

8.6.4. *Ligación*

La reacción de ligación se realizó con los 100 μl de cromatina digeridos. Para ello, se utilizaron 5 μl de Quick Ligase (2000 U/μl New England Biolabs) en una reacción de un volumen final de 700 μl. La ligación se incubó durante 1 hora a 25ºC sin agitación.

8.6.5. *Procesamiento de la cromatina*

Posteriormente a la ligación, la muestra se trató con 2 μl de RNasa (10 mg/ml) y se incubó durante 20 minutos a 37ºC. Se añadió 7 μl de SDS 10% para inactivar la RNasa y 5 μl de Proteinasa K (10mg/ml) para diferir, incubándose toda la noche a 65ºC para eliminar las proteínas. La extracción del DNA se llevó a cabo con 700 μl de fenol:cloroformo:alcohol isoamílico (25:24:1) 2 veces, centrifugando a temperatura ambiente durante 10 minutos. Finalmente, se realizó una extracción con cloroformo:isoamílico (24:1). La fase acuosa se recuperó a un tubo nuevo y el DNA se precipitó con 1/10 del volumen de acetato sódico 0,3 M pH 5,2, más 2,5 volúmenes de etanol 96% (v/v) y 2 μl de glicógeno (Roche). La precipitación se llevó a cabo desde 20 minutos a temperatura ambiente a toda la noche a -20ºC. Las muestras se centrifugaron a 13.200 r.p.m durante 20 minutos a 4^{o}C y el precipitado correspondiente se lavó con etanol 70% (v/v). El precipitado se secó por centrifugación en vacío y se resuspendió en 50 μl de TE 1X pH 8.0. La concentración de DNA se midió utilizando el sistema NanoDrop a 260nm. Para las reacciones de PCR se utilizó 250-300 ng de DNA aproximadamente.

9. Anticuerpos

Los anticuerpos usados en esta tesis se describen en la Tabla MyM6 al final de la sección.

Bibliografía

Ahn, S. H., Kim, M., Buratowski, S. **(**2004). Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3 end processing. Mol Cell 13:67–76.

Ahn, S.H., Keogh, M.C., Buratowski,S. (2009). Ctk1 promotes dissoci- ation of basal transcription factors from elongating RNA polymerase II. EMBO J 28:205–212.

Akhtar, M.S., Heidemann, M., Tietjen, J.R., Zhang, D.W., Chapman, R.D., Eick, D., and Ansari, A.Z. (2009). TFIIH kinase places bivalent marks on the carboxy- terminal domain of RNA polymerase II. Mol Cell 34, 387-393.

Akoulitchev, S., Chuikov, S., Reinberg, D. (2000). TFIIH is negatively regulated by cdk8-containing mediator complexes. Nature 407, 102-106.

Al Husini, N., Kudla, P., Ansari, A. (2013). A role for CF1A 3' end processing complex in promoter-associated transcription. PLoS Genet 9(8): e1003722.

Allison, L.A., Wong, J.K., Fitzpatrick, V.D., Moyle, M., Ingles, C.J. (1988). The C-terminal domain of the largest subunit of RNA polymerase II of Saccharomyces cerevisiae, Drosophila melanogaster, and mammals: a conserved structure with an essential function. Mol Cell Biol 8, 321-329.

Almada A.E., Wu, X., Kriz, A.J., Burge, C.B., Sharp, P.A. (2013). Promoter directionality is controlled by U1 snRNP and polyadenylation signals. Nature 499:360–363.

Amberg, D.B., and Strathern, J.N. (2005). Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual

Ansari, A., and Hampsey, M. (2005). A role for the CPF 3'-end processing machinery in RNAP II-dependent gene looping. Genes Dev 19, 2969-2978.

Archambault, J., Chambers, R.S., Kobor, M.S., Ho, Y., Cartier, M., Bolotin, D., Andrews, B., Kane, C.M., Greenblatt, J. (1997). An essential component of a C-terminal domain phosphatase that interacts with transcription factor IIF in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 94, 14300-14305.

Ardehali, M.B., and Lis, J.T. (2009) Tracking rates of transcription and splicing in vivo. Nat Struct Mol Biol 16 1123–1124.

Arigo J.T., Eyler, D.E., Carroll, K.L., Corden, J.L. (2006). Termination of cryptic unstable transcripts is directed by yeast RNA-binding proteins Nrd1 and Nab3. Mol Cell 23:841–851.

Aristizabal, M.J., Negri, G.L., Benschop, J.J., Holstege, F.C., Krogan, N.J., Kobor, M.S. (2013). High-throughput genetic and gene expression analysis of the RNAPII-CTD reveals unexpected connections to SRB10/CDK8. PLoS Genet 9(8):e1003758.

Armache, K.J., Kettenberger, H., and Cramer, P. (2003). Architecture of initiation-competent 12-subunit RNA polymerase II. Proc Natl Acad Sci U S A 100, 6964- 6968.

Armache, K.J., Mitterweger, S., Meinhart, A., Cramer, P. (2005) Structures of complete RNA polymerase II and its subcomplex, Rpb4/7. J Biol Chem 280: 7131–7134.

Baillat, D., Hakimi, M.A., Naar, A.M., Shilatifard, A., Cooch, N., Shiekhattar, R. (2005). Integrator, a multiprotein mediator of small nuclear RNA processing, associates with the C-terminal repeat of RNA polymerase II. Cell 123:265–276.

Bartkowiak, B., Liu, P., Phatnani, H.P., Fuda, N.J., Cooper, J.J., Price, D.H., Adelman, K., Lis, J.T., Greenleaf, A.L. (2010). CDK12 is a transcription elongation-associated CTD kinase, the metazoan ortholog of yeast Ctk1. Genes Dev 24, 2303-2316.

Bartkowiak, B., and Greenleaf, A.L. (2011). Phosphorylation of RNAPII: To P-TEFb or not to P-TEFb? Transcription 2, 115-119.

Basehoar, A.D., Zanton, S.J., Pugh, B.F. (2004). Identification and distinct regulation of yeast TATA box- containing genes. Cell 116, 699-709.

Baskaran, R., Chiang, G.G., Mysliwiec, T., Kruh, G.D., Wang, J.Y. (1997). Tyrosine phosphorylation of RNA polymerase II carboxyl-terminal domain by the Abl-related gene product. J Biol Chem 272, 18905-18909.

Bataille, A.R., Jeronimo, C., Jacques, P.E., Laramee, L., Fortin, M.E., Forest, A., Bergeron, M., Hanes, S.D., Robert, F. (2012). A universal RNA polymerase II CTD cycle is orchestrated by complex interplays between kinase, phosphatase, and isomerase enzymes along genes. Mol Cell 45, 158-170.

Bentley, D.L. (2014). Coupling mRNA processing with transcription in time and space. Nat Rev Genet 15: 163–175.

Beyer, A.L., and Osheim, Y.N. (1988). Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. Genes Dev 2(6):754–765.

Birse C.E., Minvielle-Sebastia L., Lee B.A., Keller W., Proudfoot N.J. (1998). Coupling termination of transcription to messenger RNA maturation in yeast. Science 280:298-301

Bouchoux, C., Hautbergue, G., Grenetier, S., Carles, C., Riva, M., Goguel, V. (2004) CTD kinase I is involved in RNA polymerase I transcription. Nucleic Acids Res 32: 5851- 5860.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14: 115-132.

Bratkowski, M., Unarta, I.C., Zhu, L., Shubbar, M., Huang, X., Liu, X. (2017). Structural dissection of an interaction between transcription initiation and termination factors implicated in promoter-terminator crosstalk. J BIol Chem

Buratowski, S. (2003). The CTD code. Nat Struct Biol 10, 679-680.

Buratowski, S. (2009). Progression through the RNA polymerase II CTD cycle. Mol Cell 36, 541-546.

Bushnell, D.A., and Kornberg, R.D. (2003). Complete, 12-subunit RNA polymerase II at 4.1-A resolution: implications for the initiation of transcription. Proc Natl Acad Sci USA 100, 6969-6973.

Bushnell, D.A., Westover, K.D., Davis, R.E., Kornberg, R.D. (2004). Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 angstroms. Science 303, 983–988

Cadena, D.L., and Dahmus, M.E. (1987) Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. J Biol Chem 262: 12468- 12474.

Calvo, O., and Manley, J.L. (2005). The transcriptional coactivator PC4/Sub1 has multiple functions in RNA polymerase II transcription. EMBO J 24: 1009-1020.

Calvo, O., and García, A. (2012). RNA Polymerase II Phosphorylation and Gene Expression Regulation (INTECH Open Access Publisher).

Calvo, O. (2017). Sub1 and RNAPII, until termination does them part. Transcription 30:e1333558.

Carles, C., Treich, I., Bouet, F., Riva, M., and Sentenac, A. (1991). Two additional common subunits, ABC10 alpha and ABC10 beta, are shared by yeast RNA polymerases. J Biol Chem 266, 24092-24096.

Carlson, M. (1997). Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD. Annu Rev Cell Dev Biol 13, 1-23.

Chambers, R.S., and Dahmus, M.E. (1994). Purification and characterization of a phosphatase from HeLa cells which dephosphorylates the C-terminal domain of RNA polymerase II. J Biol Chem 269, 26243-26248.

Chambers, R.S., and Kane, C.M. (1996). Purification and characterization of an RNA polymerase II phosphatase from yeast. J Biol Chem 271, 24498-24504.

Chapman, R.D., Heidemann, M., Albert, T.K., Mailhammer, R., Flatley, A., Meisterernst, M., Kremmer, E., Eick, D. (2007). Transcribing RNA polymerase II is phosphorylated at CTD residue serine-7. Science 318, 1780- 1782.

Chapman, R.D., Heidemann, M., Hintermair, C., Eick, D. (2008) Molecular evolution of the RNA polymerase II CTD. Trends Genet 24: 289-296.

Chédin, S., Riva, M., Schultz, P., Sentenac, A., Carles, C. (1998). The RNA cleavage activity of RNA polymerase III is mediated by an essential TFIIS-like subunit and is important for transcription termination. Genes Dev 12(24): 3857–3871.

Chen, Y., Zhang, L., Estarás, C., Choi, S.H., Moreno, L. Jr, Karn, J., Moresco, J.J., Yates, J.R. 3rd, Jones, K.A. (2014) A gene-specific role for the Ssu72 RNAPII CTD phosphatase in HIV-1 Tat transactivation. Genes Dev 28(20):2261-75.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev 15, 1078-1092.

Cho, E.J., Takagi, T., Moore, C.R., Buratowski, S. (1997). mRNA capping enzyme is recruited to the transcription complex by phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev 11, 3319-3326.

Cho, E.J., and Buratowski, S. (1999). Evidence that transcription factor IIB is required for a post-assembly step in transcription initiation. J Biol Chem 274: 25807–25813.

Cho, E. J., Kobor, M.S., Kim, M., Greenblatt, J., Buratowski, S. **(**2001). Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. Genes Dev 15**:**3319–3329.

Choder, M., and Young, R.A. (1993). A portion of RNA polymerase II molecules has a component essential for stress responses and stress survival. Mol Cell Biol 13, 6984–6991.

Chu, Y., Simic, R., Warner, M.H., Arndt, K.M., Prelich, G. (2007). Regulation of histone modification and cryptic transcription by the Bur1 and Paf1 complexes. EMBO J 26, 4646-4656.

Clemente-Blanco, A., Sen, N., Mayan-Santos, M., Sacristan, M.P., Graham, B., Jarmuz, A., Giess, A., Webb, E., Game, L., Eick, D., Bueno, A., Merkenschlager, M., Aragón, L. (2011). Cdc14 phosphatase promotes segregation of telomeres through repression of RNA polymerase II transcription. Nat Cell Biol 13, 1450-1456.

Cooper, K.F. and Strich, R. (1999) Functional analysis of the Ume3p/ Srb11p-RNA polymerase II holoenzyme interaction. Gene Expr 8(1):43-57.

Coppola, J.A., Field, A.S., Luse, D.S. (1983)- Promoter-proximal pausing by RNA polymerase II in vitro: Transcripts shorter than 20 nucleotides are not capped. Proc Natl Acad Sci 80: 1251–1255.

Core, L.J., Waterfall, J.J., Lis, J.T. (2008). Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science 322:1845–1848.

Corden, J.L., Cadena, D.L., Ahearn, J.M., Jr., Dahmus, M.E. (1985). A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II. Proc Natl Acad Sci U S A 82, 7934-7938.

Corden, J.L. (1990) Tails of RNA polymerase II. Trends Biochem Sci 15: 383-387.

Corden, J.L. (2007). Transcription. Seven ups the code. Science 318, 1735-1736.

Cramer, P., Bushnell, D.A., Fu, J., Gnatt, A.L., Maier-Davis, B., Thompson, N.E., Burgess, R.R., Edwards, A.M., David, P.R., Kornberg, R.D. (2000). Architecture of RNA polymerase II and implications for the transcription mechanism. Science 288, 640-649.

Cramer, P., Bushnell, D.A., Kornberg, R.D. (2001). Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. Science 292, 1863- 1876.

Cramer, P. (2002a). Common structural features of nucleic acid polymerases. Bioessays 24, 724-729.

Cramer, P. (2002b). Multisubunit RNA polymerases. Curr Opin Struct Biol 12, 89-97.

Cramer, P. (2004). RNA polymerase II structure: from core to functional complexes. Curr Opin Genet Dev 14, 218-226.

Cramer, P., Armache, K.J., Baumli, S., Benkert, S., Brueckner, F., Buchen, C., Damsma, G.E., Dengl, S., Geiger, S.R., Jasiak, A.J., et al. (2008). Structure of eukaryotic RNA polymerases. Annu Rev Biophys 37, 337-352.

David, C.J., and Manley, J.L. (2011). The RNA polymerase C-terminal domain: a new role in spliceosome assembly. Transcription 2:221–225.

De la Mata, M., Alonso, C.R., Kadener, S., Fededa, J.P., Blaustein, M., Pellisch, F., Cremer, P., Bentlye, D., Kornblihtt, A.R. (2003). A slow RNA polymerase II affects alternative splicing in vivo. Mol Cell 12(2):525-32

Deng, W., and Roberts, S.G. (2007) TFIIB and the regulation of transcription by RNA polymerase II. Chromosoma 116 417–429.

Descostes, N., Heidemann, M., Spinelli, L., Schüller, R., Maqbool, M.A., Fenouil, R., Koch, F., Innocenti, C., Gut, M., Gut, I., Eick, D., Andrau, J.C. (2014) Tyrosine phosphorylation of RNA polymerase II CTD is associated with antisense promoter transcription and active enhancers in mammalian cells. Elife 3:e02105.

Deshpande, S.M., Sadhale, P.P., Vijayraghavan, U. (2014). Involvement of S. cerevisiae Rpb4 in subset of pathways related to transcription elongation. Gene 545(1):126-31

Dias, J.D., Rito, T., Torlai-Triglia, E., Kukalev, A., Ferrai, C., Choralia, M., Brookes, E., Kimura, H., Pombo, A. (2015). Methylation of RNA polymerase II non-consensus Lysine residues marks early transcription in mammalian cells. eLife 4:e11215.

Dichtl, B., Blank, D., Ohnacker, M., Friedlein, A., Roeder, D., Langen, H., Keller, W. (2002). A role for SSU72 in balancing RNA polymerase II transcription elongation and termination. Mol Cell 10, 1139-1150.

Donner, A.J., Ebmeier, C.C., Taatjes, D.J., and Espinosa, J.M. (2010). CDK8 is a positive regulator of transcriptional elongation within the serum response network. Nat Struct Mol Biol *17*, 194-201.

Drogat, J., and Hermand, D. (2012). Gene-specific requirement of RNA polymerase II CTD phosphorylation. Mol Microbiol 84(6):995-1004.

Dvir, A., Conaway, R.C., Conaway, J.W. (1997). A role for TFIIH in controlling the activity of early RNA polymerase II elongation complexes. Proceedings of the National Academy of Sciences 94, 9006- 9010.

Edwards, A.M., Kane, C.M., Young, R.A., Kornberg, R.D. (1991). Two dissociable subunits of yeast RNA polymerase II stimulate the initiation of transcription at a promoter *in vitro*. J Biol Chem 266, 71-75.

Egloff, S., O'Reilly, D., Chapman, R.D., Taylor, A., Tanzhaus, K., Pitts, L., Eick, D., Murphy, S. (2007) Serine-7 of the RNA polymerase II CTD is specifically required for snRNA gene expression. Science 318: 1777-1779.

Egloff, S., and Murphy, S. (2008a). Cracking the RNA polymerase II CTD code. Trends Genet 24, 280-288.

Egloff, S., and Murphy, S. (2008b). Role of the C-terminal domain of RNA polymerase II in expression of small nuclear RNA genes. Biochem Soc Trans 36, 537-539.

Egloff, S., Szczepaniak, S.A., Dienstbier, M., Taylor, A., Knight, S., Murphy, S. (2010). The integrator complex recognizes a new double mark on the RNA polymerase II carboxyl-terminal domain. J Biol Chem 285:20564–20569.

Egloff, S., Zaborowska, J., Laitem, C., Kiss, T., Murphy, S. (2012). Ser7 phosphorylation of the CTD recruits the RPAP2 Ser5 phosphatase to snRNA genes. Mol Cell 45, 111-122.

Eychenne, T., Werner, M., Soutourina, J. (2017). Toward understanding of the mechanisms of Mediator function in vivo: Focus on the preinitiation complex assembly. Transcription 8(5):328-342.

Eick, D., and Geyer, M. (2013). The RNA polymerase II Carboxy-Terminal Domain (CTD) Code. Chem Rev 113(11): 8456–8490.

El Kaderi, B., Medler, S.,Raghunayakula, S., Ansari, A. (2009) Gene looping is conferred by activator-dependent interaction of transcription initiation and termination machineries. J Biol Chem 284, 25015–25025.

Erokhin, M., Davydova, A., Kyrchanova, O., Parshikov, A., Georgiev, P., Chetverina, D. (2011). Insulators form gene loops by interacting with promoters in Drosophila. Development 138(18):4097-106.

Espinoza, F.H., Farrell, A., Nourse, J.L., Chamberlin, H.M., Gileadi, O., Morgan, D.O. (1998). Cak1 is required for Kin28 phosphorylation and activation *in vivo*. Mol Cell Biol 18, 6365-6373.

Fabrega, C., Shen, V., Shuman, S., Lima, C.D. (2003). Structure of an mRNA capping enzyme bound to the phosphorylated carboxy-terminal domain of RNA polymerase II. Mol Cell 11: 1549–1561

Galbraith, M.D., Donner, A.J., Espinosa, J.M. (2010). CDK8: a positive regulator of transcription. Transcription 1, 4-12.

Ganem, C., Devaux, F., Torchet, C., Jacq, C., Quevillon-Cheruel, S., Labesse, G., Facca, C., Fayer, G. (2003). Ssu72 is a phosphatase essential for transcription termination of snoRNAs and specific mRNAs in yeast. EMBO J 1;22(7):1588-98.

Garavís, M., González-Polo, N., Allepuz-Fuster, P., Louro, J.A., Fernández-Tornero, C., Calvo, O. (2016). Sub1 contacts the RNA polymerase II stalk to modulate mRNA synthesis. Nucleic Acids Res 45(5):2458-2471.

Garavís, M., and Calvo, O. (2017). Sub1/PC4, a multifaceted factor: from transcription to genome stability. Current Genetics 63:1023-1035.

Garcia, A., Rosonina, E., Manley, J.L., and Calvo, O. (2010). Sub1 globally regulates RNA polymerase II C-terminal domain phosphorylation. Mol Cell Biol 30: 5180-5193.

García, A., Collin, A., Calvo, A. (2012). Sub1 associates with Spt5 and influences RNA polymerase II transcription elongation rate. Mol Biol Cell 23(21): 4297-4312.

García-López, M.C., Pelechano, V., Mirón-García, M.C., Garrido-Godino, A.I., García, A., Calvo, O., Werner, M., Pérez-Ortín, J.E., Navarro, F. (2011) The conserved foot domain of RNA pol II associates with proteins involved in transcriptional initiation and/or early elongation. Genetics 189(4):1235-48.

Garrido-Godino, A.I., García-López, M.C., Navarro, F. (2013) Correct assembly of RNA polymerase II depends on the foot domain and is required for multiple steps of transcription in Saccharomyces cerevisiae. Mol Cell Biol 33(18):3611-26.

Garrido-Godino, A.I., García-López, M.C., García-Martínez, J., Pelechano, V., Medina, D.A., Pérez-Ortín, J.E., Navarro, F. (2016). Rpb1 foot mutations demonstrate a major role of Rpb4 in mRNA stability during stress situations in yeast. Biochim Biophys Acta 1859(5):731-43.

Gelev, V., Zabolotny, J.M., Lange, M., Hiromura, M., Yoo, S.W., Orlando, J.S., Kushnir, A., Horikoshi, N., Paquet, E., Bachvarov, D., Schaffer, P.A., Usheva, A. (2014). A new paradigm for transcription factor TFIIB functionality. Sci Rep 4:3664**.**

Ghosh, A., Shuman, S., Lima, C.D. (2011). Structural insights to how mammalian capping enzyme reads the CTD code. Mol Cell 43: 299–310

Giardina, C., and Lis, J.T. (1993) Dna melting on yeast RNA polymerase II promoters. Science 6;261(5122):759-62.

Gibney, P.A., Fries, T., Bailer, S.M., Morano, K.A. (2008). Rtr1 is the Saccharomyces cerevisiae homolog of a novel family of RNA polymerase II- binding proteins. Eukaryot Cell 7, 938-948.

Gietz, R.D., Schiestl, R.H., Willems, A.R., and Woods, R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11: 355-360.

Gilbert, W., and Guthrie, C. (2004). The Glc7p nuclear phosphatase promotes mRNA export by facilitating association of Mex67p with mRNA. Mol Cell 13:201–212.

Glover-Cutter, K., Larochelle, S., Erickson, B., Zhang, C., Shokat, K., Fisher, R.P., and Bentley, D.L. (2009). TFIIH-associated Cdk7 kinase functions in phosphorylation of C-terminal domain Ser7 residues, promoter-proximal pausing, and termination by RNA polymerase II. Mol Cell Biol 29, 5455-5464.

Gnatt, A.L., Cramer, P., Fu, J., Bushnell, D.A., Kornberg, R.D. (2001). Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 A resolution. Science 292, 1876-1882.

Goler-Baron, V., Selitrennik, M., Barkai, O., Haimovich, G., Lotan, R., Choder, M. (2008). Transcription in the nucleus and mRNA decay in the cytoplasm are coupled processes. Genes Dev 22, 2022–2027

Govind, C.K., Zhang, F., Qiu, H., Hofmeyer, K., Hinnebusch, A.G. (2007). Gcn5 promotes acetylation, eviction, and methylation of nucleosomes in transcribed coding regions. Mol Cell 25, 31-42.

Grenetier, S., Bouchoux, C., Goguel, V. (2006). CTD kinase I is required for the integrity of the rDNA tandem array. Nucleic Acids Res 34, 4996-5006.

Grohmann, D., and Werner, F. (2011) Cycling through transcription with the RNA polymerase F/E (RPB4/7) complex: structure, function and evolution of archaeal RNA polymerase. Res Microbiol 162: 10–18.

Grünberg, S., and Hahn, S. (2013). Structural insights into transcription initiation by RNA polymerase II. Trends Biochem Sci 38(12):603-11.

Grzechnik, P., Tan-Wong, S.M., Proudfoot, N.J. (2014). Terminate and make a loop: regulation of transcriptional directionality. Trends Biochem Sci 39(7):319-27.

Guillamot, M., Manchado, E., Chiesa, M., Gomez-Lopez, G., Pisano, D.G., Sacristan, M.P., Malumbres, M. (2011). Cdc14b regulates mammalian RNA polymerase II and represses cell cycle transcription. Sci Rep 1, 189.

Gupta, K., Sari-Ak, D., Haffke, M., Trowitzsck, S., Berger, I. (2016). **Zooming in on** Transcription Preinitiation. J Mol Biol 428(12):2581-2591.

Haag, J.R., and Pikaard, C.S. (2011). Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. Nature reviews Molecular cell biology 12, 483-492.

Hahn, S. (2004). Structure and mechanism of the RNA polymerase II transcription machinery. Nature structural & molecular biology 11, 394.

Haimovich, G., Medina, D.A., Causse, S.Z., Garber, M., Millán-Zambrano, G., Barkai, O., Chávez, S., Pérez-Ortín, J.E., Darzacq, X., Choder, M. (2013). Gene expression is circular: factors for mRNA degradation also foster mRNA synthesis. Cell 153(5): 1000-11.

Halen, K.M., and Churchman, L.S. (2017) The code and beyond: transcription regulation by the RNA polymerase II carboxy-terminal domain. Nat Rev Mol Cell Biol 18(4):263-273

Hampsey, M. (1998). Molecular genetics of the RNA polymerase II general transcriptional machinery. Microbiol Mol Biol Rev 62, 465-503.

Hampsey, M., and Kinzy, T.G. (2007). Synchronicity: policing multiple aspects of gene expression by Ctk1. Genes Dev 21, 1288-1291.

Hanahan, D., Jessee, J., Bloom,J.R., (1991) Plasmid transformation of Escherichia coli and other bacteria. Methods Enzymol 204:63-113.

Hanes, S.D. (2014). The Ess1 prolyl isomerase: traffic cop of the RNA polymerase II transcription cycle. Biochimica et biophysica acta 1839:316–333.

Harel-Sharvit, L., Eldad, N., Haimovich, G., Barkai, O., Duek, L., Choder, M. (2010). RNA polymerase II subunits link transcription and mRNA decay to translation. Cell 143, 552-563.

Hargreaves, D.C., Horng, T., Medzhitov, R. (2009). Control of inducible gene expression by signal-dependent transcriptional elongation. Cell 138(1):129-45.

Harlen, K.M., Trotta, K.L., Smith, E.E., Mosaheb, M.M., Fuchs, S.M., Churchman, L.S. (2016). Comprehensive RNA Polymerase II Interactomes Reveal Distinct and Varied Roles for Each Phospho-CTD Residue. Cell Rep 15(10):2147-2158.

Hausmann, S., and Shuman, S. (2002). Characterization of the CTD phosphatase Fcp1 from fission yeast. Preferential dephosphorylation of serine 2 versus serine 5. J Biol Chem 277, 21213-21220.

Hawkes, N.A., and Roberts, S.G. (1999). The role of human TFIIB in start site selection in vitro and in vivo. J Biol Chem 274, 14337–14343.

He, X., Khan, A.U., Cheng, H., Pappas, D.L. Jr., Hampsey, M., Moore, C.L. (2003). Functional interactions between the transcription and mRNA 3' end processing machineries mediated by Ssu72 and Sub1. Genes Dev 17(8):1030-42.

Heidemann, M., Hintermair, C., Voss, K., Eick, D. (2012). Dynamic phosphorylation patterns of RNA polymerase II CTD during transcription. Biochim Biophys Acta.

Hengartner, C.J., Myer, V.E., Liao, S.M., Wilson, C.J., Koh, S.S., Young, R.A. (1998). Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. Mol Cell. 2: 43-53.

Henriques, T., Ji, Z., Tan-Wong, S.M., Carmo, A.M., Tian, B., Proudfoot, N.J., Moreira, A. (2012). Transcription termination between polo and snap, two closely spaced tandem genes of D. melanogaster. Transcription 3(4):198-212.

Henry, N.L., Bushnell, D.A., and Kornberg, R.D. (1996). A yeast transcriptional stimulatory protein similar to human PC4. J Biol Chem *271*, 21842-21847.

Hintermair, C., Heidemann, M., Koch, F., Descostes, N., Gut, M., Gut, I., Fenouil, R., Ferrier, P., Flatley, A., Kremmer, E., Champman, R.D., Andrau, J.C., Eick, D. (2012).

Threonine-4 of mammalian RNA polymerase II CTD is targeted by Polo-like kinase 3 and required for transcriptional elongation. EMBO J 31, 2784-2797.

Hirayoshi K., and Lis J.T. (1999). Nuclear run-on assays: assessing transcription by measuring density of engaged RNA polymerases. Methods Enzymol. 304:351-62

Hirose, Y., and Manley, J.L. (2000). RNA polymerase II and the integration of nuclear events. Genes Dev 14, 1415-1429.

Hirose, Y., and Ohkuma, Y. (2007). Phosphorylation of the C-terminal domain of RNA polymerase II plays central roles in the integrated events of eucaryotic gene expression. Journal of biochemistry 141, 601- 608.

Hirst, M., Kobor, M.S., Kuriakose, N., Greenblatt, J., Sadowski, I. (1999). GAL4 is regulated by the RNA polymerase II holoenzyme-associated cyclin-dependent protein kinase SRB10/CDK8. Mol Cell 3, 673-678.

Ho, C.K., and Shuman, S. (1999). Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. Mol Cell 3, 405-411.

Holstege, F.C., Fiedler, U., Timmers, H.T.M. (1997). Three transitions in the RNA polymerase II transcription complex during initiation. The EMBO journal 16, 7468-7480.

Holstege, F.C., Jennings, E.G., Wyrick, J.J., Lee, T.I., Hengartner, C.J., Green, M.R., Golub, T.R., Lander, E.S., Young, R.A. (1998). Dissecting the regulatory circuitry of a eukaryotic genome. Cell 95, 717-728.

Houseley, J., and Tollervey, D. (2009). The many pathways of RNA degradation. Cell 136: 763-776.

Hsin, J.P., Sheth, A., Manley, J.L. (2011). RNAP II CTD phosphorylated on threonine-4 is required for histone mRNA 3' end processing. Science 334, 683- 686.

Hsin, J.P., and Manley, J.L. (2012). The RNA polymerase II CTD coordinates transcription and RNA processing. Genes Dev 26, 2119-2137.

Hsin, J.P., Xiang, F., Manley, J.L. (2014). Function and control of RNA polymerase II C-terminal domain phosphorylation in vertebrate transcription and RNA processing. Mol Cell Biol 34(13):2488-98.

Hsu, P.L., Yang, F., Smith-Kinnaman, W., Yang, W., Song, J.E, Mosley, A.L., Verani, G. (2014). Rtr1 is a dual specificity phosphatase that dephosphorylates Tyr1 and Ser5 on the RNA polymerase II CTD. J Mol Biol 426(16):2970-81

Hurt, E., Luo, M.J., Rother, S., Reed, R., Strasser, K. (2004). Cotranscriptional recruitment of the serine-arginine-rich (SR)-like proteins Gbp2 and Hrb1 to nascent mRNA via the TREX complex. Proc Natl Acad Sci U S A. 101: 1858-1862.

Irani, S., Yogesha, S.D., Mayfield, J., Zhang, M., Zhang, Y., Matthews, W.L., Nie, G., Prescott, N.A., Zhang, Y.J. (2016). Structure of Saccharomyces cerevisiae Rtr1 reveals an active site for an atypical phosphatase. Sci Signal 9(417):ra24.

Jasiak, A.J., Hartmann, H., Karakasili, E., KAlocsay, M., Flatley, A., Kremmer, E., Strässer, K., Martin, D.E., Söding, J., Cramer, P. (2008). Genome-associated RNA polymerase II includes the dissociable Rpb4/7 subcomplex. J Biol Chem 283: 26423-26427.

Jeronimo, C., Bataille, A.R., and Robert, F. (2013). The writers, readers, and functions of the RNA polymerase II C-terminal domain code. Chemical reviews 113, 8491-8522.

Jeronimo, C., and Robert, F. Kin28 regulates the transient association of Mediator with core promoters. Nat Struct Mol Biol 21, 449–455 (2014).

Jiang, Y., Yan, M., Gralla, J.D. (1996). A three-step pathway of transcription initiation leading to promoter clearance at an activation RNA polymerase II promoter. Mol Cell Biol 16, 1614-1621.

Jimeno, S., Rondon, A.G., Luna, R., Aguilera, A. (2002). The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability. EMBO J 21: 3526-3535.

Jones, J.C., Phatnani, H.P., Haystead, T.A., MacDonald, J.A., Alam, S.M., Greenleaf, A.L. (2004). C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats. J Biol Chem 279, 24957-24964.

Jonkers, I., and Lis, J.T. (2015). Getting up to speed with transcription elongation by RNA polymerase II. Nat Rev Mol Cell Biol 16, 167-177.

Kamenski, T., Heilmeier, S., Meinhart, A., Cramer, P. (2004) Structure and mechanism of RNA polymerase II CTD phosphatases. Mol Cell 15(3):399-407.

Keogh, M.C., Cho, E.J., Podolny, V., Buratowski, S. (2002). Kin28 is found within TFIIH and a Kin28-Ccl1-Tfb3 trimer complex with differential sensitivities to T- loop phosphorylation. Mol Cell Biol 22, 1288-1297.

Keogh, M.C., Podolny, V., Buratowski, S. (2003). Bur1 kinase is required for efficient transcription elongation by RNA polymerase II. Mol Cell Biol 23, 7005- 7018.

Kim, M., Ahn, S.H., Krogan, N.J., Greenblatt, J.F., Buratowski, S. (2004). Transitions in RNA polymerase II elongation complexes at the 3' ends of genes. EMBO J 23, 354-364.

Kim, M., Suh, H., Cho, E.J., Buratowski, S. (2009). Phosphorylation of the yeast Rpb1 C-terminal domain at serines 2, 5, and 7. J Biol Chem 284, 26421-26426.

Kim, T.S., Liu, C.L., Yassour, M., Holik, J., Friedman, N., Buratowski, S., Rando, O.J. (2010). RNA polymerase mapping during stress responses reveals widespread nonproductive transcription in yeast. Genome Biol 11(7):R75.

Kimmelman. J., Kaldis, P., Hengartner, C.J., Laff, G.M., Koh, S.S., Young, R.A., Solomon, M.J. (1999) Activating phosphorylation of the Kin28p subunit of yeast TFIIH by Cak1p. Mol Cell Biol 19:4774–4787.

Kimura, M., Suzuki, H., Ishihama, A. (2002) Formation of a carboxy-terminal domain phosphatase (Fcp1)/TFIIF/RNA polymerase II (pol II) complex in Schizosaccharomyces pombe involves direct interaction between Fcp1 and the Rpb4 subunit of pol II. Mol Cell Biol 22(5):1577-88.

Kimura, M., and Ishihama, A. (2004). Tfg3, a subunit of the general transcription factor TFIIF in Schizosaccharomyces pombe, functions under stress conditions. Nucleic Acids Res 32, 6706-6715.

Knaus, R., Pollock, R., Guarente, L. (1996). Yeast SUB1 is a suppressor of TFIIB mutations and has homology to the human co-activator PC4. EMBO J 15:1933–1940.

Kobor, M.S., Archambault, J., Lester, W., Holstege, F.C., Gileadi, O., Jansma, D.B., Jennings, E.G., Kouyoumdjian, F., Davidson, A.R., Young, R.A., Greenblat, J. (1999). An unusual eukaryotic protein phosphatase required for transcription by RNA polymerase II and CTD dephosphorylation in *S. cerevisiae*. Mol Cell 4, 55-62.

Kolodziej, P.A., Woychik, N., Liao, S.M., Young, R.A. (1990) RNA polymerase II subunit composition, stoichiometry, and phosphorylation. Mol Cell Biol 10(5):1915-20.

Komarnitsky, P., Cho, E.J., Buratowski, S. (2000) Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev 14, 2452-2460.

Kornberg, R.D. (2005). Mediator and the mechanism of transcriptional activation. Trends Biochem Sci 30, 235-239.

Kong, S.E., Kobor, M.S., Krogan, N.J., Somesh, B.P., Sogaard, T.M., Greenblatt, J.F., Svejstrup, J.Q. (2005). Interaction of Fcp1 phosphatase with elongating RNA polymerase II holoenzyme, enzymatic mechanism of action, and genetic interaction with elongator. J Biol Chem 280, 4299-4306.

Kostrewa, D., Zeller, M.E., Armache, K.J., Seizl, M., Leike, K., Thomm, M., Cramer, P. (2009). RNA polymerase II-TFIIB structure and mechanism of transcription initiation. Nature 462, 323-330.

Koyanagi, K.O., Hagiwara, M., Itoh, T., Gojobori, T., Imanishi, T. (2005). Comparative genomics of bidirectional gene pairs and its implications for the evolution of a transcriptional regulation system. Gene 353:169–176.

Krishnamurthy, S., He, X., Reyes-Reyes, M., Moore, C., Hampsey, M. (2004). Ssu72 Is an RNA polymerase II CTD phosphatase. Mol Cell 14, 387-394.

Krishnamurthy, S., Ghazy, M.A., Moore, C., Hampsey, M. (2009). Functional interaction of the Ess1 prolyl isomerase with components of the RNA polymerase II initiation and termination machineries. Mol Cell Biol 29: 2925-2934.

Kubicek, K., Cerna, H., Holub, P., Pasulka, J., Hrossova, D., Loehr, F., Hofr, C., Vanacova, S., Stefi, R. (2012). Serine phosphorylation and proline isomerization in RNAP II CTD control recruitment of Nrd1. Genes Dev 26(17): 1891-6.

Kuehner J.N. and Brow D.A. (2008). Regulation of a eukaryotic gene by GTP-dependent start site selection and transcription attenuation. Mol cell 31:201-11.

Kuehner, J.N., Pearson, E.L., Moore, C. (2011). Unravelling the means to an end: RNA polymerase II transcription termination. Nature reviews Molecular cell biology 12, 283-294.

Kwak, H., and Lis, J.T. (2013). Control of transcriptional elongation. Annu Rev Genet 47:483-508.

Kwapisz, M., Beckouet, F., and Thuriaux, P. (2008). Early evolution of eukaryotic DNA-dependent RNA polymerases. Trends Genet 24, 211-215.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lainé, J.P., Singh, B.N., Krishnamurthy, S., Hampsey, M. (2009). A physiological role for gene loops in yeast. Genes Dev 23(22):2604-9.

Landrieux, E., Alic, N., Ducrot, C., Acker, J., Riva, M., Carles, C. (2006). A subcomplex of RNA polymerase III subunits involved in transcription termination and reinitiation. EMBO 25:118-28.

Langer, D., Hain, J., Thuriaux, P., and Zillig, W. (1995). Transcription in archaea: similarity to that in eucarya. Proceedings of the National Academy of Sciences 92: 5768-5772.

Lara-Gonzalez, S., Birktoft, J.J., Lawson, C.L. (2010). Structure of the Escherichia coli RNA polymerase alpha subunit C-terminal domain. Acta Crystallogr D Biol Crystallogr 66:806-12

Larschan, E., and Winston, F. (2005). The Saccharomyces cerevisiae Srb8-Srb11 complex functions with the SAGA complex during Gal4-activated transcription. Mol Cell Biol 25, 114-123.

Li, S., and Smerdon, M.J. (2002). Rpb4 and Rpb9 mediate subpathways of transcription-coupled DNA repair in Saccharomyces cerevisiae. EMBO J 21(21): 5921-9.

Li, H., Zhang, Z., Wang, B., Zhang, J., Zhao, Y., Jin, Y. (2007). Wwp2-mediated ubiquitination of the RNA polymerase II large subunit in mouse embryonic pluripotent stem cells. Mol Cell Biol 27: 5296–5305

Liang, C., and Stillman, B. (1997). Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev 11(24):3375-86.

Liao, S.M., Zhang, J., Jeffery, D.A., Koleske, A.J., Thompson, C.M., Chao, D.M., Viljoen, M., van Vuuren, H.J., and Young, R.A. (1995). A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature *374*, 193-196.

Lindstrom, D.L., and Hartzog, G.A. (2001). Genetic interactions of Spt4-Spt5 and TFIIS with the RNA polymerase II CTD and CTD modifying enzymes in Saccharomyces cerevisiae. Genetics 159: 487-497.

Liou, Y.C., Zhou, X.Z., Lu, K.P. (2011). Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins. Trends in biochemical sciences 36:501–514.

Liu, P., Kenney, J.M., Stiller, J.W., Greenleaf, A.L. (2010) Genetic organization, length conservation, and evolution of RNA polymerase II carboxyl-terminal domain. Mol Biol Evol. 27: 2628-2641.

Liu, Y., Kung, C., Fishburn, J., Ansari, A.Z., Shokat, K.M., Hahn, S. (2004). Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. Mol Cell Biol 24, 1721-1735.

Liu, X., Bushnell, D.A., Silva, D.A., Huang, X., Kornberg, R.D. (2011). Initiation complex structure and promoter proofreading. Science 333, 633-637.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

Lopez, K.J., and Henderson, O. (2015). Yeast Ssu72 Protein as a Mediator of Gene Loop Formation at Bidirectional. Journal of Student Research Volume 4, Issue 1: pp. 29-35

Lotan, R., Goler-Baron, V., Harel-Sharvit, L., Duek, L., Melamed, D., Choder, M. (2005). The RNA polymerase II subunit Rpb4p mediates decay of a specifi c class of mRNAs. Genes Dev 19 :3004–3016.

Lotan, R., Goler-Baron, V., Duek, L. Haimovich, G., Choder, M. (2007). The Rpb7p subunit of yeast RNA polymerase II plays roles in the two major cytoplasmic mRNA decay mechanisms. J Cell Biol 178(7): 1133-43.

Lu, H., Flores, O., Weinmann, R., Reinberg, D. (1991). The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. Proc Natl Acad Sci U S A 88, 10004-10008.

Lu, K.P., and Zhou, X.Z. (2007). The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. Nat Rev Mol Cell Biol 8, 904-916.

Lu, K.P., Finn, G., Lee, T.H., Nicholson, L.K. (2007). Prolyl cis-trans isomerization as a molecular timer. Nat Chem Biol 3, 619-629.

Lunde, B.M., Reichow, S.L., Kim, M., Suh, H., Leeper, T.C., Yang, F., Mutschler, H., Buratowski, S., Meinhart, A., Varani, G. (2010). Cooperative interaction of transcription

termination factors with the RNA polymerase II C-terminal domain. Nat Struct Mol Biol 17: 1195–1201.

Luse, D.S. (2013). Promoter clearance by RNA polymerase II. Biochim Biophys Acta 1829(1):63-8.

Ma, Z., Atencio, D., Barnes, C., DeFiglio, H., Hanes, S.D. (2012) Multiple Roles for the Ess1 Prolyl Isomerase in the RNA Polymerase II Transcription Cycle Mol Cell Biol 32(17): 3594–3607.

Mandal, S.S., Cho, H., Kim, S., Cabane, K., Reinberg, D. (2002). FCP1, a phosphatase specific for the heptapeptide repeat of the largest subunit of RNA polymerase II, stimulates transcription elongation. Mol Cell Biol 22, 7543-7552.

Mapendano, C.K., Lykke-Anderse, S., Kjems, J., Bertrand, E., Jensen, T.H. (2010). Crosstalk between mRNA 3' end processing and transcription initiation. Mol Cell 40(3): 410-22

Mason, P.B., and Struhl, K. (2005). Distinction and relationship between elongation rate and processivity of RNA polymerase II in vivo. Mol cell 17, 831-840.

Mayer, A.,Lidschreiber, M., Siebert, M., Leike, K., Söding, J., Cramer, P. (2010). Uniform transitions of the general RNA polymerase II transcription complex. Nat Struct Mol Biol 17(10):1272-8

Mayer, A., Heidemann, M., Lidschreiber, M., Schreieck, A., Sun, M., Hintermair, C., Kremmer, E., Eick, D., Cramer, P. (2012). CTD tyrosine phosphorylation impairs termination factor recruitment to RNA polymerase II. Science 336, 1723- 1725.

Mayfield, J.E., Fan, S., Wei, S., Zhang, M., Li, B., Ellington, A.D., Etzkorn, F.A., Zhang, Y.J. (2015). Chemical Tools To Decipher Regulation of Phosphatases by Proline Isomerization on Eukaryotic RNA Polymerase II. ACS chemical biology

McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S.D., Wickens, M., Bentley, D.L. (1997a). The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. Nature 385, 357-361.

McCracken, S., Fong, N., Rosonina, E., Yankulov, K., Brothers, G., Siderovski, D., Hessel, A., Foster, S., Shuman, S., Bentley, D.L. (1997b). 5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. Genes Dev 11: 3306-3318.

McKune, K., Richards, K.L., Edwards, A.M., Young, R.A., Woychik, N.A. (1993). RPB7, one of two dissociable subunits of yeast RNA polymerase II, is essential for cell viability. Yeast 9:295–299.

Medler, S., Al Husini, N., Raghunayakula, S., Mukundan, B., Aldea, A., Ansari, A. (2011). Evidence for a complex of transcription factor IIB with poly(A) polymerase and cleavage factor 1 subunits required for gene looping. J Biol Chem 30;286(39):33709-18.

Medler, S. and Ansari, A. (2015). Gene looping facilitates TFIIH kinase-mediated termination of transcription. Sci Rep 5:12586.

Meinhart, A., Silberzahn, T., Cramer, P. (2003). The mRNA transcription/processing factor Ssu72 is a potential tyrosine phosphatase. J Biol Chem. 278: 15917-15921.

Miller, C., Schwalb, B., Maier, K., Schulz, D., Dümcke, S., Zacher, B., Mayer, A., Sydow, J., Marcinowski, L., Dölke, L., et al. (2011). Dynamic transcriptome analysis measures rates of mRNA synthesis and decay in yeast. Mol Syst Biol 7: 458

Mirón-García, M.C., Carrido-Godina, A.I., García-Molinero, V., Hernández-Torres, F., Rodríguez-Navarro, S., Navarro, F. (2013). The Prefoldin Bud27 Mediates the Assembly of the Eukaryotic RNA Polymerases in an Rpb5-Dependent Manner. Plos Genet 9(2): e1003297.

Mitsuzawa, H., Kanda, E., Ishihama, A. (2003). Rpb7 subunit of RNA polymerase II interacts with an RNA-binding protein involved in processing of transcripts. Nucleic Acids Res 31(16): 4696–4701.

Miyao, T., Barnett, J.D., Woychik, N.A. (2001). Deletion of the RNA polymerase subunit RPB4 acts as a global, not stress-specific, shut-off switch for RNA polymerase II transcription at high temperatures. J Biol Chem 276:46408–46413.

Moabbi, A.M., Agarwal, N., El Kaderi, B., Ansari, A. (2012). Role for gene looping in intron-mediated enhancement of transcription. Proc Natl Acad Sci U S A 109(22): 8505–8510.

Mosley, A.L., Pattenden, S.G., Carey, M., Venkatesh, S., Gilmore, J.M., Florens, L., Workman, J.L., Washburn, M.P. (2009). Rtr1 is a CTD phosphatase that regulates RNA polymerase II during the transition from serine 5 to serine 2 phosphorylation. Mol Cell 34, 168-178.

Mosley, A.L., Hunter, G.O., Sardiu, M.E., Smolle, M., Workman, J.L., Florens, L., Washburn, M.P. (2013) Quantitative proteomics demonstrates that the RNA polymerase II subunits Rpb4 and Rpb7 dissociate during transcriptional elongation. Mol Cell Proteom 12:1530–1538.

Moteki, S., and Price, D. (2002). Functional coupling of capping and transcription of mRNA. Mol Cell 10(3):599-609.

Mukundan, B. and Ansari, A. (2013) Srb5/Med18-mediated termination of transcription is dependent on gene looping. J Biol Chem 288(16):11384-94.

Muñoz, M.J., de la Mata, M., Kornblihtt, A.R. (2010). The carboxy terminal domain of RNA polymerase II and alternative splicing. Trends in biochemical sciences 35, 497-504.

Murakami, K., Elmlund, H., Kalisman, N., Bushnell, D.A., Adams, C.M., Azubel, M., Elmlund, D., Levi-Kalisman, Y., Liu, X., Gibbons, B.J., Levitt, M., Kornber, R.D. (2013). Architecture of an RNA polymerase II transcription pre-initiation complex. Science 342(6159):1238724.

Murray, S., Udupa, R., Yao, S., Hartzog, G., Prelich, G. (2001). Phosphorylation of the RNA polymerase II carboxy-terminal domain by the Bur1 cyclin-dependent kinase. Mol Cell Biol 21, 4089-4096.

Myers, L. C., Gustafsson, C.M., Lui, M., Erdjument-Bromage, H., Tempst, P., Kornberg, R.D. (1998) The Med proteins of yeast and their function through the RNA polymerase II carboxy- terminal domain. Genes Dev 12, 45–54.

Naji, S., Grunberg, S., Thomm, M. (2007). The RPB7 orthologue E' is required for transcriptional activity of a reconstituted archaeal core enzyme at low temperatures and stimulates open complex formation. J Biol Chem 282: 11047–11057.

Nechaev, S., and Adelman, K. (2011). Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms 1809, 34-45.

Nedea, E., He, X., Kim, M., Prootoolal, J., Zhong, G., Canadien, V., Hughes, T., Buratowski, S., Moore, C.L., Greenblatt, J. (2003). Organization and function of APT, a subcomplex of the yeast cleavage and polyadenylation factor involved in the formation of mRNA and small nucleolar RNA 3′-ends. J. Biol. Chem 278:33000–33010.

Nedea, E., Nalbant, D., Xia, D., Theoharis, N.T., Suter, B., Richardson, C.J., Tatchell, K., Greenblatt, J.F., Nagy, P.L. (2008). The Glc7 phosphatase subunit of the cleavage and polyadenylation factor is essential for transcription termination on snoRNA genes. Mol Cell 29:577–587.

Neil, H., Malabat, C, d'Aubenton-Carafa, Y, Xu, Z, Steinmetz, LM, Jacquier, A. (2009). Widespread bidirectional promoters are the major source of cryptic transcripts in yeast. Nature 457:1038–1042.

Nelson, C., Goto, S., Lund, K., Hung, W., Sadowski, I. (2003). Srb10/Cdk8 regulates yeast filamentous growth by phosphorylating the transcription factor Ste12. Nature 421, 187-190.

Ng, H.H., Robert, F., Young, R.A., Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol Cell 11, 709-719.

Ni, Z., Schwartz, B.E., Werner, J., Suarez, J.R., Lis, J.T. (2004). Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. Mol Cell 13, 55-65.

Ni, Z., Olsen, J.B., Guo, X., Zhong, G., Ruan, E.D., Marcon, E., Young, P., Guo, H., Li, J., Moffat, J., Emili, A., Greenblat, J.F. (2011). Control of the RNA polymerase II phosphorylation state in promoter regions by CTD interaction domain-containing proteins RPRD1A and RPRD1B. Transcription 2, 237-242.

Nonet, M., Scafe, C., Sexton, J., Young, R. (1987). Eukaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol Cell Biol 7:1602–1611

Nonet, M., Sweetser, D., Young, R.A. (1987). Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. Cell 50:909–915.

Ohler, U., Liao, G.C., Niemann, H., Rubin, G.M. (2002). Computational analysis of core promoters in the Drosophila genome. Genome Biol 3(12): RESEARCH0087

O'Reilly, D., and Greaves, D.R. (2007). Cell-type-specific expression of the human CD68 gene is associated with changes in Pol II phosphorylation and short-range intrachromosomal gene looping. Genomics 90(3):407-15.

Orlicky, S.M., Tran, P.T., Sayre, M.H., Edwards, A.M. (2001). Dissociable Rpb4-Rpb7 subassembly of RNA polymerase II binds to single-strand nucleic acid and mediates a post-recruitment step in transcription initiation. J Biol Chem 276:10097–10102.

Ostapenko, D., and Solomon, M.J. (2003). Budding yeast CTDK-I is required for DNA damage-induced transcription. Eukaryot Cell 2, 274-283.

O'Sullivan, J.M., Tan-Wong, S.M., Morillon, A., Lee, B., Coles, J., Mellor, J., Proudfoot, N.J. (2004). Gene loops juxtapose promoters and terminators in yeast. Nat Genet 36, 1014-1018.

Pal, M., Ponticelli, A.S., Luse, D.S. (2005). The role of the transcription bubble and TFIIB in promoter clearance by RNA polymerase II. Mol Cell 19, 101-110.

Palancade, B., and Bensaude, O. (2003). Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation. Eur J Biochem 270, 3859-3870.

Pappas, D.L. Jr. and Hampsey, M. (2000) Functional interaction between Ssu72 and the Rpb2 subunit of RNA polymerase II in Saccharomyces cerevisiae. Mol Cell Biol 20(22):8343-51.

Patturajan, M., Schulte, R.J., Sefton, B.M., Berezney, R., Vincent, M., Bensaude, O., Warren, S.L., Corden, J.L. (1998). Growth-related changes in phosphorylation of yeast RNA polymerase II. J Biol Chem 273, 4689-4694.

Payne, J.M., Laybourn, P.J., Dahmus, M.E. (1989) The transition of RNA polymerase II from initiation to elongation is associated with phosphotylation od the carboxy-terminal domain of subunit IIa. J Biol Chem. 25;264(33):19621-9.

Perales, R., and Bentley, D. (2009). "Cotranscriptionality": the transcription elongation complex as a nexus for nuclear transactions. Mol Cell 36, 178-191.

Perkins, K.J., Lusic, M., Mitar, I., Giacca, M., Proudfoot, N.J. (2008). Transcription-dependent gene looping of the HIV-1 provirus is dictated by recognition of pre-mRNA processing signals. Mol Cell 29:56–68.

Phatnani, H.P., and Greenleaf, A.L. (2006). Phosphorylation and functions of the RNA polymerase II CTD. Genes Dev 20, 2922-2936.

Pillai, B., Sampath, V., Sharma, N., Sadhale, P. (2001). Rpb4, a non-essential subunit of core RNA polymerase II of Saccharomyces cerevisiae is important for activated transcription of a subset of genes. J Biol Chem 276:30641–30647.

Pillai, B., Verma, J., Abraham, A., Francis, P., Kumar, Y., Tatu, U., Brahmachari, S.K., Sadhale, P.P. (2003). Whole genome expression profiles of yeast RNA polymerase II core subunit, Rpb4, in stress and nonstress conditions. J Biol Chem 278(5):3339-46.

Pinto, I., Wu, W.H., Na, J.G., Hampsey, M. (1994). Characterization of *sua7* mutations defines a domain of TFIIB involved in transcription start site selection in yeast. J Biol Chem 269:30569–30573.

Pokholok, D.K., Hannett, N.M., Young, R.A. (2002) Exchange of RNA polymerase II initiation and elongation factors during gene expression in vivo. Mol Cell 9: 799-809.

Porrua, O., and Libri, D. (2015). Transcription termination and the control of the transcriptome: why, where and how to stop. Nat Rev Mol Cell Biol 16(3):190-202.

Powell, W., and Reines, D. (1996). Mutations in the Second Largest Subunit of RNA Polymerase II Cause 6-Azauracil Sensitivity in Yeast and Increased Transcriptional Arrest *in Vitro* J Biol Chem 271(12): 6866–6873.

Preker, P., Nielsen, J., Kammler, S., Lykke-Andersen, S., Christensen, M.S., Mapendano, C.K., Schierup, M.H., Jensen, T.H. (2008). RNA exosome depletion reveals transcription upstream of active human promoters. Science 322:1851–1854.

Prelich, G. (2002). RNA polymerase II carboxy-terminal domain kinases: emerging clues to their function. Eukaryot Cell 1, 153-162.

Proudfoot, N.J., Furger, A., Dye, M.J. (2002). Integrating mRNA processing with transcription. Cell 108, 501-512.

Qiu, H., Hu, C., Hinnebusch. A.G. **(**2009). Phosphorylation of the Pol II CTD by KIN28 enhances BUR1/BUR2 recruitment and Ser2 CTD phos- phorylation near promoters. Mol Cell 33:752–762.

Ramanathan, Y., Rajpara, S.M., Reza, S.M., Lees, E., Shuman, S., Mathews, M.B., Pe'ery, T. (2001). Three RNA polymerase II carboxyl-terminal domain kinases display distinct substrate preferences. J Biol Chem 276, 10913-10920.

Ranish, J.A., Yudkovsky, N., Hahn, S. (1999). Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. Genes Dev 13:49–63.

Ranuncolo, S.M., Ghosh, S., Hanover, J.A., Hart, G.W., Lewis, B.A. (2012). Evidence of the involvement of O-GlcNAc-modified human RNA polymerase II CTD in transcription in vitro and in vivo. J Biol Chem 287, 23549-23561.

Rasmussen, E.B., and Lis, J.T. (1993). In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. Proc Natl Acad Sci USA 90(17):7923-7.

Reyes-Reyes, M. and Hampsey, M. (2007) Role for the Ssu72 C-terminal domain phosphatase in RNA polymerase II transcription elongation. Mol Cell Biol 27(3):926-36.

Rhee, H.S., and Pugh, B.F. (2012). Genome-wide structure and organization of eukaryotic pre-initiation complexes. Nature 483(7389): 295-301.

Rodriguez, C.R., Cho, E.J., Keogh, M.C., Moore, C.L., Greenleaf, A.L., Buratowski, S. (2000). Kin28, the TFIIH-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II. Mol Cell Biol 20, 104-112.

Rosado-Lugo, J.D. and Hampsey, M. (2014). The Ssu72 phosphatase mediates the RNA polymerase II initiation-elongation transition. J Biol Chem 289(49): 33916-26.

Rosenheck, S., and Choder, M. (1998). Rpb4, a subunit of RNA polymerase II, enables the enzyme to transcribe at temperature extremes *in vitro*. J Bacteriol 180, 6187–6192.

Rother, S., and Strasser, K. (2007). The RNA polymerase II CTD kinase Ctk1 functions in translation elongation. Genes Dev 21, 1409-1421.

Ruprich-Robert, G., and Thuriaux, P. (2010). Non-canonical DNA transcription enzymes and the conservation of two-barrel RNA polymerases. Nucleic Acids Res 38, 4559-4569.

Runner, V.M., Podolny, V., Buratowski, S. (2008) The Rpb4 subunit of RNA polymerase II contributes to cotranscriptional recruitment of 3' processing factors. Mol Cell Biol 28:1883–1891.

Sainsbury, S., Niesser, J., Cramer., P. (2013). Structure and function of the initially transcribing RNA polymerase II-TFIIB complex. Nature 493(7432):437-40.

Sampath, V., Rekha, N., Srinivasan, N., Sadhale,P. (2003). The conserved and non-conserved regions of Rpb4 are involved in multiple phenotypes in Saccharomyces cerevisiae. J Biol Chem 278:51566–51576.

Sampath, V., Balakrishnan, B., Verma-Gaur, J., Onesti, S., Sadhale, P.P. (2008). Unstructured N terminus of the RNA polymerase II subunit Rpb4 contributes to the interaction of Rpb4.Rpb7 subcomplex with the core RNA polymerase II of Saccharomyces cerevisiae. J Biol Chem 283(7):3923-31.

Saunders, A., Core, L.J., Lis, J.T. (2006). Breaking barriers to transcription elongation. Nat Rev Mol Cell Biol 7, 557-567.

Scafe, C., Chao, D., Lopes, J., Hirsch, J.P., Henry, S., Young, R.A. (1990). RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. Nature 347:491–4.

Sheffer, A., Varon, M., Choder, M. (1999). Rpb7 can interact with RNA polymerase II and support transcription during some stresses independently of Rpb4. Mol Cell Biol 19: 2672-2680.

Schmitt, M.E., Brown, T.A., and Trumpower, B.L. (1990). A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res 18: 3091-3092.

Schreieck, A., Easter, A.D., Etzold, S., Wiederhold, K., Lidshreiber, M., Cramer, P., Passmore, L.A. (2014). RNA polymerase II termination involves C-terminal-domain tyrosine dephosphorylation by CPF subunit Glc7. Nat Struct Mol Biol 21(2):175-9.

Schroeder, S.C., Schwer, B., Shuman, S., Bentley, D. (2000). Dynamic association of capping enzymes with transcribing RNA polymerase II. Genes Dev 14, 2435-2440.

Schüller, R., Forné, I., Straub, T., Schreieck, A., Texier, Y., Shah, N., Decker, T.M., Cramer, P., Imhof, A., Eick, D. (2016). Heptad-Specific Phosphorylation of RNA Polymerase II CTD. Mol Cell 61(2):305-14.

Schulz, D., Schwalb, B., Kiesel, A., Baejen, C., Torkler, P., Gagneur, J., Soeding, J., Cramer, P. (2013). Transcriptome surveillance by selective termination of noncoding RNA synthesis. Cell 155:1075–1087.

Schulz, D., Pirkl, N., Lehmann, E., Cramer, P. (2014). Rpb4 subunit functions mainly in mRNA synthesis by RNA polymerase II. J Biol Chem 289(25):17446-52.

Schwartz, L.B., and Roeder, R.G. (1975). Purification and subunit structure of deoxyribonucleic acid-dependent ribonucleic acid polymerase II from the mouse plasmacytoma, MOPC 315. J Biol Chem 250, 3221-3228.

Schwer, B., and Shuman, S. (2011) Deciphering the RNA polymerase II CTD code in fission yeast. Mol Cell 43:311–8.

Seila A.C., Calabrese, J.M., Levine, S.S., Yeo, G.W., Rahl, P.B., Flynn, R.A., Young, R.A., Sharp, P.A.(2008). Divergent transcription from active promoters. Science 322:1849-1851.

Selitrennik, M., Duek, L., Lotan, R., Choder, M. (2006). Nucleocytoplasmic shuttling of the Rpb4p and Rpb7p subunits of Saccharomyces cerevisiae RNA polymerase II by two pathways. Eukaryot Cell *5*, 2092-2103.

Shalem, O., Groisman, B., Choder, M., Dahan, O., Pilpel, Y. (2011). Transcriptome kinetics is governed by a genome-wide coupling of mRNA production and degradation: a role for RNA Pol II. PLoS Genet *7*, e1002273.

Shandilya, J., and Roberts, S.G. (2012). The transcription cycle in eukaryotes: From productive initiation to RNA polymerase II recycling. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms 1819, 391-400.

Sharma, A., and Kumari, R. (2013). Rpb4 and Rpb7: multifunctional subunits of RNA polymerase II. Crit Rev Microbiol 39(4):362-72.

Sharma, N., and Sadhale, P.P. (1999). Overexpression of the gene for Rpb7 subunit of yeast RNA polymerase II rescues the phenotypes associated with absence of the largest, nonessential subunit Rpb4. J Genet 78, 149–156.

Sheffer, A., Varon, M., Choder, M. (1999). Rpb7 can interact with RNA polymerase II and support transcription during some stresses independently of Rpb4. Mol Cell Biol 19, 2672–2680.

Sikorski, T.W., and Buratowski, S. (2009) The basal initiation machinery: beyond the general transcription factors. Curr Opin Cell Biol 21: 344-351.

Simon, M., Seraphin, B., Faye, G. (1986). KIN28, a yeast split gene coding for a putative protein kinase homologous to CDC28. EMBO J 5, 2697-2701.

Singh, S.R., Pillai, B., Balakrishnan, B., Naorem, A., Sadhale, P.P. (2007). Relative levels of RNA polII subunits differentially affect starvation response in budding yeast. Biochem Biophys Res Commun 356(1):266-72.

Singh, B.N., and Hampsey, M. (2007). A transcription-independent role for TFIIB in gene looping. Mol Cell 27, 806-816.

Singh, B.N., Ansari, A., Hampsey, M. (2009). Detection of gene loops by 3C in yeast. Methods 48(4): 361-367

Singh, N., Ma, Z., Gemmill, T., Wu, X., Defiglio, H., Rossettini, A., Rabeler, C., Beane, O., Morse, R.H., Palumbo, M.J., Hanes, S.D. (2009). The Ess1 prolyl isomerase is required for transcription termination of small noncoding RNAs via the Nrd1 pathway. Mol cell 36:255–266.

Skaar, D.A., and Greenleaf, A.L. (2002). The RNA polymerase II CTD kinase CTDK-I affects pre-mRNA 3' cleavage/polyadenylation through the processing component Pti1p. Mol Cell 10, 1429-1439.

Smith, E., and Shilatifard, A. (2010) The chromatin signaling pathway: diverse mechanisms of recruitment of histone-modifying enzymes and varied biological outcomes. Mol Cell 10;40(5):689-701.

Spitalny, P., and Thomm, M. (2008). A polymerase III-like reinitiation mechanism is operating in regulation of histone expression in archaea. Mol Microbiol 67:958-70

Srivastava, R., and Ahn, S.H. (2015). Modifications of RNA polymerase II CTD: Connections to the histone code and cellular function. Biotech Adv 33, 856-872.

Steinmetz, E.J., and Brow, D.A. (1996). Repression of gene expression by an exogenous sequence element acting in concert with a heterogeneous nuclear ribonucleoprotein-like protein, Nrd1, and the putative helicase Sen1. Mol Cell Biol 16, 6993-7003.

Steinmetz, E.J., Conrad, N.K., Brow, D.A., Corden, J.L. (2001). RNA-binding protein Nrd1 directs poly(A)-independent 3′-end formation of RNA polymerase II transcripts. Nature 413: 327–331.

Steinmetz, E.J., and Brow, D.A. (2003). Ssu72 protein mediates both poly(A)-coupled and poly(A)-independent termination of RNA polymerase II transcription. Mol Cell Biol 23, 6339-6349.

Steinmetz E.J., Ng S.B., Cloute J.P., Brow D.A. (2006) cis- and trans-Acting determinants of transcription termination by yeast RNA polymerase II. Mol Cell Biol 26:2688-96

Stock, J.K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A.G., Pombo, A. (2007) Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat Cell Biol 9(12):1428-35.

Stiller, J.W., McConaughy, B.L., Hall, B.D. Evolutionary complementation for polymerase II CTD function. Yeast 2000;16:57–64

Suh, H., Ficarro, S.B., Kang, U.B., Chun, Y., Marto, J.A., Buratowski, S. (2016). Direct Analysis of Phosphorylation Sites on the Rpb1 C-Terminal Domain of RNA Polymerase II. Mol Cell 61(2):297-304

Sun, Z.W., and Hampsey, M. (1996). Synthetic enhancement of a TFIIB defect by a mutation in SSU72, an essential yeast gene encoding a novel protein that affects transcription start site selection in vivo. Mol Cell Biol 16, 1557-1566.

Sun, M., Schwalb, B., Schulz, D., Pirki, N., Etzold, S., Larivière, L., Maier, K.C., Seizl, M., Tresch, A., Cramer, P. (2012). Comparative dynamic transcriptome analysis (cDTA) reveals mutual feedback between mRNA synthesis and degradation. Genome Res 22:1350-1359.

Sun, M., Schwalb, B., Pirkl, N., Maier, K.C., Schenk, A., Failmezger, H., Tresh, A., Cramer, P. (2013). Global analysis of eukaryotic mRNA degradation reveals Xrn1-dependent buffering of transcript levels. Mol Cell 52(1): 52-62.

Tan, Q., Prysak, M.H., Woychik, N.A. (2003). Loss of the Rpb4/Rpb7 subcomplex in a mutant form of the Rpb6 subunit shared by RNA polymerases I, II, and III. Mol Cell Biol (9):3329-38.

Tanny, J.C. (2014). Chromatin modification by the RNA Polymerase II elongation complex. Transcription 5(5): e988093.

Tan-Wong, S.M., French, J.D., Proudfoot, N.J., Brown, M.A. (2008). Dynamic interactions between the promoter and terminator regions of the mammalian BRCA1 gene. Proc Natl Acad Sci 105:5160–5165.

Tan-Wong, S.M., Wijayatilake, H.D., Proudfoot**,** N.J. (2009). Gene loops function to maintain transcriptional memory through interaction with the nuclear pore complex Genes Dev 23(22): 2610–2624.

Tan-Wong, S.M., Zaugg, J.B., Camblong, J., Xu, Z., Zhang, D.W., Mischo, H.E., Ansari, A.Z., Luscombe, N.M., Steinmetz, L.M., Proudfoot, N.J. (2012). Gene loops enhance transcriptional directionality. Science 338:671-675.

Thomas, B.J., and Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. Cell 56: 619-630

Thomas, M.C., and Chiang, C.M. (2006) The general transcription machinery and general cofactors. Crit Rev Biochem Mol Biol 41: 105-178.

Thompson, N.E., Aronson, D.B., Burgess, R.R. (1990) Purification of eukaryotic RNA polymerase II by immunoaffinity chromatography. Elution of active enzyme with

protein stabilizing agents from a polyol-responsive monoclonal antibody. J Biol Chem 265:7069–7077.

Tietjen, J.R., Zhang, D.W., Rodriguez-Molina, J.B., White, B.E., Akhtar, M.S., Heidemann, M., Li, X., Chapman, R.D., Shokat, K., Keles, S., Eick, D., Ansari, A.Z. (2010). Chemical-genomic dissection of the CTD code. Nat Struct Mol Biol 17, 1154-1161.

Tombácz, I., Schauer, T., Juhasz, I., Komonyi, O., Boros, I. (2009) The RNA Pol II CTD phosphatase Fcp1 is essential for normal development in Drosophila melanogaster. Gene 446:58–67.

Trinklein, N.D., Aldred, S.F., Hartman, S.J., Schroeder, D.I., Otillar, R.P., Myers, R.M. (2004). An abundance of bidirectional promoters in the human genome. Genome Res 14:62–66.

Turowski, T.W. (2013). The impact of transcription on posttranscriptional processes in yeast. Gene 526(1): 23-9.

Ujvari, A., and Luse, D.S. (2006) RNA emerging from the active site of RNA polymerase II interacts with the Rpb7 subunit. Nat. Struct Mol Biol 13: 49–54.

Ursic, D., Chinchilla, K., Finkel, J.S., Culbertson, M.R. (2004). Multiple protein/protein and protein/RNA interactions suggest roles for yeast DNA/RNA helicase Sen1p in transcription, transcription-coupled DNA repair and RNA processing. Nucleic Acids Res 32: 2441–2452.

Van Dijk, E., Chen, C., d'Aubenton-Carafa, Y., Gourvennec, S., Kwapisz, M., Roche, V., Bertrand, C., Silvain, M., Legoix-Ne, P., and Loeillet, S. (2011). XUTs are a class of Xrn1-sensitive antisense regulatory non- coding RNA in yeast. Nature 475: 114-117.

Vannini, A., and Cramer, P. (2012). Conservation between the RNA polymerase I, II, and III transcription initiation machineries. Mol Cell 45(5): 439-46.

Vasiljeva, L., and Buratowski, S. (2006). Nrd1 interacts with the nuclear exosome for 3′ processing of RNA polymerase II transcripts. Mol Cell 21: 239–248.

Venters, B.J., and Pugh, B.F. (2013). Genomic organization of human transcription initiation complexes. Nature 502(7469):53-8.

Verma-Gaur, J., Rao, S.N., Taya, T., Sadhale, P. (2008) Genomewide recruitment analysis of Rpb4, a subunit of polymerase II in Saccharomyces cerevisiae, reveals its involvement in transcription elongation. Eukaryot Cell 7(6):1009-18.

Vincent, O., Kuchin, S., Hong, S.P., Townley, R., Vyas, V.K., Carlson, M. (2001). Interaction of the Srb10 kinase with Sip4, a transcriptional activator of gluconeogenic genes in Saccharomyces cerevisiae. Mol Cell Biol 21, 5790-5796.

Visconti, R., Palazzo, L., Della Monica, R., Grieco, D. (2012) Fcp1-dependent dephosphorylation is required for M-phase-promoting factor inactivation at mitosis exit. Nat Commun 12;3:894.

Voss, K., Forné, I., Descostes, N., Hintermair, C., Schüller, R., Maqbool, M.A., Heidemann, M., Flatley, A., Imhof, A., Gut, M., Gut, I., Kremmer, E., Andrau, J.C., Eick, D. (2015). Site-specific methylation and acetylation of lysine residues in the C-terminal domain (CTD) of RNA polymerase II. Transcription 6(5):91-101.

Wada, T., Takagi, T., Yamaguchi, Y., Watanabe, D., and Handa, H. (1998). Evidence that P-TEFb alleviates the negative effect of DSIF on RNA polymerase II- dependent transcription in vitro. EMBO J 17, 7395-7403.

Wang, W., Carey, M., Gralla, J.D. (1992). Polymerase II promoter activation: closed complex formation and ATP-driven start site opening. Science 255, 450- 453.

Wang, Q., Wan, L., Li, D., Zhu, L., Qian, M., Deng, M. (2009). Searching for bidirectional promoters in *Arabidopsis thaliana*. BMC Bioinform 10 (Suppl.1):S29.

Werner, F. (2008). Structural evolution of multisubunit RNA polymerases. Trends in microbiology 16, 247-250.

Werner, F., and Grohmann, D. (2011). Evolution of multisubunit RNA polymerases in the three domains of life. Nature Reviews Microbiology 9, 85-98.

Westover, K.D., Bushnell, D.A., Kornberg, R.D. (2004). Structural basis of transcription: separation of RNA from DNA by RNA polymerase II. Science 303, 1014-1016.

Wintzerith, M., Acker, J., Vicaire, S., Vigneron, M., Kedinger, C. (1992). Complete sequence of the human RNA polymerase II largest subunit. Nucleic Acids Res 20, 910.

Wong, C.M., Qiu, H., Hu, C., Dong, J., Hinnebusch, A.G. (2007). Yeast cap binding complex impedes recruitment of cleavage factor IA to weak termination sites. Mol Cell Biol 27, 6520-6531.

Wong, K. H., Jin, Y., Struhl, K. (2014) TFIIH phosphorylation of the Pol II CTD stimulates mediator dissociation from the preinitiation complex and promoter escape. Mol Cell 54, 601–612

Woychik, N.A., Lane, W.S., Young, R.A. (1991). Yeast RNA polymerase II subunit RPB9 is essential for growth at temperature extremes. J Biol Chem, 266, 19053–19055.

Woychik, N.A., and Young, R.A. (1989). RNA polymerase II subunit RPB4 is essential for high- and low-temperature yeast cell growth. Mol Cell Biol 9, 2854–2859.

Woychik, N.A., and Young, R.A. (1990). RNA polymerase II: subunit structure and function. Trends in biochemical sciences 15, 347-351.

Woychik, N.A., Liao, S.M., Kolodziej, P.A., Young, R.A. (1990). Subunits shared by eukaryotic nuclear RNA polymerases. Genes Dev 4, 313-323.

Wu, X., Wilcox, C.B., Devasahayam, G., Hackett, R.L., Arevalo-Rodriguez, M., Cardenas, M.E., Heitman, J., Hanes, S.D. (2000). The Ess1 prolyl isomerase is linked

to chromatin remodeling complexes and the general transcription machinery. EMBO J 19, 3727-3738.

Wu, W.H., Pinto, I., Chen, B.S., Hampsey, M. (1999). Mutational analysis of yeast TFIIB. A functional relationship between Ssu72 and Sub1/Tsp1 defined by allele-specific interactions with TFIIB. Genetics 153, 643-652.

Xiao, T., Shibata, Y., Rao, B., Laribee, R.N., O'Rourke, R., Buck, M.J., Greenblatt, J.F., Krogan, N.J., Lieb, J.D., Strahl, B.D. (2007). The RNA polymerase II kinase Ctk1 regulates positioning of a 5' histone methylation boundary along genes. Mol Cell Biol 27, 721-731.

Xiang, K., Nagaike, T., Xiang, S., Kilic, T., Beh, M.M., Manley, J.L., Tong, L. (2010). Crystal structure of the human symplekin-Ssu72-CTD phosphopeptide complex. Nature 467, 729-733.

Xiang, K., Manley, J.L., Tong, L. (2012). The yeast regulator of transcription protein Rtr1 lacks an active site and phosphatase activity. Nat Commun 3, 946.

Xu, Y.X., Hirose, Y., Zhou, X.Z., Lu, K.P., and Manley, J.L. (2003). Pin1 modulates the structure and function of human RNA polymerase II. Genes Dev 17, 2765- 2776.

Xu, Y.X., and Manley, J.L. (2007). Pin1 modulates RNA polymerase II activity during the transcription cycle. Genes Dev 21, 2950-2962.

Xu, Z., Wei, W., Gagneur, J., Perocchi, F., Clauder-Münster, S., Camblong, J., Guffanti, E., Stutz, F., Huber, W., and Steinmetz, L.M. (2009). Bidirectional promoters generate pervasive transcription in yeast. Nature 457: 1033-1037.

Yang, C., Bolotin, E., Jiang, T., Sladek, F.M., Martinez, E. (2007). Prevalence of the initiator over the TATA box in human and yeast genes and identification of DNA motifs enriched in human TATA-less core promoters. Gene 389(1):52-65.

Yao, S., and Prelich, G. (2002). Activation of the Bur1-Bur2 cyclin-dependent kinase complex by Cak1. Mol Cell Biol 22, 6750-6758.

Young, R.A. (1991). RNA polymerase II. Annu Rev Biochem 60, 689-715.

Yudkovsky, N., Ranish, J.A., Hahn, S. (2000) A transcription reinitiation intermediate that is stabilized by activator. Nature 408, 225–229.

Yurko, N.M., and Manley, J.L. (2017) The RNA polymerase II CTD “orphan” residues: Emerging insights into the functions of Tyr-1, Thr-4, and Ser-7. Transcription 3:1-11.

Zanton, S.J. and Pugh, B.F. (2006) Full and partial genome-wide assembly and disassembly of the yeast transcription machinery in response to heat shock. Genes Dev 20(16):2250-65.

Zhang, J., and Corden, J.L. (1991) Phosphorylation causes a conformational change in the carboxyl-terminal domain of the mouse RNA polymerase II largest subunit. J Biol Chem 266: 2297-2302.

Zhang, D.W., Mosley, A.L., Ramisetty, S.R., Rodriguez-Molina, J.B., Washburn, M.P., Ansari, A.Z. (2012). Ssu72 phosphatase-dependent erasure of phospho-Ser7 marks on the RNA polymerase II C-terminal domain is essential for viability and transcription termination. J Biol Chem 287, 8541-8551.

Zhao, D.Y., Gish, G., Braunschweig, U., Li, Y, Ni, Z., Schmitges, F.W., Zhong, G., Liu, K., Li, W., Moffat, J., Vedadi, M., Min, J., Pawson, T.J., Blencowe, B.J., Greenblatt, J.F. (2016). SMN and symmetric arginine dimethylation of RNA polymerase II C-terminal domain control termination. Nature 529(7584):48-53.

Zhou, K., Kuo, W.H., Fillingham, J., Greenblatt, J.F. (2009). Control of transcriptional elongation and cotranscriptional histone modification by the yeast BUR kinase substrate Spt5. Proc Natl Acad Sci U S A 106, 6956-6961.

Anexos

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Rpb4/7 facilitates RNA polymerase II CTD dephosphorylation

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ABSTRACT

The Rpb4 and Rpb7 subunits of eukaryotic RNA polymerase II (RNAPII) participate in a variety of processes from transcription, DNA repair, mRNA export and decay, to translation regulation and stress response. However, their mechanism(s) of action remains unclear. Here, we show that the Rpb4/7 heterodimer in *Saccharomyces cerevisiae* plays a key role in controlling phosphorylation of the carboxy terminal domain (CTD) of the Rpb1 subunit of RNAPII. Proper phosphorylation of the CTD is critical for the synthesis and processing of RNAPII transcripts. Deletion of *RPB4*, and mutations that disrupt the integrity of Rpb4/7 or its recruitment to the RNAPII complex, increased phosphorylation of Ser2, Ser5, Ser7 and Thr4 within the CTD. *RPB4* interacted genetically with genes encoding CTD phosphatases (*SSU72*, *FCP1*), CTD kinases (*KIN28*, *CTK1*, *SRB10*) and a prolyl isomerase that targets the CTD (*ESS1*). We show that Rpb4 is important for Ssu72 and Fcp1 phosphatases association, recruitment and/or accessibility to the CTD, and that this correlates strongly with Ser5P and Ser2P levels, respectively. Our data also suggest that Fcp1 is the Thr4P phosphatase in yeast. Based on these and other results, we suggest a model in which Rpb4/7 helps recruit and potentially stimulate the activity of CTD-modifying enzymes, a role that is central to RNAPII function.

INTRODUCTION

Eukaryotic RNA polymerase II (RNAPII) contains 12 subunits, Rpb1 to Rpb12, that in *Saccharomyces cerevisiae* dissociates into a 10-subunit core and a Rpb4/Rpb7 heterodimer (1). Rpb4 and Rpb7 are conserved from yeast to humans, and orthologs found in archaea also function in transcription (2). Rpb7, but not Rpb4, is essential for viability (3,4). Early studies suggested a role for the Rpb4/7 heterodimer in the transcriptional response to stress (3,5), however, it is now clear that Rpb4/7 participates in a broad range of activities under a variety of conditions (6,7)

In *S. cerevisiae*, the Rpb4/7 heterodimer is required for promoter-dependent transcription *in vitro*, is involved in elongation and termination (7) and is important for co-transcriptional recruitment of factors required for 3′-end formation of mRNA and snoRNA genes (8). The association of Rpb4/7 with elongating RNAPII may be transient and regulated by elongation factors (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, and once in the cytoplasm, stimulate translation initiation and subsequent deadenylation and mRNA decay (10–12). However, it has been argued that these cytoplasmic effects on gene expression are indirect, and occur in response to widespread defects in mRNA synthesis (13). So while Rpb4/7 is implicated in a number of important gene regulatory functions, its mechanism(s) of action remains unclear.

Structure studies reveal that the Rpb4/7 heterodimer forms a stalk-like protrusion extending from the main body of the RNAPII complex (14). Similar structures are found on eukaryotic RNA polymerases I and III, composed of subunits Rpa14/43 and Rpc17/25, respectively (15), and on archaeal RNA polymerase (subunits F/E) (2). Bacterial RNA polymerases do not contain the analogous structure and lack the homologous subunits. On the eukaryotic and archaeal polymerases, the stalk-like protrusion is positioned adjacent to the RNA exit channel, and in archaea, the F/E complex binds RNA and is important for elongation processivity (16). In the RNAPII complex, the Rpb4/7 heterodimer is also adjacent to the carboxy-terminal domain (CTD) of largest subunit, Rpb1. The proximity of Rpb4/7

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to both the nascent RNA and the CTD suggests it might play a role in the recruitment of factors important for RNA biogenesis and/or CTD modification. Indeed, Rpb7 can be cross-linked to the emerging RNA transcript in human cells (17), and in *Schizosaccharomyces pombe* Rpb7 interacts with Seb1, a termination factor homologous to Nrd1, which in budding yeast binds both the nascent RNA and the Ser5P form of the CTD (18,19). In *S. pombe* and *Drosophila melanogaster*, *in vitro* binding and yeast two-hybrid assays, respectively, showed that Rpb4 interacts with Fcp1, a Ser2P CTD phosphatase (20,21). Structural and biochemical studies suggested that in addition to direct recognition of the phospho-CTD substrate, *S. cerevisiae* Fcp1 might also interact with RNAPII through Rpb4/7 (22). These findings suggest the Rpb4/7 might recruit Fcp1 to regulate CTD modification.

The CTD of Rpb1 is unique to eukaryotes and is composed of a repeated heptapeptide motif with a consensus sequence of Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (23,24). The CTD can be phosphorylated at Ser2, Ser5 and Ser7, as well as Tyr1 and Thr4 (25–27), and in mammals, it can be acetylated (28), glycosylated (29,30) and methylated (31). In addition, the prolyl bonds within the CTD can be isomerized by the peptidyl prolyl *cis-trans* isomerase, Ess1 in yeast (Pin1 in humans) (32–34). These modifications are dynamic and collectively constitute a 'CTD code' (35), whereby the various forms of the CTD differentially recruit co-factors required for transcriptional efficiency and RNA processing (36,37).

CTD phosphorylation is regulated by the action of kinases and phosphatases (38). In *S. cerevisiae*, the CTD is phosphorylated by the cyclin-dependent kinases (CDKs), Srb10, Kin28, Ctk1 and Bur1 (27,39). Srb10 (mammalian Cdk8), a Ser2/Ser5 kinase, is part of the Mediator complex and inactivates RNAPII prior to pre-initiation complex formation (40), and, together with Kin28 (a Ser5/Ser7 kinase), facilitates transcription and formation of the scaffold complex (41). Kin28 (mammalian Cdk7), part of the TFIIH initiation complex (42), phosphorylates Ser5 to promote co-transcriptional 5′ mRNA capping (43–45), and in mammals also phosphorylates Ser7 during promoter-proximal pausing and perhaps during termination (46–48). Ctk1, the main Ser2 kinase in coding and 3′-end regions, is required for co-transcriptional recruitment of the polyadenylation machinery (49,50). In mammalian cells, Ser2P elongation marks are placed by Cdk9/CyclinT (P-TEFb) (51) and Cdk12 (52). Finally, Bur1/Bur2 phosphorylates Ser2 near promoters and stimulates Ser2 phosphorylation by Ctk1 to promote elongation (50,53). Bur1 seems also to place Ser7P marks later in the transcription cycle (54).

CTD dephosphorylation is carried out by four phosphatases: Rtr1, Ssu72, Glc7 and Fcp1 (38,55). Rtr1 works early in the CTD cycle and dephosphorylates Ser5P to promote the transition from Ser5P to Ser2P (56,57). Human Rtr1, RPAP2, is recruited to snRNA genes via Ser7P (58). Rtr1 lacks a recognizable catalytic domain and may play a non-catalytic role in CTD dephosphorylation (59). In yeast, Rtr1 has been proposed to be a Tyr1P phosphatase (60). Ssu72 and Glc7 are components of the 3′-end processing machinery; Ssu72 targets Ser5P and Ser7P for dephosphorylation (61–64), while Glc7 targets Tyr1P (55), a mark important to avoid premature recruitment of termination factors within gene bodies (65). Fcp1 is essential and is specific for Ser2P dephosphorylation *in vivo* and opposes Ctk1 (50) to ensure proper levels of Ser2P during elongation (50,61,66). Fcp1 dephosphorylation of Ser2P after termination is assisted prior to the action of Ssu72 (61). Additionally, in mammals, Fcp1 has been described as the Thr4P phosphatase (67).

The phosphorylation state of the CTD is also regulated by the Ess1/Pin1 prolyl isomerase. In yeast, Ess1 promotes dephosphorylation of Ser5P (and Ser7P) (34,63,68). It does so by providing a kinetic advantage to the binding and catalytic activity of Ssu72, which requires a *cis*-conformation of the CTD Ser5P-Pro6 bond (69). In mammalian cells, Pin1 promoted Ser2/Ser5 phosphorylation of the CTD via inhibition of Fcp1 and stimulation of Cdc2 kinase (70).

How co-factors are recruited to RNAPII to facilitate transcription and RNA processing remains poorly understood. Prior work suggested a role for Rpb4/7 in recruitment of co-factors important for RNA processing and/or modification of the CTD. Here, we sought to determine whether the Rpb4/7 heterodimer influences CTD phosphorylation, and if so, by what mechanism. Results of genetic and biochemical experiments reveal that Rpb4/7 is important to maintain proper levels of RNAPII phosphorylation, and that it functions by a mechanism(s) that involves Rpb4/7-dependent association, recruitment and/or accessibility, of key CTD modifying enzymes.

MATERIALS AND METHODS

Yeast strains, media, plasmids and oligonucleotides

The strains used are listed in Supplementary Table I. Strain construction and other genetic manipulations were performed following standard procedures (71). Plasmids and oligonucleotide sequences used in this study are listed in Supplementary Table II and III, respectively.

Chromatin isolation

Chromatin isolation was performed as described (72) with modifications. Briefly, about 5×10^8 cells growing exponentially ($OD_{600} \sim 0.6$–0.8) were resuspended in 3 ml of 100 mM PIPES/KOH (pH 9.4) containing 10 mM DTT and 0.1% sodium azide and incubated at room temperature for 10 min. After concentration by centrifugation, cells were resuspended in 2 ml of 50 mM phosphate buffer (pH 7.5), containing 0.6 M Sorbitol, 10 mM DTT and 4 μl of 20 mg/ml zymolyase and were incubated 10 min at 37°C. Spheroplasts were pelleted at 4°C, washed with 50 mM HEPES-HOK buffer (pH 7.5) containing 100 mM KCl, 2.5 mM $MgCl_2$ and 0.4 M Sorbitol, and resuspended in equal volume (~80 μl) of EBX buffer (50 mM HEPES/KOH, pH 7.5), containing 100 mM KCl, 2.5 mM $MgCl_2$, 0.25% Triton-X100, 0.5 mM PMSF, 0.5 mM DTT and 1× protease inhibitor cocktail (Complete; Roche) and incubated for 3 min on ice. Spheroplasts break under these conditions and the resulting whole-cell extracts were added to 400 μl of EBX-S buffer (EBX with 30% sucrose) and centrifuged at 12 000 revolutions per minute (rpm) for 10 min. After centrifugation, a chromatin pellet which was

visible, was washed with 400 μl of EBX buffer and resuspended in 50 μl of 1.5× Tris-Glycine SDS Sample Buffer and incubated for 2 min at 85°C, followed by centrifugation at 10 000 rpm for 30 s. A 1:3 dilution of the chromatin pellet was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western analysis using anti-Histone H3 (ab1791; Abcam), anti-phosphoglycerate kinase (459250; Invitrogen), Y-80 anti-Rpb1 (sc-25758, Santacruz), anti-CTD-Ser5 (anti-RNA polymerase II; CTD4H8, Millipore), anti-CTD-Ser2 (anti-RNA polymerase II; ab5095, Abcam), anti-CTD-Ser7 (anti-RNA polymerase II; 4E12, ChromoTek), anti-Rpb4 (2Y14; Santa Cruz) or 9E10 anti-C-Myc (Santa Cruz). Intensities of immunoreactive bands on western blots were quantified by densitometry of scanned images using TOTALLAB software. The data are the results of at least three independent experiments.

Co-immunoprecipitation (Co-IP) and western blot analysis

Rpb3 IP was performed as follows: wild-type and *rpb4*Δ cells were grown in rich medium to an OD_{600} of 0.8, harvested, washed with water and resuspended in 250 μl lysis buffer (10 mM HEPES-KOH at pH 7.5, 140 mM NaC1, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM PMSF and 1× protease inhibitor cocktail (Complete; Roche). Cell lysis was achieved at 4°C using a FastPrep System. Anti-Rpb3 antibody (WPO12, Neoclone) coupled to protein A Sepharose was incubated for 1 h at 4°C and after several washes of the clarified whole cell extracts (WCE) were added and immunoprecipitated for 3 h at 4°C. The IPs were extensively washed and resuspended in SDS-PAGE sample buffer. Western blot analysis was performed using the appropriate antibodies, detailed above. The enhanced chemiluminescence (ECL) reagents were used for detection. The signal was acquired on film and/or with a ChemiDoc XRS (Bio-Rad) system and quantified with the Quantity One software (Bio-Rad). The data plotted correspond to values means from at least three different experiments, and the error bars represent standard deviations.

Chromatin immunoprecipitation (ChIP)

Chromatin purification, IP, quantitative real-time polymerase chain reaction (qPCR) amplification and data analysis were performed as described (73). Briefly, PCR of purified chromatin, following IP, was performed by real-time qPCR with and CFX96 Detection System (Bio-Rad Laboratories, Inc.), using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) 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. Real-time PCR reactions were performed in triplicate and with at least three independent ChIPs. Quantitative analysis was carried out using the CFX96 Manager Software (version 3.1, Bio-Rad). The values obtained for the IP's PCR products were compared to those of the total input, and the ratio of the values from each PCR product from transcribed genes to a non-transcribed region of CVII was calculated. Numbers on the *y*-axis of graphs are detailed in the corresponding figure legend.

RNA isolation and reverse transcriptase-qPCR (RT-qPCR)

Total RNA was extracted as described (74), and reverse transcribed using the iScript RT reagent Kit (Bio-Rad.), following the manufacturer's instructions. Real-time RT-qPCR was performed using a CFX-384 Real-Time PCR instrument (BioRad) and EvaGreen detection system 'SsoFast™ EvaGreen® Supermix' (BioRad). Reactions were performed in 10 μl total volume containing cDNA corresponding to 0.1 ng of total RNA. PCR reactions were performed at least three times with three independent biological replicates. The 18S rRNA gene was used for normalization.

RESULTS

Deletion of *RPB4* or mutations impairing Rpb4/7 heterodimer association increase Rpb1-CTD phosphorylation

Previous work showed that levels of Ser2P CTD increase in the absence of *RPB4* (*rpb4*Δ) (8) . This increase had been attributed to an overall increase *RPB1* mRNA and Rpb1 protein levels. Here, we examined the phosphorylation of Ser2 CTD in more detail, and extended our analysis to other phosphorylation sites, Ser5 and Ser7. In agreement with the published work (8), we observed an increase in Ser2P levels in *rpb4*Δ cells, as well as increased total Rpb1 protein, as detected using an antibody (Y-80) that recognizes the N-terminus of Rpb1, independent of CTD phosphorylation (Figure 1A). However, we do not detect an increase in *RPB1* mRNA levels in *rpb4*Δ cells (Figure 1D), although its relative abundance may increase relative to that of other mRNAs, which show a significant decrease (Supplementary Figure S1 and (13). The increase in bulk CTD phosphorylation exceeded, albeit modestly, the increase in Rpb1 protein levels, suggesting that in *rpb4*Δ cells, Ser2P levels increase relative to that in wild-type cells (Figure 1A, right panel).

Examination of Ser5P and Ser7P levels in *rpb4*Δ mutants showed similar results, a strong increase in phosphorylation, particularly for Ser7, that exceeded the increase in Rpb1 levels (Figure 1A). Moreover, two different *rpb7* mutants (*rpb7–33* (10) and *rpb7-ΔC3* (a gift from P. Thuriaux)) also displayed increases in Ser2P, Ser5P and Ser7P (Figure 1B). These *rpb7* mutants show reduced Rpb4 levels, but notably, the levels of Rpb1 were not increased (Figure 1B and D). Finally, cells bearing the *rpb6-Q100R* mutation, which reduces the association of the Rpb4/7 heterodimer with RNAPII (75) also showed enhanced CTD phosphorylation without altering Rpb1 protein or *RPB1* mRNA levels (Figure 1C and D). From these data, we conclude that proper phosphorylation of the RNAPII CTD depends on a functional Rpb4/7 heterodimer.

We also analyzed the mRNA expression pattern of three constitutively transcribed genes in *rpb4Δ*, *rpb7-ΔC3* and *rpb6-Q100R* mutant cells, as well as in their corresponding isogenic wild-type strains, at the permissive temperature of 28°C (Supplementary Figure S1). The *rpb7-ΔC3* mutation caused a significant reduction in steady-state levels of mRNA, although not to the level of an *RPB4* deletion. In contrast, the *rpb6-Q100R* mutant did not significantly affect mRNA levels. For *rpb4Δ* and *rpb7-ΔC3* mutants, the lower mRNA levels might be due to reduced initiation, consistent

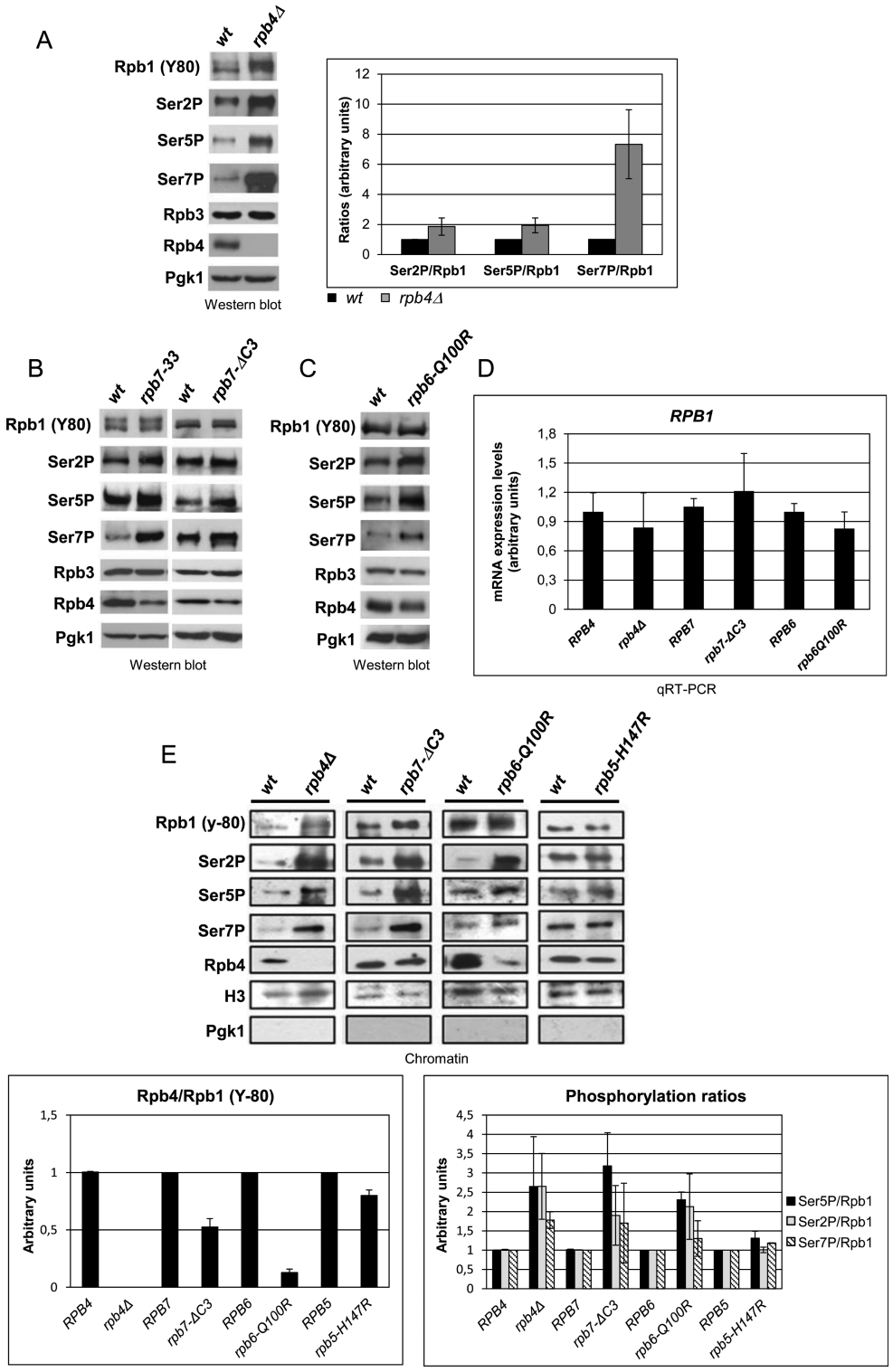


Figure 1. Deletion of *RPB4* or mutations that reduce Rpb4/7 heterodimer association increase Rpb1-CTD phosphorylation. (A) Left panel: WCE were prepared from wild-type (*wt*) and *rpb4*Δ strains and analyzed by **w**estern blotting using the following antibodies: anti-Rpb1 (Y-80), anti-CTD-Ser5P (4H8), anti-CTD-Ser2P (ab5095), anti-CTD-Ser7P (4E12) and anti-Rpb4 (2Y14). Anti-Rpb3 was used as control for RNAPII levels and anti-Pgk1 (phosphoglycerate kinase) as a control for total protein. Right panel: Plots of Ser2P/Rpb1, Ser5P/Rpb1 and Ser7P/Rpb1 ratios determined by quantitation (by densitometry) of immunoreactive bands on western blots. The mean values and standard deviations are from three independent experiments. (B and C) WCE from *rpb7–33*, *rpb7-ΔC3* and *rpb6-Q100R* mutants and isogenic *wt* controls were analyzed as in (A). (D) qRT-PCR of *RPB1* mRNA levels in the indicated strain backgrounds. The 18S rRNA gene was used as a control. (E) Western analysis of Rpb1 phosphorylation in chromatin fractions from *rpb4*Δ, *rpb7-ΔC3* and *rpb6-Q100R* mutant strains and their isogenic *wt* controls. *rpb5-H147R* served as control for no effect on RNAPII phosphorylation, and histone H3 and Pgk1 were used as controls for nuclear and cytoplasmic proteins, respectively. Lower panels: Ratios calculated by quantitation of immunoreactive bands on western blots.

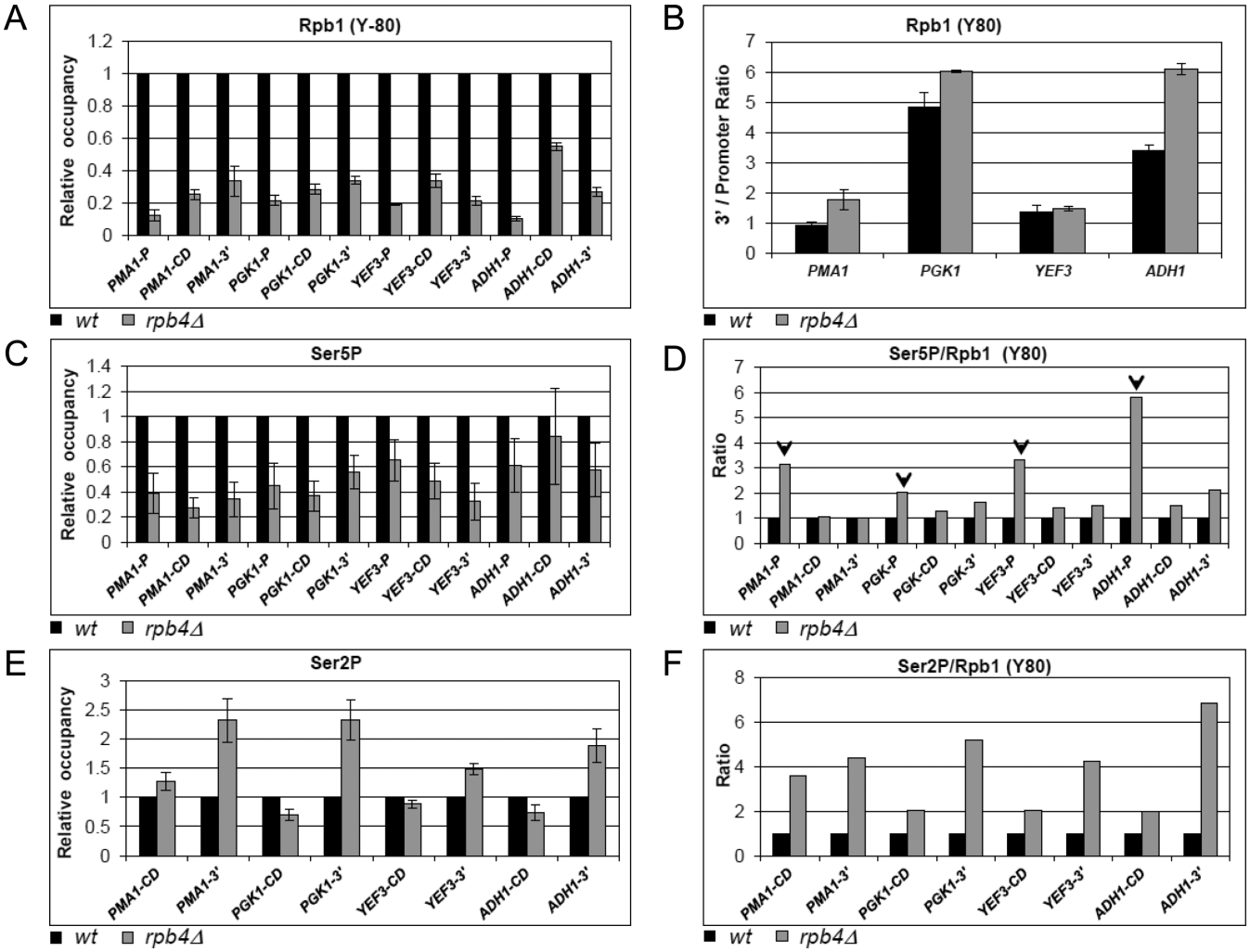
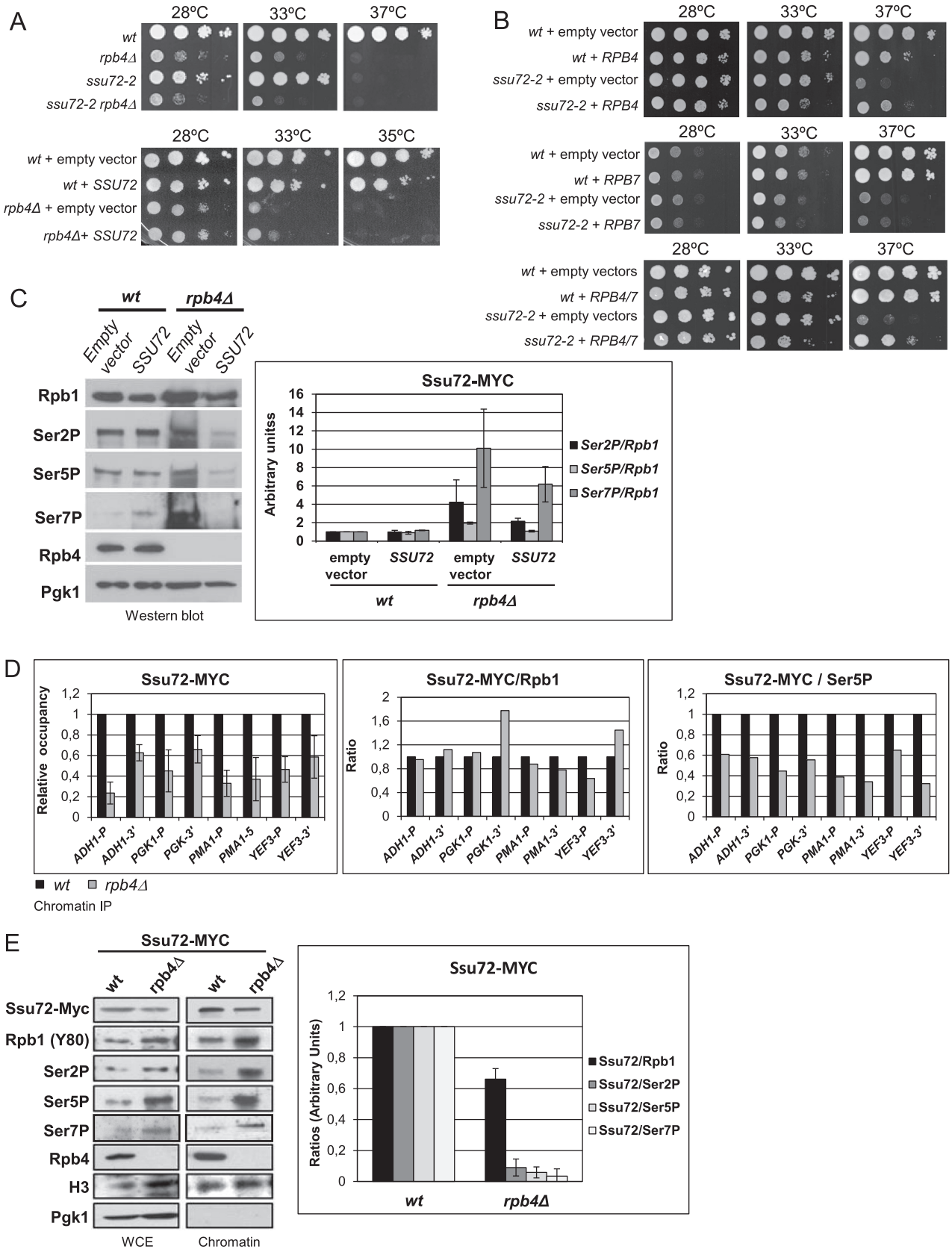


Figure 2. Rpb1-CTD Ser5P and Ser2P occupancy in *rpb4Δ* cells. (A) ChIP analyses was performed using *wt* and *rpb4*Δ strains. Rpb1 binding to the 5′ promoter (P), coding region (CD) and just prior to the transcription termination site (3′), of four constitutively expressed genes, *PMA1*, *PGK1*, *YEF3* and *ADH1* was examined by qPCR. Results were quantitated (see Materials and Methods), and Rpb1 binding in *rpb4*Δ cells is plotted relative to that in *wt* cells (set equal to 1). (B) Rpb1 occupancy expressed as a ratio of 3′-end/Promoter levels, indicating an enrichment near the termination site on three of four genes analyzed. (C) Rpb1-CTD Ser5P occupancy in *wt* and *rpb4*Δ strains analyzed by ChIP as in (A). (D) Rpb1-CTD Ser5P/Rpb1 ratios based on data in (A) and (C). (E) Rpb1-CTD Ser2P occupancy in *wt* and *rpb4*Δ strains analyzed by ChIP as in (A). (F) Rpb1-CTD Ser2P/Rpb1 ratios based on data in (A) and (E).

with strongly reduced Rpb1 ChIP signals at promoters and throughout the rest of the gene as observed in *rpb4Δ* cells (Figure 2A). These mutants may also have defects in elongation, termination and/or pre-mRNA processing due to RNAPII hyperphosphorylation (Figure 1A–C). In fact, it has been shown that Rpb4 contributes to cotranscriptional recruitment of 3′-end processing factors (8).

Next, we investigated the phosphorylation status of the CTD of RNAPII bound to chromatin. Chromatin was purified from *rpb4*Δ, *rpb7-ΔC3* and *rpb6-Q100R* mutant cells, and the levels of Rpb4, Rpb1, Ser2P, Ser5P and Ser7P were measured by western analysis (Figure 1E). As a control, we included the *rpb5-H174R* mutant, which has no effect on CTD phosphorylation (unpublished data). The results show that in chromatin fractions from *rpb4*Δ, *rpb6-Q100R* and *rpb7-Δ33* strains, in which Rpb4 levels are absent or reduced, the levels of Ser2P, Ser5P and Ser7P were increased (Figure 1E, upper and bottom left panels). The increased CTD phosphorylation cannot be explained solely by increased chromatin-bound Rpb1, as indicated by the ratios of CTD phosphorylation versus Rpb1 (Figure 1E, right graph). These data suggest that chromatin-bound RNAPII requires a functional Rpb4/7 heterodimer to be appropriately phosphorylated.

To analyze the effects of *RPB4* deletion on RNAPII occupancy and CTD modification along individual constitutively transcribed genes, we used ChIP (Figure 2). Two antibodies were used for Rpb1, Y80, which recognizes the N-terminal domain (Figure 2A, left panel), and 8WG16 (Supplementary Figure S2B), which recognizes mostly unphosphorylated CTD but whose epitope may be masked by Ser2 phosphorylation. In fact, under conditions where Ser2P levels are increased, an artificial decrease in Rpb1 has been observed using 8WG16 (50,61). Using ChIP probes in


A
28ºC
33ºC
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ssu72-2
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wt + empty vector
wt + SSU72
rpb4Δ + empty vector
rpb4Δ+ SSU72
B
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wt + RPB4
ssu72-2 + empty vector
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Relative occupancy
ADH1-P
ADH1-3'
PGK1-P
PGK-3'
PMA1-P
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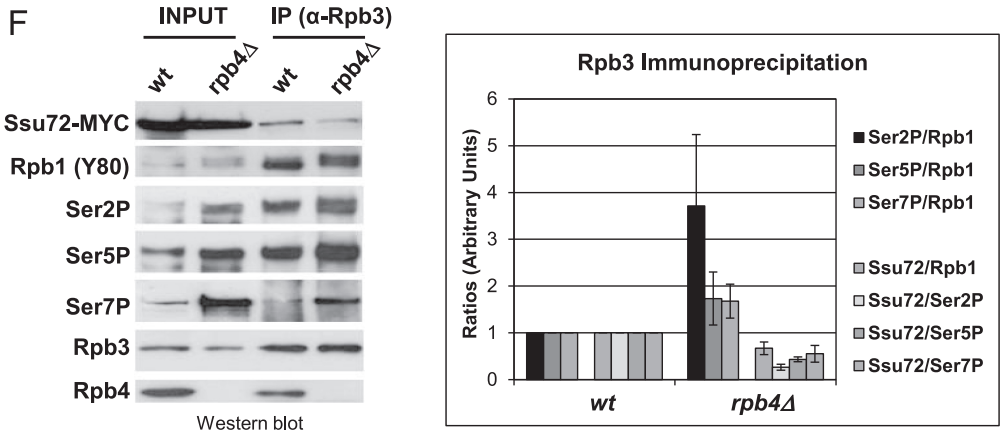

Figure 3. Ssu72 is functionally dependent on Rpb4. (A) Genetic interaction between *RPB4* and *SSU72*. Serial dilutions (1:10) of *wt* and mutant strains were spotted on rich medium and grown for 2–3 days at the indicated temperatures. *RPB4* deletion exacerbates *ssu72–2* slow growth at 28°C and 33°C, while overexpression of *SSU72* partially suppresses the slow growth phenotype of *rpb4*Δ strain. Growth was on SC media for 2–3 days. (B) Overexpression of *RPB4* or *RPB4*/*RPB7* (coexpression), but not *RPB7*, suppress the thermosensitivity of the *ssu72–2* strain. The corresponding strains (*wt* and *ssu72–2*) were transformed with empty vectors or with high-copy plasmids bearing *RPB4* and/or *RPB7* and grown on SC media for 2–3 days at 28°C, 33°C and 37°C. (C) Overexpression of *SSU72* reduces Ser5P levels in *rpb4*Δ mutant cells. Reductions are also detected for Ser2P and Ser7P (see text). Western blot analysis was performed using WCE from *wt* and *rpb4*Δ cells transformed with an empty vector or a high-copy plasmid bearing *SSU72* and grown to mid-logarithmic phase in SC. Graph on the right represents the mean values of Ser2P/Rpb1, Ser5P/Rpb1 and Ser7P/Rpb1 ratios from at least three independent experiments. Error bars are standard deviations from the mean. (D) ChIP analysis of Ssu72-MYC chromosomally integrated in *wt* and *rpb4*Δ cells. ChIP was performed using anti-MYC antibodies. Ssu72-MYC crosslinking to the promoter and 3′-end regions of *ADH1*, *PGK1*, *PMA1* and *YEF3* was analyzed by qPCR and occupancy in the *rpb4*Δ mutant was normalized to that in *wt* cells. The graph show mean values of three independent experiments, and error bars represent standard deviations. Middle panel, Ssu72/Rpb1 ratios are graphed and show that similar levels of Ssu72 and Rpb1 are recruited to promoter and 3′-end regions. Right panel, Ssu72/Ser5P ratios are depicted, and indicate a decrease of Ssu72 in promoter and 3′ regions relative to the CTD-Ser5P substrate. (E) Western analysis of CTD phosphorylation levels on chromatin fractions of *wt* and *rpb4*Δ cells expressing Ssu72-MYC. Histone H3 and Pgk1 are included as controls as in Figure 1E. Right panel, ratios of Ssu72 to Rpb1, Ser2P, Ser5P and Ser7P indicate a depletion of Ssu72 in chromatin relative to multiple RNAPII forms. (F) Co-IP performed on WCEs from Ssu72-MYC cells (*wt* and *rpb4*Δ) using an anti-Rpb3 antibody. Inputs and IPs were analyzed by western blotting with antibodies to the indicated proteins. Right panel, mean values from the quantification of immunoreactive signals from three experiments, where error bars represent standard deviations.

the promoter, coding region, and 3′-end of *PMA1*, *PGK1*, *YEF3* and *ADH1* (Supplementary Figure S2A) we observed a significant reduction in Rpb1 occupancy (using Y80) in all three regions in *rpb4*Δ cells (Figure 2A). Similar results were obtained using the 8WG16 antibody, despite the presumed increase in Ser2P levels, and an antibody against another RNAPII subunit (Rpb3) (Supplementary Figure S2B). Thus, in the absence of Rpb4, RNAPII occupancy was reduced, even though total Rpb1 was increased ((8); Figure 1, this study). These findings suggest that impairment of the Rpb4/7 heterodimer results in an excess of RNAPII not engaged in productive transcription. The ChIP data also revealed a more pronounced decrease in Rpb1 occupancy at the promoters compared with the 3′-ends of the genes examined (Figure 2B).

We also analyzed Ser5P and Ser2P levels in *rpb4*Δ mutants using ChIP (Figure 2C–F). Surprisingly, while Ser5P occupancy during transcription was reduced in *rpb4*Δ cells, the relative levels of Ser5P versus Rpb1 (Ser5P/Rpb1) clearly increased at the promoter regions of all the genes tested. No major changes in the Ser5P/Rpb1 ratios in coding and 3′-end regions were observed, with the exception of the *ADH1* gene, where the Ser5P/Rpb1 ratio is higher at the 3′-end in *rpb4*Δ versus wild-type cells. In the case of Ser2P, only coding and 3′-end regions were analyzed, because Ser2P is almost undetectable at promoters (43,50). A significant enrichment of Ser2P at 3′-end regions was observed, which become more evident by the Ser2P/Rpb1 ratios (Figure 2E and F). Similar results were obtained using 8WG16 antibodies to immunoprecipitate Rpb1 and using anti-Rpb3 with the exception of the *ADH1* gene that shows a slightly different pattern (Supplementary Figure S3). Taken together, the results demonstrate that the Rpb4/7 heterodimer is required to maintain normal RNAPII CTD phosphorylation levels. Below, we investigate potential mechanism(s) for how Rpb4/7 might function to regulate CTD phosphorylation.

Ssu72 is functionally dependent on Rpb4

Results thus far indicate that compromising Rpb4/7 function leads to abnormally high levels of Ser2P, Ser5P and Ser7P. To investigate the possibility that high Ser5/7P levels result from misregulation of Ssu72, a Ser5P (and Ser7P)-specific phosphatase (61), we carried out genetic interaction experiments. We used the temperature-sensitive *ssu72–2* mutant, which displays impaired phosphatase activity *in vitro*, accumulation of Ser5P *in vivo* and defects in transcription elongation (76,77). At permissive temperatures (28°C, 33°C), the *rpb4*Δ *ssu72–2* double mutants grew slower than either mutant alone (Figure 3A, upper panel). In contrast, overexpression of *SSU72* partially rescued growth of *rpb4*Δ at 33°C (Figure 3A, lower panel), while overexpression of *RPB4* partially rescued growth of *ssu72–2* at 37°C (Figure 3B, upper panel). A stronger rescue of *ssu72–2* was observed by co-overexpressing *RPB4* and *RPB7* (Figure 3B, lower panel). In contrast, *RPB7* alone had no effect (Figure 3B,

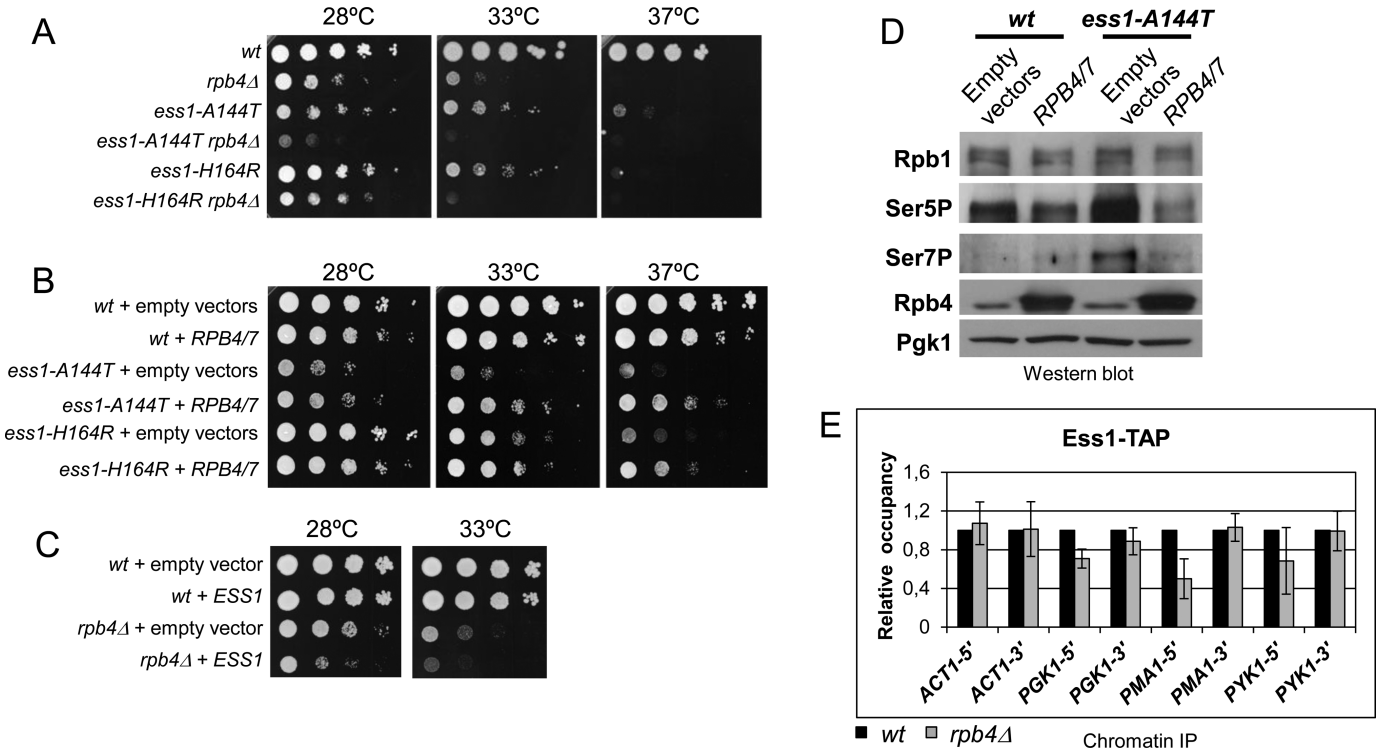

Figure 4. Ess1 and Rpb4/7 are genetically linked. (A) Genetic interaction between *RPB4* and *ESS1*. Serial dilutions of *wt* and mutant strains were spotted on rich medium and grown for 2–3 days at the indicated temperatures. *RPB4* deletion further slows growth of *ess1-A144T* and *ess1-H164R* strains. (B) Overexpression of *RPB4/7*, partially suppresses the growth defect of the *ess1-T144A* strain at all temperatures tested, while it suppresses the *ess1-H164R* strain detectably only at 37°C. Wild-type and *ess1-T144A* cells were transformed with the corresponding empty vectors or with high-copy plasmids bearing *RPB4* and/or *RPB7* and grown on SC at the indicated temperatures. (C) Overexpression of *ESS1* exacerbates the slow growth of *rpb4*Δ cells at 28°C and 33°C. Cells were grown on SC media for 2–3 days at the indicated temperatures. (D) Co-expression of *RPB4/7* reduces Ser5P and Ser7P levels in *ess1-T144A* mutant. Western blot analysis with the indicated antibodies were performed using WCE from *wt* and *ess1-T144A* cells transformed with empty vectors or with *RPB4* and *RPB7* high-copy plasmids, and grown to mid-logarithmic phase at 28°C in SC. (E) ChIP analysis of Ess1-TAP chromosomally integrated in *wt* and *rpb4*Δ cells. Mean values of the relative occupancy of Ess1 at 5′ and 3′ ends at the indicated genes are plotted, where *wt* values are considered 1. Error bars represent standard deviations.

middle panel). These genetic data suggest that Rpb4/7 and Ssu72 functions are closely related.

We next determined whether suppression of the *rpb4*Δ growth defect by *SSU72* was accompanied by a reduction of CTD-Ser5P and CTD-Ser7P levels. Western analysis showed that overexpression of *SSU72* reduced CTD-Ser5P and Ser7 levels in *rpb4*Δ cells toward wild-type levels (Figure 3C), consistent with the genetic data. Surprisingly, *SSU72* overexpression also reduced Ser2P levels.

Genome-wide studies show that Ssu72 is present primarily at the 3′ ends of genes with some detectable at transcription start sites (61,64). To determine whether Rpb4/7 is required for localization of Ssu72, we carried out ChIP using wild-type and *rpb4*Δ mutant cells (Figure 3D). Ssu72 association with promoter and 3′-end regions was significantly reduced in *rpb4*Δ cells, when compared with Ser5P CTD levels (Figure 3D). If instead, Ssu72 association in *rpb4*Δ cells is compared with total Rpb1 levels, the difference is no longer apparent, clouding the issue of Rpb4-dependent recruitment of Ssu72. To resolve this issue, we carried out additional experiments. We found that Ssu72 levels are in fact decreased in bulk chromatin in *rpb4*Δ mutants, even when compared to the levels of Rpb1, and compared to Ser2P, Ser5P and Ser7P, which increased significantly (Figure 3E). And, using Co-IP with Rpb3, we found that Ssu72 interaction with RNAPII is decreased in *rpb4*Δ mutant (Figure 3F). These results are consistent with the idea that increased CTD-Ser5P phosphorylation in *rpb4*Δ cells is a consequence of a defect in recruitment of the Ssu72 phosphatase to the RNAPII complex. These results do not, however, rule out additional effects of Rpb4 on the access of Ssu72 to the CTD substrate within chromatin or its activity.

Ess1 and Rpb4/7 are linked, likely via Ssu72

Similar to *rpb4*Δ mutant described above, mutations in the *ESS1* gene increase Ser5 and Ser7 phosphorylation levels, while overexpression decreases Ser5P (Ser7P was not tested) (63,68,78). Mechanistically, the Ess1 isomerase stimulates Ssu72 phosphatase binding and catalytic activity (69). In addition, overexpression of *RPB7* from *Candida albicans* suppresses *ess1* mutants (34). Therefore, we tested whether Rpb4/7 and Ess1 are functionally related. Indeed, genetic interaction experiments showed that *RPB4* deletion enhanced the growth defect of two *ts*-mutants, *ess1-A144T* and *ess1-H164R,* (34), whereas co-overexpression of *RPB4/7* (but not of either gene alone) suppressed the growth defect (Figure 4A and B). Overexpression of *ESS1*, which decreases Ser5P levels (78), did not rescue *rpb4*Δ cells

and seemed to further reduce the growth of *rpb4*Δ cells (Figure 4C).

We next determined whether suppression of the growth defect of *ess1* mutant cells by overexpression of *RPB4/7* was correlated with CTD-Ser5P and CTD-Ser7P levels, which are known to be elevated in these mutants (63,68,78). Indeed, western analysis revealed that overexpression of *RPB4/7* restored CTD-Ser5P and Ser7P levels in *ess1-A144T* cells back down to wild-type levels (Figure 4D), consistent with the observed genetic suppression. We noted that in some experiments total Rpb1 levels were increased in *ess1* mutants (data not shown), and this increase was reversed by *RPB4/7*. Together, these data suggest that Rpb4/7 suppresses *ess1* mutants by controlling overall levels of Rpb1 and its phosphorylation, particularly on CTD-Ser5 and CTD-Ser7.

Finally, recruitment of Ess1 to the 5′ and 3′ ends of genes was not significantly reduced in *rpb4*Δ cells (Figure 4E), ruling out the simple explanation for the genetic interaction results (failure to recruit Ess1). Together these data reveal a functional interaction between Ess1 and Rpb4/7, most likely via a common target, Ssu72, and suggest that coordination between isomerization and CTD phosphorylation is important during the transcription cycle.

Rpb4 is required for proper Fcp1 association and Ser2P and Thr4P dephosphorylation

Given that *rpb4*Δ cells have increased Ser2P levels and that Fcp1 has been identified as the major Ser2P phosphatase, we investigated whether Rpb4 promotes Fcp1 function. Consistent with this idea, we found that by increasing *FCP1* expression, the growth defects of *rpb4*Δ cells were partially suppressed (Figure 5A, upper panel). In the reciprocal experiment *RPB4* overexpression partially suppressed the growth defects of *fcp1–1* (Figure 5A, bottom panel), which lacks phosphatase activity leading to increased Ser2P levels (79,80). It has been shown that in mammalian cells, MCF inactivation at mitosis exit requires Fcp1 (81). Therefore, it is possible that in yeast Fcp1 has also other functions in addition to its role in CTD dephosphorylation. This may, in part, account for the inability of *fcp1–1* growth defects to be fully suppressed by *RPB4* overexpression. As expected, increased expression of *FCP1* reduced the levels of Ser2P phosphorylation in *rpb4Δ* cells back to wild-type levels (Figure 5B). In contrast, Ser5P levels were affected very little, indicating that Fcp1 retained its specificity (Figure 5B).

In mammals, Fcp1 was also shown to dephosphorylate CTD-Thr4P (67). If Rpb4 is important for Fcp1 to dephosphorylate Thr4P, then Thr4P levels should increase in *rpb4*Δ mutant. Indeed, this is what we observed (Figure 5C, left panel), and this effect seems to be mediated by Fcp1, because Thr4P levels are specifically increased in the *fcp1–1* mutant (as are Ser2P levels), but not in the *ssu72–2* mutant (Figure 5C). These results suggest that Rpb4 facilitates dephosphorylation of Thr4P and Ser2P by Fcp1.

Similar to results with Ssu72, the occupancy of Fcp1 as measured by ChIP (in coding and 3′ regions) in the four query genes was strongly decreased in *rpb4*Δ cells, especially when compared to levels of its substrate target modification, CTD-Ser2P (Figure 5D). However, similar to the case with Ssu72, normalization to Rpb1 does not reveal a large difference in occupancy of Fcp1, supporting the alternative model (not mutually exclusive) that Rpb4/7 might play a role subsequent to Fcp1 binding, such as positioning of the phosphatase (providing access to its substrate) or controlling its activity. Using a Myc-tagged Fcp1 strain, we could Co-IP significant amounts of Rpb4 using anti-Myc antibodies (Figure 5E). And, Fcp1 co-immunoprecipitated much more Ser2P in *rpb4*Δ cells than in the wild type. Together, the results suggest that in the absence of Rpb4/7, Fcp1 associates with RNAPII more weakly, and that the Fcp1 that is bound is not able to efficiently de-phosphorylate Ser2P. In summary, the results are consistent with a positive role for Rpb4 on Fcp1 activity toward the RNAII CTD.

Finally, we performed Co-IP assays to determine whether the association of Fcp1 with RNAPII *in vivo* is Rpb4-dependent. Less Fcp1 was associated with RNAPII in *rpb4*Δ cells as detected by IP with an anti-Rpb3 antibody (Figure 5F, left panel). This reduction is apparent when comparing Fcp1 to levels of Rpb1 in *rpb4*Δ cells, and especially when comparing to levels of Ser2P-, Ser5P- and Ser7-modified RNAPII (Figure 5F, right panel).

Effect of *RPB4* deletion on Kin28 and Ctk1 functions

Our data thus far do not exclude the possibility that, in addition to the effects on CTD phosphatases, Rpb4 also influences the binding or activity of CTD kinases. For example, it is possible that Kin28, Ctk1 and Srb10 kinases contribute to the increased phosphorylation of Ser5, Ser2 and Ser7. To investigate this possibility, we analyzed the genetic relationships between *RPB4* and *KIN28*, *CTK1* and *SRB10*. *RPB4* was deleted from strains containing two *kin28* alleles, *kin28-T17D* and *kin28-T162A* (carried on a plasmid), or chromosomal *ctk1Δ* and *srb10Δ* mutations. The *kin28-T17D* mutant has decreased CTD kinase activity, a defect in capping enzyme recruitment and is slow growing at 25°C and 37°C. The *kin28-T162A* allele, which does not display a slow growth phenotype, has reduced activity *in vitro*, but does not detectably affect CTD phosphorylation and capping enzymes recruitment *in vivo* (44,82,83).

A simple prediction would be that mutations in CTD kinases in *rpb4*Δ cells might restore CTD phosphorylation levels (which are elevated) and thus suppress *rpb4*Δ growth defects. However, this was not the case with two of the three kinases. Instead, combining *rpb4*Δ with *kin28* resulted in synthetic lethality at the permissive temperature (*kin28-T17D*; Figure 6A, left and middle panels) or enhancement of the slow growth phenotypes at 28°C and 33°C (*kin28-T162A*; Figure 6A, right panel). The *rpb4*Δ *ctk1*Δ double mutant also showed synthetic growth defects (Figure 6B). Only *srb10*Δ, which is thought to play a negative role in transcription (40), suppressed, at least mildly, *rpb4*Δ growth defects (Figure 6C). Thus, although there are genetic interactions between *rpb4*Δ and genes encoding three CTD kinases, the interpretation of these results is not straightforward. This might be because Rpb4/7 functions during multiple steps in transcription/CTD modification and that the CTD kinases may have other important substrates be-

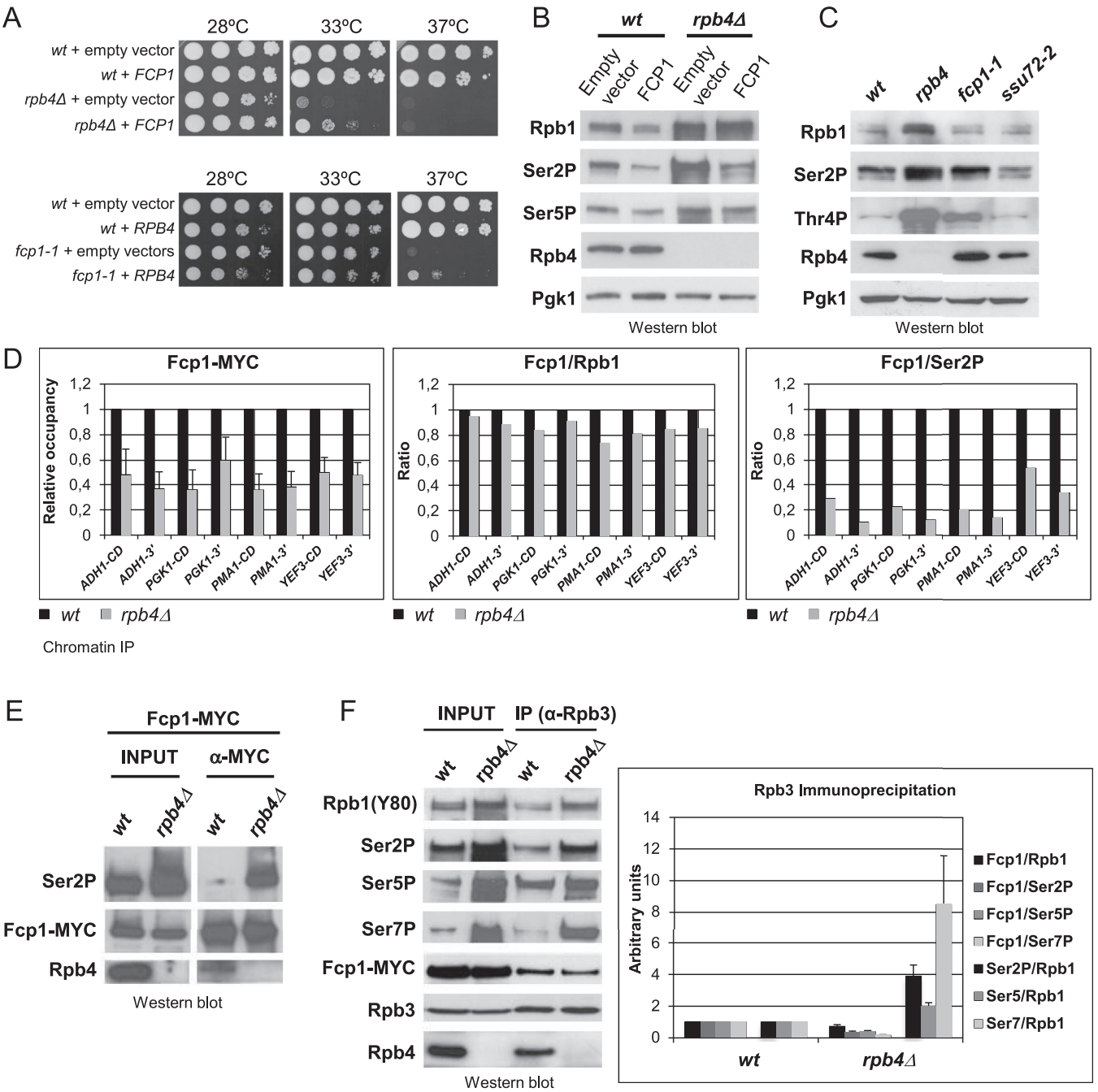

Figure 5. Rpb4 is required for proper Fcp1 association and Ser2 dephosphorylation. (A) Genetic interactions between *RPB4* and *FCP1*. Expression of *FCP1* from a centromeric plasmid partially suppresses the slow growth of *rpb4*Δ cells at 33°C, while overexpression of *RPB4* partially suppresses the thermosensitivity of the *fcp1–1* mutant strain. Growth was on SC media for 2–3 days. (B) Expression of *FCP1* reduces Ser2P levels (but not Ser5P levels) in *rpb4*Δ mutant cells. Western analysis was performed using WCE from *wt* and *rpb4*Δ cells transformed with an empty vector or a centromeric plasmid bearing *FCP1* and grown to mid-logarithmic phase in SC. (C) *RPB4* deletion increases CTD-Thr4P levels. Western analysis of WCE from *wt*, *rpb4*Δ, *fcp1–1* and *ssu72–2* cells, using anti-Rpb1 (Y80), anti-Ser2P, anti-Thr4P (Novus biological), anti-Rpb4 and anti-Pgk1. (D) ChIP analysis using anti-MYC antibodies of *wt* and *rpb4*Δ strains bearing chromosomally integrated Fcp1-MYC. Fcp1-MYC occupancy on coding and 3′-end regions of *ADH1*, *PGK1*, *PMA1* and *YEF3* was analyzed in *rpb4*Δ mutant cells by qPCR and normalized to that in *wt* cells. Middle panel, Fcp1/Rpb1 ratios indicate that Fcp1 occupancy is similar to that of Rpb1. Right panel, Fcp1/Ser2P ratios indicate reduced Fcp1 at coding and 3′ regions relative to its CTD-Ser2P substrate (Ser2P ChIP, data not shown). (E) Co-IP using WCE from Fcp1-MYC cells (*wt* and *rpb4*Δ) and anti-MYC antibody. Inputs and IPs were analyzed by western blot with antibodies to the indicated proteins. (F) Co-IP using WCE from Fcp1-MYC cells (*wt* and *rpb4*Δ) and anti-Rpb3 antibody. Inputs and IPs were analyzed by western blot with antibodies to the indicated proteins. Right panel, mean values from the quantification of immunoreactive signals from three experiments, where error bars represent standard deviations.

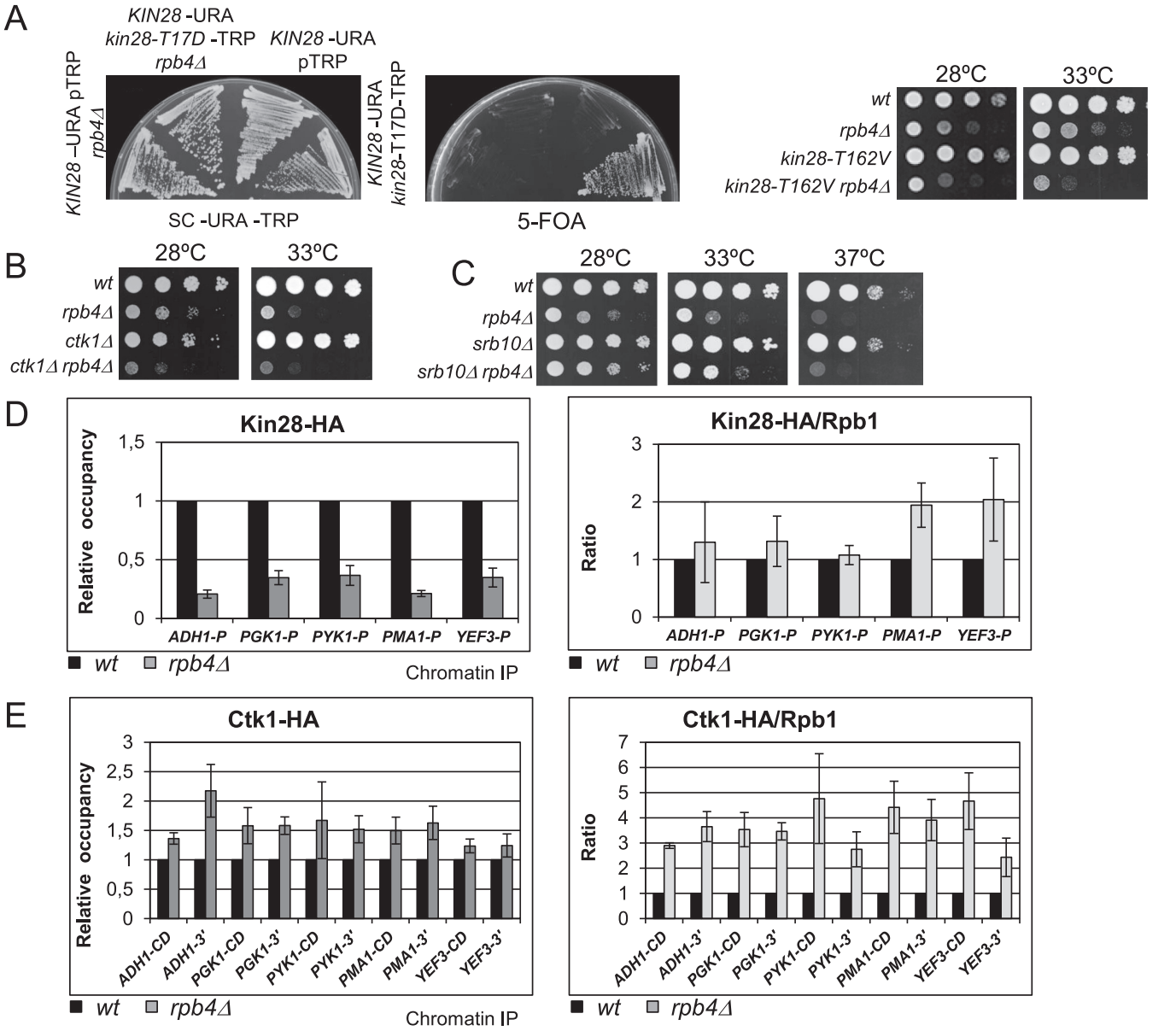

Figure 6. Effect of *RPB4* deletion on Kin28 and Ctk1 functions. (A) Genetic interaction between *RPB4* and *KIN28*. Left and middle panels: Growth of *RPB4* and *rpb4*Δ cells containing plasmid-borne copies of wild-type *KIN28* (*CEN*, *URA3*) and either an empty vector (pTRP1) or *kin28-T17D* (*CEN*, *TRP1*). Cells were streaked to SC media (left) or SC media containing 5-FOA (right) to select against the *KIN28* plasmid. Combining the *kin28-T17D* and *rpb4*Δ mutations results in synthetic lethality (*kin28-T17D* only supports growth in the $RPB4^{+}$ background). Right panel, serial dilutions of *wt* and mutant strains were spotted on SC medium and grown for 2–3 days at the indicated temperatures. *RPB4* deletion in combination with *kin28-T162V* causes a significant slow growth defect. (B and C) Genetic interaction between *RPB4* and *CTK1* and *SRB10.* Serial dilutions of *wt* and mutant strains were spotted on rich medium and grown for 2–3 days at the indicated temperatures. Deletion of *CTK1* exacerbates the slow growth of *rpb4*Δ at 28°C and 33°C, while deletion of *SRB10* partially rescues the slow growth of *rpb4Δ.* (D) ChIP analysis of Kin28-HA chromosomally integrated in *wt* and *rpb4*Δ cells. ChIP was performed using anti-HA antibody. Kin28-HA crosslinking to promoter regions of *ADH1*, *PGK1*, *PMA1* and *YEF3* was analyzed by qPCR and occupancy in the *rpb4*Δ mutant was normalized to that in *wt* cells, considered as 1. Kin28/Rpb1 ratios (Rpb1 ChIP data not shown) showed slightly increased occupancy of Kin28 at promoters relative to Rpb1. (E) Ctk1-HA occupancy was carried out by ChIP and ratios were determined as in (D) above.

sides the CTD. Another possibility was suggested by further analysis of *rpb4*Δ *ctk1*Δ double mutants, which showed very low levels of Rpb1 (Supplementary Figure S4), perhaps below a threshold required to sustain vigorous growth. These data also indicate that elevated Ser2P levels in the *rpb4*Δ mutant are dependent on Ctk1, because this mark was nearly absent in the *rpb4*Δ *ctk1*Δ cells, compared with Ser5P, which was present (Supplementary Figure S4).

To determine whether the effects on CTD phosphorylation by Rpb4/7 are predominantly through the CTD phosphatases or CTD kinases, we examined recruitment of Kin28 (to promoters), Ctk1 (to coding and 3′ regions) and Srb10 (to UAS regions) of active genes using ChIP. Kin28 occupancy on promoters was reduced in *rpb4*Δ cells when compared to wild-type cells (Figure 6D), consistent with the genetic interaction. However, when normalized to the

amount of Rpb1, the occupancy of Kin28 in *rpb4*Δ cells was not significantly higher in three of the five genes tested (Figure 6D, right panel). This suggests that the increase in Ser5P and Ser7P in *rpb4*Δ cells is more likely the result of failure to dephosphorylate these serines (by Ssu72) than an 'over-recruitment' of the corresponding kinase (Kin28).

In contrast, ChIP analysis of Ctk1 revealed a strong increase in relative Ctk1 recruitment in *rpb4*Δ cells (Figure 6E). The increased occupancy of Ctk1/Rpb1 on coding and 3′ regions, in coordination with decrease in Fcp1 association and/or access may contribute to the observed hyperphosphorylation of Ser2 in *rpb4*Δ cells. Furthermore, the high Ser5P levels could lead to increased Ser2P because, as previously shown, Ctk1 acts on the CTD already phosphorylated on Ser5P (84). Finally, ChIP analysis of Srb10 recruitment did not show significant changes in *rpb4*Δ cells (data not shown).

In summary, our results show that Rpb4/7 heterodimer modulates RNAPII phosphorylation, and that it likely functions primarily by facilitating the association, recruitment and/or accessibility of the CTD phosphatases, Ssu72 and Fcp1.

DISCUSSION

In this study, we showed that impairing Rpb4/7 heterodimer function by deleting Rpb4 or mutating Rpb7 subunits, or preventing its recruitment to RNAPII, cause elevated CTD phosphorylation at Ser2, Ser5, Ser7 and Thr4. Consistent with these changes in phosphorylation of RNAPII, we found genetic interactions between *RPB4* and genes encoding the CTD phosphatases Ssu72 and Fcp1, and the CTD kinases Kin28, Ctk1 and Srb10. We also show genetic interactions between *RPB4* and *ESS1*, which encodes a prolyl isomerase that targets the CTD and affects its phosphorylation. Finally, using fractionation, ChIP and Co-IP, we found that Rpb4 helps recruit both Fcp1 and Ssu72 to RNAPII, and that association of these enzymes with RNAPII and their access/activity toward their substrates is decreased in *rpb4*Δ mutants, explaining, in large part, the increased phosphorylation.

Our findings lead to the following model. Rpb4/7 is recruited to the core RNAPII complex via Rpb6 (and Rpb1) as previously shown (85,86). The Rpb6 subunit is conserved in RNAPI and RNAPIII, and archaeal RNAP (K), and might play an analogous role in recruiting the corresponding stalk subunits. There is even an Rpb6 ortholog in bacteria (ω) that plays a role in assembly of the other four core RNAP subunits (87). Once recruited, the Rpb4/7 complex is positioned near the RNA exit channel and the Rpb1-CTD (14), where it likely carries out two sets of functions. First are the *CTD-independent* functions shared with other 'stalk-containing' polymerases (RNAPI, RNAPIII and archaeal RNAP). These would potentially include DNA melting during initiation (88), stabilization of the elongation complex by an RNA binding-dependent mechanism (leading to increased processivity) and contributions to site-specific termination (2). The second set of Rpb4/7 functions would be *CTD-dependent* functions, specific to RNAPII and would include association, recruitment and/or accessibility, of CTD-modifying enzymes and CTD-binding co-factors required for RNA processing. Regulating levels of CTD phosphorylation may not have been the primary role of Rpb4/7. Instead, this activity could have been acquired as the role of the CTD became integral for coupling of co-transcriptional processes, and facilitated by the proximity of the Rpb4/7 heterodimer to the CTD as well as the RNA exit chanel (14). Here, we showed that Rpb4/7 helps recruit and potentially position/activate the CTD phosphatases, Ssu72 and Fcp1, and may inhibit the binding of the CTD kinases Kin28 and Ctk1. Thus, Rpb4/7 would promote an overall dephosphorylation of the CTD, and help keep Ser2P, Ser5P, Ser7P and Thr4P levels in check. Recruitment of the Rpb4/7 heterodimer to elongating RNAPII may itself be regulated (9), so its effects on CTD phosphorylation may be more complex, and possibly gene-specific. Rpb4/7 may also impact RNAPII processivity and recycling efficiency by increasing RNAPII occupancy via its effects on CTD phosphorylation levels. Consistent with this model, increased CTD phosphorylation (on Ser2, Ser5, Ser7) relative to total Rpb1 was observed not only in *rpb4Δ* and *rpb7* mutants, but also in a *rpb6* and a *rpb1* missense mutants that reduce Rpb4/7 recruitment to the RNAPII core (Figure 1 and (75,89). The increased Ser5P levels in *rpb4*Δ cells (Figure 2C and D) correlates well with the strongly decreased recruitment of the Ser5P-specific phosphatase Ssu72 (Figure 3D), and to the slightly increased of the Ser5 kinase Kin28 to promoters (Figure 6D). Likewise, the increased Ser2P levels in coding and 3′ regions in *rpb4*Δ cells (Figure 2E and F) correlates well with the decreased recruitment of the Ser2P-specific phosphatase Fcp1, particularly in 3′ regions (Figure 5D), and to the increased Ser2 kinase Ctk1 in these regions (Figure 6E). These findings are consistent with earlier work showing that Rpb4 binds Fcp1 in *S. pombe* and in *Drosophila* (20). The increases in Ser2P levels in the *rpb4Δ* mutant (in which Fcp1 recruitment is compromised) are Ctk1-dependent, as indicated by the fact that Ser2P levels were dramatically reduced in *rpb4*Δ*ctk1* Δ double mutants (Supplementary Figure S4). Notably, Ser7P, which is a target of Ssu72 is also significantly increased in *rpb4*Δ, *rpb7* and *rpb6* mutants (Figure 1), and Thr4P (Figure 5C), which is a target of Fcp1 in human cells (67) is extraordinarily enhanced in *rpb4*Δ mutants, consistent with a conserved function for Fcp1 as the Thr4P phosphatase from yeast to mammals.

Our genetic evidence also supports the idea that recruitment of CTD phosphatases is a major factor in governing the Rpb4/7-dependent control of CTD phosphorylation. For example, overexpression of either *SSU72* or *FCP1* suppressed the slow growth of *rpb4*Δ cells (Figures 3A and 5A), and restored (lowered) Ser5P/Ser7P and Ser2P levels, respectively (Figures 3C and 5B). Note that overexpression of *SSU72*, which should be Ser5/Ser7P-specific, also lowered Ser2P levels (Figure 3C), perhaps due to a reduced substrate specificity when expressed at the higher concentrations, or from Ssu72 stimulating Fcp1 activity as previously observed (61). In addition, the synthetic-lethality of *rpb4*Δ *ess1*^{ts} mutants, and rescue of *ess1*^{ts} mutants by *RPB4/7* overexpression are consistent with a defective recruitment of CTD phosphatases in *rpb4*Δ, as corroborated by the fact that *RPB4/7* overexpression reduces the high levels of Ser5P and Ser7P in *ess1* mutant cells. Ess1 increases

Ssu72 activity by isomerizing its substrate, Ser5P-Pro6 in the CTD (69). Therefore, the combination of reduced recruitment of Ssu72 (due to *rpb4*Δ) and reduced activity (due to *ess1*^{ts}) predicts a more severe phenotype, as was observed (Figure 4A), while increased Rpb4/7, which should enhance Ssu72 recruitment, compensated for the *ess1*^{ts} defect (Figure 4B).

Genetic interactions with the CTD kinases (Figure 6) were more difficult to interpret, and suggested that multiple pathways were affected in double mutant cells. For example, the synthetic lethality of *rpb4*Δ *kin28* double mutants was not predicted, because these mutations should have opposite effects on CTD phosphorylation. However, both Kin28 and Rpb4/7 have functions during transcription initiation (i.e. (41,90,91), that can be significantly impaired when the two mutations are combined. In the *rpb4*Δ*ctk1*Δ double mutants, the slow growth may have been caused by the nearly undetectable levels of Rpb1, possibly due to protein degradation, because *RPB1* transcription is not altered (data not shown).

Finally, fractionation and Co-IP experiments strongly support a phosphatase recruitment model. Both Ssu72 and Fcp1 were associated with chromatin fractions and interacted directly (or indirectly) with the RNAPII complex in a manner that was dependent upon Rpb4 (Figures 3E and F and 5C and E). And as mentioned above, ChIP data showed that the access/activity of Ssu72 and Fcp1 may also be dependent on Rpb4 (Figures 3D and 5D).

In agreement with published work (8), we found a significant increase in total cellular Rpb1 levels in *rpb4*Δ cells, while at the same time a decrease in Rpb1 occupancy at the 3′ ends of genes (Figures 1 and 2A). In contrast, we also observe a reduction in Rpb1 occupancy at promoters in *rpb4*Δ cells, even when compared to 3′ occupancy (Figure 2A and B). This difference might be due to increased sensitivity in our ChIP assays. One interpretation of our results is that in the absence of Rpb4, release of Rpb1 could be impaired after termination, which would in turn reduce RNAPII recycling, explaining the reduction of Rpb1 occupancy at the promoter. This could be a consequence of the CTD dephosphorylation defect in *rpb4*Δ cells, similar to the reduced Rpb1 crosslinking to promoters and increased Ser2P observed in *fcp1* mutants, which are proposed to have a recycling defect (50,66). Finally, the aberrant CTD phosphorylation (higher Ser5P) and lower Ssu72 at termination regions that we observed might also contribute to the known termination defects in *rpb4*Δ mutants (8,64).

In summary, our results show that the Rpb4/7 heterodimer controls covalent modification of the RNAPII, specifically, the phosphorylation of the Rpb1 CTD. The most likely mechanism is by enhancing recruitment of Ssu72 and Fcp1 phosphatases to the chromatin-bound RNAPII and or positioning them allowing their access to their substrate. Although the exact mechanism of recruitment is not known, Rpb4/7 could exert effects directly on the CTD, making it more accessible to binding by Ssu72 and Fcp1. Additionally, it could work indirectly through some other polymerase-associated proteins, and/or by or excluding the CTD kinases. Finally, our data do not rule out the possibility of 'pre-binding' of Rpb4/7 to Ssu72 and Fcp1 to facilitate their entry into the RNAPII complex. Further work will be needed to dissect the mechanism of action of Rpb4/7 on CTD phosphorylation, its regulation and how Rpb4/7 effects CTD phosphorylation fit into the larger picture of 'CTD code' regulation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Cramer,P. (2004) RNA polymerase II structure: from core to functional complexes. *Curr. Opin. Genet. Dev.*, **14**, 218–226.
2. Grohmann,D. and Werner,F. (2011) Cycling through transcription with the RNA polymerase F/E (RPB4/7) complex: structure, function and evolution of archaeal RNA polymerase. *Res. Microbiol.*, **162**, 10–18.
3. McKune,K., Richards,K.L., Edwards,A.M., Young,R.A. and Woychik,N.A. (1993) RPB7, one of two dissociable subunits of yeast RNA polymerase II, is essential for cell viability. *Yeast*, **9**, 295–299.
4. Woychik,N.A. and Young,R.A. (1989) RNA polymerase II subunit RPB4 is essential for high- and low-temperature yeast cell growth. *Mol. Cell. Biol.*, **9**, 2854–2859.
5. Choder,M. and Young,R.A. (1993) A portion of RNA polymerase II molecules has a component essential for stress responses and stress survival. *Mol. Cell. Biol.*, **13**, 6984–6991.
6. Choder,M. (2004) Rpb4 and Rpb7: subunits of RNA polymerase II and beyond. *Trends Biochem. Sci.*, **29**, 674–681.
7. Sharma,N. and Kumari,R. (2013) Rpb4 and Rpb7: multifunctional subunits of RNA polymerase II. *Crit. Rev. Microbiol*, **39**, 362–372.
8. Runner,V.M., Podolny,V. and Buratowski,S. (2008) The Rpb4 subunit of RNA polymerase II contributes to cotranscriptional recruitment of 3' processing factors. *Mol. Cell. Biol.*, **28**, 1883–1891.
9. Mosley,A.L., Hunter,G.O., Sardiu,M.E., Smolle,M., Workman,J.L., Florens,L. and Washburn,M.P. (2013) Quantitative proteomics demonstrates that the RNA polymerase II subunits Rpb4 and Rpb7 dissociate during transcriptional elongation. *Mol. Cell. Proteom.*, **12**, 1530–1538.
10. Harel-Sharvit,L., Eldad,N., Haimovich,G., Barkai,O., Duek,L. and Choder,M. (2010) RNA polymerase II subunits link transcription and mRNA decay to translation. *Cell*, **143**, 552–563.
11. Dahan,N. and Choder,M. (2013) The eukaryotic transcriptional machinery regulates mRNA translation and decay in the cytoplasm. *Biochim. Biophys. Acta*, **1829**, 169–173.
12. Haimovich,G., Choder,M., Singer,R.H. and Trcek,T. (2013) The fate of the messenger is pre-determined: a new model for regulation of gene expression. *Biochim. Biophys. Acta*, **1829**, 643–653.
13. Schulz,D., Pirkl,N., Lehmann,E. and Cramer,P. (2014) Rpb4 subunit functions mainly in mRNA synthesis by RNA polymerase II. *J. Biol. Chem.*, **289**, 17446–17452.

14. Armache,K.J., Mitterweger,S., Meinhart,A. and Cramer,P. (2005) Structures of complete RNA polymerase II and its subcomplex, Rpb4/7. *J. Biol. Chem.*, **280**, 7131–7134.
15. Vannini,A. and Cramer,P. (2012) Conservation between the RNA polymerase I, II, and III transcription initiation machineries. *Mol. Cell*, **45**, 439–446.
16. Hirtreiter,A., Grohmann,D. and Werner,F. (2010) Molecular mechanisms of RNA polymerase–the F/E (RPB4/7) complex is required for high processivity in vitro. *Nucleic Acids Res.*, **38**, 585–596.
17. Ujvari,A. and Luse,D.S. (2006) RNA emerging from the active site of RNA polymerase II interacts with the Rpb7 subunit. *Nat. Struct. Mol. Biol.*, **13**, 49–54.
18. Mitsuzawa,H., Kanda,E. and Ishihama,A. (2003) Rpb7 subunit of RNA polymerase II interacts with an RNA-binding protein involved in processing of transcripts. *Nucleic Acids Res.*, **31**, 4696–4701.
19. Kubicek,K., Cerna,H., Holub,P., Pasulka,J., Hrossova,D., Loehr,F., Hofr,C., Vanacova,S. and Stefl,R. (2012) Serine phosphorylation and proline isomerization in RNAP II CTD control recruitment of Nrd1. *Genes Dev.*, **26**, 1891–1896.
20. Kimura,M., Suzuki,H. and Ishihama,A. (2002) Formation of a carboxy-terminal domain phosphatase (Fcp1)/TFIIF/RNA polymerase II (pol II) complex in Schizosaccharomyces pombe involves direct interaction between Fcp1 and the Rpb4 subunit of pol II. *Mol. Cell. Biol.*, **22**, 1577–1588.
21. Tombacz,I., Schauer,T., Juhasz,I., Komonyi,O. and Boros,I. (2009) The RNA Pol II CTD phosphatase Fcp1 is essential for normal development in Drosophila melanogaster. *Gene*, **446**, 58–67.
22. Kamenski,T., Heilmeier,S., Meinhart,A. and Cramer,P. (2004) Structure and mechanism of RNA polymerase II CTD phosphatases. *Mol. Cell*, **15**, 399–407.
23. Eick,D. and Geyer,M. (2013) The RNA polymerase II carboxy-terminal domain (CTD) code. *Chem. Rev.*, **113**, 8456–8490.
24. Heidemann,M., Hintermair,C., Voss,K. and Eick,D. (2013) Dynamic phosphorylation patterns of RNA polymerase II CTD during transcription. *Biochim. Biophys. Acta*, **1829**, 55–62.
25. Chapman,R.D., Heidemann,M., Hintermair,C. and Eick,D. (2008) Molecular evolution of the RNA polymerase II CTD. *Trends Genet.*, **24**, 289–296.
26. Cho,E.J. (2007) RNA polymerase II carboxy-terminal domain with multiple connections. *Exp. Mol. Med.*, **39**, 247–254.
27. Meinhart,A., Kamenski,T., Hoeppner,S., Baumli,S. and Cramer,P. (2005) A structural perspective of CTD function. *Genes Dev.*, **19**, 1401–1415.
28. Schroder,S., Herker,E., Itzen,F., He,D., Thomas,S., Gilchrist,D.A., Kaehlcke,K., Cho,S., Pollard,K.S., Capra,J.A. *et al.* (2013) Acetylation of RNA polymerase II regulates growth-factor-induced gene transcription in mammalian cells. *Mol. Cell*, **52**, 314–324.
29. Kelly,W.G., Dahmus,M.E. and Hart,G.W. (1993) RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc. *J. Biol. Chem.*, **268**, 10416–10424.
30. Ranuncolo,S.M., Ghosh,S., Hanover,J.A., Hart,G.W. and Lewis,B.A. (2012) Evidence of the involvement of O-GlcNAc-modified human RNA polymerase II CTD in transcription in vitro and in vivo. *J. Biol. Chem.*, **287**, 23549–23561.
31. Sims,R.J. 3rd, Rojas,L.A., Beck,D., Bonasio,R., Schuller,R., Drury,W.J. 3rd, Eick,D. and Reinberg,D. (2011) The C-terminal domain of RNA polymerase II is modified by site-specific methylation. *Science*, **332**, 99–103.
32. Hanes,S.D.(2014) The Ess1 prolyl isomerase: traffic cop of the RNA polymerase II transcription cycle. Biochim. Biophys. Acta., **1839**, 316–333.
33. Verdecia,M.A., Bowman,M.E., Lu,K.P., Hunter,T. and Noel,J.P. (2000) Structural basis for phosphoserine-proline recognition by group IV WW domains. *Nat. Struct. Biol.*, **7**, 639–643.
34. Wu,X., Wilcox,C.B., Devasahayam,G., Hackett,R.L., Arevalo-Rodriguez,M., Cardenas,M.E., Heitman,J. and Hanes,S.D. (2000) The Ess1 prolyl isomerase is linked to chromatin remodeling complexes and the general transcription machinery. *EMBO J.*, **19**, 3727–3738.
35. Buratowski,S. (2003) The CTD code. *Nat. Struct. Biol.*, **10**, 679–680.
36. Buratowski,S. (2009) Progression through the RNA polymerase II CTD cycle. *Mol. Cell*, **36**, 541–546.
37. Hsin,J.P. and Manley,J.L. (2012) The RNA polymerase II CTD coordinates transcription and RNA processing. *Genes Dev.*, **26**, 2119–2137.
38. Jeronimo,C., Bataille,A.R. and Robert,F. (2013) The writers, readers, and functions of the RNA polymerase II C-terminal domain code. *Chem. Rev.*, **113**, 8491–8522.
39. Phatnani,H.P. and Greenleaf,A.L. (2006) Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev.*, **20**, 2922–2936.
40. Hengartner,C.J., Myer,V.E., Liao,S.M., Wilson,C.J., Koh,S.S. and Young,R.A. (1998) Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. *Mol. Cell*, **2**, 43–53.
41. Liu,Y., Kung,C., Fishburn,J., Ansari,A.Z., Shokat,K.M. and Hahn,S. (2004) Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. *Mol. Cell. Biol.*, **24**, 1721–1735.
42. Keogh,M.C., Cho,E.J., Podolny,V. and Buratowski,S. (2002) Kin28 is found within TFIIH and a Kin28-Ccl1-Tfb3 trimer complex with differential sensitivities to T-loop phosphorylation. *Mol. Cell. Biol.*, **22**, 1288–1297.
43. Komarnitsky,P., Cho,E.J. and Buratowski,S. (2000) Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. *Genes Dev.*, **14**, 2452–2460.
44. Rodriguez,C.R., Cho,E.J., Keogh,M.C., Moore,C.L., Greenleaf,A.L. and Buratowski,S. (2000) Kin28, the TFIIH-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II. *Mol. Cell. Biol.*, **20**, 104–112.
45. Schroeder,S.C., Schwer,B., Shuman,S. and Bentley,D. (2000) Dynamic association of capping enzymes with transcribing RNA polymerase II. *Genes Dev.*, **14**, 2435–2440.
46. Akhtar,M.S., Heidemann,M., Tietjen,J.R., Zhang,D.W., Chapman,R.D., Eick,D. and Ansari,A.Z. (2009) TFIIH kinase places bivalent marks on the carboxy-terminal domain of RNA polymerase II. *Mol. Cell*, **34**, 387–393.
47. Glover-Cutter,K., Larochelle,S., Erickson,B., Zhang,C., Shokat,K., Fisher,R.P. and Bentley,D.L. (2009) TFIIH-associated Cdk7 kinase functions in phosphorylation of C-terminal domain Ser7 residues, promoter-proximal pausing, and termination by RNA polymerase II. *Mol. Cell. Biol.*, **29**, 5455–5464.
48. Kim,M., Suh,H., Cho,E.J. and Buratowski,S. (2009) Phosphorylation of the yeast Rpb1 C-terminal domain at serines 2, 5, and 7. *J. Biol. Chem.*, **284**, 26421–26426.
49. Ahn,S.H., Kim,M. and Buratowski,S. (2004) Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. *Mol. Cell*, **13**, 67–76.
50. Cho,E.J., Kobor,M.S., Kim,M., Greenblatt,J. and Buratowski,S. (2001) Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. *Genes Dev.*, **15**, 3319–3329.
51. Price,D.H. (2000) P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Mol. Cell. Biol.*, **20**, 2629–2634.
52. Bartkowiak,B., Liu,P., Phatnani,H.P., Fuda,N.J., Cooper,J.J., Price,D.H., Adelman,K., Lis,J.T. and Greenleaf,A.L. (2010) CDK12 is a transcription elongation-associated CTD kinase, the metazoan ortholog of yeast Ctk1. *Genes Dev.*, **24**, 2303–2316.
53. Yao,S., Neiman,A. and Prelich,G. (2000) BUR1 and BUR2 encode a divergent cyclin-dependent kinase-cyclin complex important for transcription in vivo. *Mol. Cell. Biol.*, **20**, 7080–7087.
54. Tietjen,J.R., Zhang,D.W., Rodriguez-Molina,J.B., White,B.E., Akhtar,M.S., Heidemann,M., Li,X., Chapman,R.D., Shokat,K., Keles,S. *et al.* (2010) Chemical-genomic dissection of the CTD code. *Nat. Struct. Mol. Biol.*, **17**, 1154–1161.
55. Schreieck,A., Easter,A.D., Etzold,S., Wiederhold,K., Lidschreiber,M., Cramer,P. and Passmore,L.A. (2014) RNA polymerase II termination involves C-terminal-domain tyrosine dephosphorylation by CPF subunit Glc7. *Nat. Struct. Mol. Biol.*, **21**, 175–179.
56. Gibney,P.A., Fries,T., Bailer,S.M. and Morano,K.A. (2008) Rtr1 is the Saccharomyces cerevisiae homolog of a novel family of RNA polymerase II-binding proteins. *Eukaryot. Cell*, **7**, 938–948.
57. Mosley,A.L., Pattenden,S.G., Carey,M., Venkatesh,S., Gilmore,J.M., Florens,L., Workman,J.L. and Washburn,M.P. (2009) Rtr1 is a CTD

phosphatase that regulates RNA polymerase II during the transition from serine 5 to serine 2 phosphorylation. *Mol. Cell*, **34**, 168–178.
58. Egloff,S., Zaborowska,J., Laitem,C., Kiss,T. and Murphy,S. (2012) Ser7 phosphorylation of the CTD recruits the RPAP2 Ser5 phosphatase to snRNA genes. *Mol. Cell*, **45**, 111–122.
59. Xiang,K., Manley,J.L. and Tong,L. (2012) The yeast regulator of transcription protein Rtr1 lacks an active site and phosphatase activity. *Nat. Commun.*, **3**, 946.
60. Hsu,P.L., Yang,F., Smith-Kinnaman,W., Yang,W., Song,J.E., Mosley,A.L. and Varani,G. (2014) Rtr1 is a dual specificity phosphatase that dephosphorylates Tyr1 and Ser5 on the RNA polymerase II CTD. J. Mol. Biol., **426**, 2970–2981.
61. Bataille,A.R., Jeronimo,C., Jacques,P.E., Laramee,L., Fortin,M.E., Forest,A., Bergeron,M., Hanes,S.D. and Robert,F. (2012) A universal RNA polymerase II CTD cycle is orchestrated by complex interplays between kinase, phosphatase, and isomerase enzymes along genes. *Mol. Cell*, **45**, 158–170.
62. He,X., Khan,A.U., Cheng,H., Pappas,D.L. Jr, Hampsey,M. and Moore,C.L. (2003) Functional interactions between the transcription and mRNA 3' end processing machineries mediated by Ssu72 and Sub1. *Genes Dev.*, **17**, 1030–1042.
63. Krishnamurthy,S., Ghazy,M.A., Moore,C. and Hampsey,M. (2009) Functional interaction of the Ess1 prolyl isomerase with components of the RNA polymerase II initiation and termination machineries. *Mol. Cell. Biol.*, **29**, 2925–2934.
64. Zhang,D.W., Mosley,A.L., Ramisetty,S.R., Rodriguez-Molina,J.B., Washburn,M.P. and Ansari,A.Z. (2012) Ssu72 phosphatase-dependent erasure of phospho-Ser7 marks on the RNA polymerase II C-terminal domain is essential for viability and transcription termination. *J. Biol. Chem.*, **287**, 8541–8551.
65. Mayer,A., Heidemann,M., Lidschreiber,M., Schreieck,A., Sun,M., Hintermair,C., Kremmer,E., Eick,D. and Cramer,P. (2012) CTD tyrosine phosphorylation impairs termination factor recruitment to RNA polymerase II. *Science*, **336**, 1723–1725.
66. Cho,H., Kim,T.K., Mancebo,H., Lane,W.S., Flores,O. and Reinberg,D. (1999) A protein phosphatase functions to recycle RNA polymerase II. *Genes Dev.*, **13**, 1540–1552.
67. Hsin,J.P., Xiang,K. and Manley,J.L. (2014) Function and control of RNA polymerase II C-terminal domain phosphorylation in vertebrate transcription and RNA processing. *Mol. Cell. Biol.*, **34**, 2488–2498.
68. Ma,Z., Atencio,D., Barnes,C., DeFiglio,H. and Hanes,S.D. (2012) Multiple roles for the Ess1 prolyl isomerase in the RNA polymerase II transcription cycle. *Mol. Cell. Biol.*, **32**, 3594–3607.
69. Werner-Allen,J.W., Lee,C.J., Liu,P., Nicely,N.I., Wang,S., Greenleaf,A.L. and Zhou,P. (2011) cis-Proline-mediated Ser(P)5 dephosphorylation by the RNA polymerase II C-terminal domain phosphatase Ssu72. *J. Biol. Chem.*, **286**, 5717–5726.
70. Xu,Y.X., Hirose,Y., Zhou,X.Z., Lu,K.P. and Manley,J.L. (2003) Pin1 modulates the structure and function of human RNA polymerase II. *Genes Dev.*, **17**, 2765–2776.
71. Burke,D., Dawson,D. and Stearns,T. (2000) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, NY.
72. Liang,C. and Stillman,B. (1997) Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev.*, **11**, 3375–3386.
73. Garcia,A., Rosonina,E., Manley,J.L. and Calvo,O. (2010) Sub1 globally regulates RNA polymerase II C-terminal domain phosphorylation. *Mol. Cell. Biol.*, **30**, 5180–5193.
74. Schmitt,M.E., Brown,T.A. and Trumpower,B.L. (1990) A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. *Nucleic Acids Res.*, **18**, 3091–3092.
75. Tan,Q., Prysak,M.H. and Woychik,N.A. (2003) Loss of the Rpb4/Rpb7 subcomplex in a mutant form of the Rpb6 subunit shared by RNA polymerases I, II, and III. *Mol. Cell. Biol.*, **23**, 3329–3338.
76. Pappas,D.L. Jr and Hampsey,M. (2000) Functional interaction between Ssu72 and the Rpb2 subunit of RNA polymerase II in Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **20**, 8343–8351.
77. Reyes-Reyes,M. and Hampsey,M. (2007) Role for the Ssu72 C-terminal domain phosphatase in RNA polymerase II transcription elongation. *Mol. Cell. Biol.*, **27**, 926–936.
78. Singh,N., Ma,Z., Gemmill,T., Wu,X., Defiglio,H., Rossettini,A., Rabeler,C., Beane,O., Morse,R.H., Palumbo,M.J. *et al.* (2009) The Ess1 prolyl isomerase is required for transcription termination of small noncoding RNAs via the Nrd1 pathway. *Mol. Cell*, **36**, 255–266.
79. Ahn,S.H., Keogh,M.C. and Buratowski,S. (2009) Ctk1 promotes dissociation of basal transcription factors from elongating RNA polymerase II. *EMBO J.*, **28**, 205–212.
80. Kobor,M.S., Archambault,J., Lester,W., Holstege,F.C., Gileadi,O., Jansma,D.B., Jennings,E.G., Kouyoumdjian,F., Davidson,A.R., Young,R.A. *et al.* (1999) An unusual eukaryotic protein phosphatase required for transcription by RNA polymerase II and CTD dephosphorylation in S. cerevisiae. *Mol. Cell*, **4**, 55–62.
81. Visconti,R., Palazzo,L., Della Monica,R. and Grieco,D. (2012) Fcp1-dependent dephosphorylation is required for M-phase-promoting factor inactivation at mitosis exit. *Nat. Commun.*, **3**, 894.
82. Espinoza,F.H., Farrell,A., Nourse,J.L., Chamberlin,H.M., Gileadi,O. and Morgan,D.O. (1998) Cak1 is required for Kin28 phosphorylation and activation in vivo. *Mol. Cell. Biol.*, **18**, 6365–6373.
83. Kimmelman,J., Kaldis,P., Hengartner,C.J., Laff,G.M., Koh,S.S., Young,R.A. and Solomon,M.J. (1999) Activating phosphorylation of the Kin28p subunit of yeast TFIIH by Cak1p. *Mol. Cell. Biol.*, **19**, 4774–4787.
84. Qiu,H., Hu,C. and Hinnebusch,A.G. (2009) Phosphorylation of the Pol II CTD by KIN28 enhances BUR1/BUR2 recruitment and Ser2 CTD phosphorylation near promoters. *Mol. Cell*, **33**, 752–762.
85. Armache,K.J., Kettenberger,H. and Cramer,P. (2003) Architecture of initiation-competent 12-subunit RNA polymerase II. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 6964–6968.
86. Bushnell,D.A. and Kornberg,R.D. (2003) Complete, 12-subunit RNA polymerase II at 4.1-A resolution: implications for the initiation of transcription. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 6969–6973.
87. Minakhin,L., Bhagat,S., Brunning,A., Campbell,E.A., Darst,S.A., Ebright,R.H. and Severinov,K. (2001) Bacterial RNA polymerase subunit omega and eukaryotic RNA polymerase subunit RPB6 are sequence, structural, and functional homologs and promote RNA polymerase assembly. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 892–897.
88. Naji,S., Grunberg,S. and Thomm,M. (2007) The RPB7 orthologue E' is required for transcriptional activity of a reconstituted archaeal core enzyme at low temperatures and stimulates open complex formation. *J. Biol. Chem.*, **282**, 11047–11057.
89. Garrido-Godino,A.I., Garcia-Lopez,M.C. and Navarro,F. (2013) Correct assembly of RNA polymerase II depends on the foot domain and is required for multiple steps of transcription in Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **33**, 3611–3626.
90. Edwards,A.M., Kane,C.M., Young,R.A. and Kornberg,R.D. (1991) Two dissociable subunits of yeast RNA polymerase II stimulate the initiation of transcription at a promoter in vitro. *J. Biol. Chem.*, **266**, 71–75.
91. Jensen,G.J., Meredith,G., Bushnell,D.A. and Kornberg,R.D. (1998) Structure of wild-type yeast RNA polymerase II and location of Rpb4 and Rpb7. *EMBO J.*, **17**, 2353–2358.

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Sub1 contacts the RNA polymerase II stalk to modulate mRNA synthesis

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ABSTRACT

Biogenesis of messenger RNA is critically influenced by the phosphorylation state of the carboxy-terminal domain (CTD) in the largest RNA polymerase II (RNAPII) subunit. Several kinases and phosphatases are required to maintain proper CTD phosphorylation levels and, additionally, several other proteins modulate them, including Rpb4/7 and Sub1. The Rpb4/7 heterodimer, constituting the RNAPII stalk, promote phosphatase functions and Sub1 globally influences CTD phosphorylation, though its mechanism remains mostly unknown. Here, we show that Sub1 physically interacts with the RNAPII stalk domain, Rpb4/7, likely through its C-terminal region, and associates with Fcp1. While Rpb4 is not required for Sub1 interaction with RNAPII complex, a fully functional heterodimer is required for Sub1 association to promoters. We also demonstrate that a complete CTD is necessary for proper association of Sub1 to chromatin and to the RNAPII. Finally, genetic data show a functional relationship between Sub1 and the RNAPII clamp domain. Altogether, our results indicate that Sub1, Rpb4/7 and Fcp1 interaction modulates CTD phosphorylation. In addition, Sub1 interaction with Rpb4/7 can also modulate transcription start site selection and transcription elongation rate likely by influencing the clamp function.

INTRODUCTION

Eukaryotic RNA polymerase II (RNAPII) contains 12 subunits, Rpb1–Rpb12. Rpb1, the largest subunit, has a unique and highly conserved carboxy-terminal domain (CTD) with an essential role in transcription regulation *in vivo* (1–4). The RNAPII CTD is required for efficient capping, splicing and cleavage/polyadenylation of pre-mRNAs (5–7). It recruits RNA processing, export and histone modifying factors to the transcription complex, coupling mRNA biogenesis to other nuclear processes (3,8). Nearby the CTD and the RNA exit channel, two RNAPII subunits, Rpb4 and Rpb7, form a heterodimer that protrudes from the enzyme core like a stalk. From this location, the Rpb4/7 heterodimer regulates the interaction with factors important for RNA biogenesis, such as several components of the PIC and the Mediator (9–11), and for CTD modification. Indeed, we have shown that the Rpb4/7 heterodimer plays a key role in controlling phosphorylation of the CTD (12).

The CTD is composed of a repeated heptapeptide motif (26-52 depending upon the organism) with a consensus sequence of Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (13,14). Five of the seven residues can be phosphorylated, though Ser2 and Ser5 phosphorylation seem to be the predominant modifications (5,7,15). In mammals, the CTD can be also acetylated (16), glycosylated (17,18) and methylated (19). The multitude of possible CTD modifications, especially Ser phosphorylations, in combination with the numerous repetitions, generates a wide range of phosphorylation patterns that have been proposed to comprise a "CTD code" (20). The CTD modifications are dynamic and differentially orchestrate the recruitment of a number of factors required for transcriptional efficiency and RNA processing (21,22).

Several kinases and phosphatases regulate the levels of CTD phosphorylation (23). In *Saccharomyces cerevisiae*, four cyclin-dependent kinases (CDKs), Srb10, Kin28, Ctk1 and Bur1 (3,7) as well as four phosphatases, Rtr1, Ssu72, Glc7 and Fcp1 (23,24) determine CTD phosphorylation along the transcription cycle. Three additional yeast factors have been involved in the modulation of CTD phosphorylation: Ess1, Sub1 and Rpb4/7 (12,25,26). The peptidyl prolyl *cis–trans* isomerase Ess1 promotes the function of Ssu72 by isomerising CTD prolines in yeast, while human Pin1 isomerase assists Fcp1 activity (26–28). More recently, we have shown that Rpb4/7 heterodimer is important for recruiting Ssu72 and Fcp1 phosphatases (12). Sub1 globally modulates CTD phosphorylation all along the transcription cycle (25) although its mechanism remains essentially unknown. Sub1 was originally identified as a transcriptional stimulatory protein, homologous to the human positive coactiva-

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tor PC4 (29–33) that physically interacts with TFIIB, arguing for a role as a coactivator in transcription initiation (34,35). Indeed, Sub1 has been identified as a component of the pre-initiation (PIC) complex (36), and having a role in the selection of transcription start site (TSS) (37). Additionally, Sub1 has also been implicated in other aspects of mRNA biogenesis, such as elongation (38), transcription termination, and 3′end formation (39–41). Here, we present novel data demonstrating that Sub1 directly interacts with the Rpb4/7 heterodimer via Rpb7 and, in association with Fcp1, modulates the CTD phosphorylation levels. Moreover, our data suggest a role for Sub1 related to the Rpb1 clamp domain, in agreement with the role of Sub1 in the transcription start site (TSS) selection and in the regulation of the RNAPII transcription rate and its interaction with the elongation factor Spt5 (38).

MATERIALS AND METHODS

Yeast strains and media

The strains used are listed in Table 1 (Supplementary data). Strain construction and other genetic manipulations were performed following standard procedures (42). Oligonucleotide sequences are available upon request.

TAP purification and mass spectrometric analysis

Purification of Sub1–TAP was performed as described in (43). Sub1–TAP and associated proteins were recovered from cell extracts by affinity selection on an IgG matrix. After washing, TEV protease was added to release the bound material. The eluate was incubated with calmodulin-coated beads in the presence of calcium. After washing, the bound material was released by incubation with EGTA. The eluate was analyzed by Nano-ESI ion-trap mass spectrometry, or by trichloroacetic acid precipitation and analyzed by Western blotting.

Co-immunoprecipitation and western blot analysis

Cells containing TAP-tagged Sub1, and HA-tagged Rpb4/Rpb7 were grown in 200 ml of rich medium to an OD_{600} of 1.0, harvested, washed with water, followed by suspension in 2.5 ml of lysis buffer (20 mM HEPES pH 7.6, 200 mM potassium acetate, 1 mM EDTA pH 8.0, glycerol 10 %) containing protease and phosphatase inhibitors. The cell suspension was flash frozen in liquid nitrogen, and then ground in a chilled mortar to a fine powder. Afterwards, the cell lysate was thawed slowly on ice and transferred to pre-chilled tubes and centrifuged at 13 200 rpm for 20 min. The supernatant was collected and total protein concentration was estimated measuring absorbance at 280 nm in a nanodrop. In experiments where Sub1–TAP was precipitated, the volume of each cell extract containing 25 mg of protein was incubated with 50 μl of IgG Sepharose 6FF (GE Healthcare) slurry for 2 h at 4°C. In experiments where Rpb3 was immunoprecipitated, cell extracts were incubated with 5 μl of anti-Rpb3 antibody for 2 h at 4°C, followed by binding to 30 μl of Protein A Sepharose CL-4B (GE Healthcare) for another 2 h at 4°C. The IPs were extensively washed with lysis buffer and beads were suspended in SDS-PAGE sample buffer. Thereafter they were incubated at 65°C for 20 min and supernatants were loaded onto a SDS-PAGE gel. In the case of Rpb3 IP in whole cell extracts from *wt* and *rpb4*Δ, and *wt* and *rpb1-CTD11* cells containing Sub1–TAP, we proceeded similarly, except that cell lysis was achieved at 4°C using a FastPrep System and 5 mg of total protein was immunoprecipitated. This was also the procedure used to IP Sub1–HA to study Fcp1-MYC/Sub1–HA association. In addition, RNase A and DNAse I treatments were done by incubating washed beads in lysis buffer with 10 mg/ml RNase A at room temperature for 15 minutes or with 10 units/μl DNAse I for 20 min at 37°C in its own buffer.

Western blot analysis was performed using the appropriate antibodies in each case. Anti-phosphoglycerate kinase (Pgk1, 459250; Invitrogen), anti-HA (12CA5, Roche); anti-TAP (CAB1001, Open Biosystems); anti-Rpb1: Y-80 (sc-25758, Santacruz) and 8WG16 (8WG16 (nonP-CTD, Covance); anti-Rpb4 (2Y14; Santa Cruz); anti-Rpb3 (1Y26; Santa Cruz); anti-MYC (9E10, Santa Cruz) were acquired from the indicated vendors. The ECL reagents were used for detection. The signal was acquired on film and/or with a ChemiDoc XRS (Bio-Rad) system and when necessary quantified with the Quantity One software (Bio-Rad).

Expression and purification of recombinant proteins

Recombinant Sub1.

Transfection, virus amplification and protein expression. Sub1 recombinant bacmid was transfected into adherent High Five™ insect cells (Invitrogen). For virus amplification, recombinant baculovirus stocks were used to infect insect cells for four days. The amplified virus stock (25 ml) was obtained by cell pipetting and centrifugation at 2000 rpm for 5 min and then stored at 4°C. For protein expression, the virus amplification procedure was followed except that the flasks containing adherent insect cells were infected each with 70 μl of the amplified virus stock. Finally, the cells were harvested and frozen.

Purification. Frozen cell pellets were thawed and resuspended in lysis buffer (100 mM Tris pH 7.4, 300 mM NaCl, 6 mM $MgCl_2$, 10% (v/v) glycerol, half tablet of EDTA-free Protease Inhibitor Cocktail (Complete™, Roche) and DNase I (Roche)). Cells were sonicated, centrifuged and the supernatant loaded into a HisTrap HP (GE Healthcare) affinity chromatography column equilibrated in binding buffer (100 mM Tris pH 7.4, 300 mM NaCl, 10% (v/v) glycerol). The column was extensively washed with HTA buffer (100 mM Tris pH 7.4, 300 mM NaCl, 10% (v/v) glycerol, 20 mM Imidazole) and washing buffer (100 mM Tris pH 7.4, 1.5 M NaCl, 10% (v/v) glycerol, 20 mM Imidazole), and then re-equilibrated in HTA buffer. Protein was eluted by running a gradient from 0% to 100% HTB buffer (100 mM Tris pH 7.4, 300 mM NaCl, 10% (v/v) glycerol, 250 mM imidazole) in 20 column volumes and collected fractions were analyzed by SDS-PAGE. Selected fractions were pooled, concentrated in a 10 kDa cut-off centrifugal filter (Millipore) and loaded onto a Superdex 200 10/300 GL (GE Healthcare) column equilibrated in GF buffer (20 mM

Tris pH 7.4, 100 mM NaCl). The fractions containing the eluted protein were collected and analyzed by SDS-PAGE. The concentrated pool of the fractions containing the protein was frozen in liquid nitrogen and stored at −80°C.

To eliminate the His6-tag at the C-terminus, the protein was incubated with TEV protease (ratio Sub1:TEV 1:50) overnight at 4°C in a mixture containing 1 mM DTT. Digestion was confirmed by SDS-PAGE given the different electrophoretic mobilities of the His-tagged and untagged proteins. The digested protein was purified by recovering it from the supernatant after incubation of the reaction mix with a Ni resin (Ni Sepharose High Performance, Amersham Biosciences), where both undigested protein and TEV protease were captured through their polyhistidine tails. Fractions containing the digested protein were pooled and concentrated using Millipore centrifugal devices.

rRpb4/Rpb7-6His

We co-expressed and purified Rpb4/7 complex as described (44) from a plasmid containing Rpb4 and Rpb7, where a six-histidine tail was fused to the C-terminus of Rpb7.

Pull down-assays

Recombinant Rpb4/Rpb7-6His proteins (3 μg) were incubated in a 20 μl slurry of HisPur Cobalt Resine (Thermo Scientific) in binding buffer (20 mM Tris pH 7.5, 50 mM NaCl) for 2 h at 4°C. Thereafter, the resin with attached Rpb4/Rpb7-6His was washed three times with binding buffer and then Sub1 (3 μg) was added and incubated with the resin for 3 more hours at 4°C in binding buffer. As a negative control, the same amount of Sub1 was incubated in parallel under the same conditions with 20 μl slurry of Cobalt resin without Rpb4/7 proteins. After incubation, the resin was washed four times with washing buffer (20 mM Tris pH 7.5, 100 mM NaCl) and then treated with 15 μl of elution buffer (20 mM Tris pH 7.5, 100 mM NaCl, 150 mM imidazole) for 5 minutes at room temperature. The eluate was collected and analysed by SDS-PAGE. The gel was stained by immersion into Blue Safe staining solution (NZY tech) for several hours.

Chromatin immunoprecipitation (ChIP)

Chromatin purification, immunoprecipitation, quantitative real-time PCR (qPCR) amplification and data analysis were performed as described (12,25,45). Briefly, PCR of purified chromatin, following immunoprecipitation, was performed by quantitative real-time PCR with the CFX96 Detection System (Bio-Rad Laboratories, Inc.), using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) following the 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. Real-time PCR reactions were performed in triplicate using at least three independent ChIPs. Quantitative analysis was carried out using the CFX96 Manager Software (version 3.1, Bio-Rad). The values obtained for the IP'd PCR products were compared to those of the total input, and the ratio of the values from each PCR product from transcribed genes to a non-transcribed region of chromosome VII was calculated. Numbers on the y-axis of graphs are detailed in the corresponding figure legend.

RNA isolation and RT-PCR

Total RNA was extracted as described (46) and RT-PCR was performed using the iScript RT reagent Kit (Bio-Rad), following the manufacturer's instructions. PCR reactions were performed in triplicate with at least three independent cDNA samples.

Sub1 mutagenesis

The *sub1-FRN54-56AGG* triple mutation was generated by site-directed mutagenesis of a wild-type copy of *SUB1* cloned into a centromeric plasmid under the control of its own promoter and with a 6x-HA epitope at the C-terminus of the protein. Plasmid expressing the ssDNA binding domain mutation or a *wt* copy of *SUB1* was used to transform the *sub1Δ* mutant and to generate the *sub1-FRN54-56AGG* strain. As a control, the *sub1Δ* strain was transformed with an empty plasmid.

RESULTS

Sub1 interacts with RNAPII through the Rpb4/7 heterodimer

Sub1 influences RNAPII CTD phosphorylation via all four CTD kinases: Kin28, Srb10, Bur1 and Ctk1 (25). This was observed both genetically and biochemically, including Sub1 effects on kinase activity and/or recruitment to chromatin. Thus, Sub1 can act throughout the transcription cycle as a general regulator of CTD phosphorylation, though we do not understand the biochemical basis of the influence of Sub1 on the activity of all four CTD kinases. We considered two possible explanations: (i) Sub1 enhances the association (or dissociation) of an unidentified, common regulator with the kinases; or (ii) Sub1 influences kinase accessibility to the CTD.

To further explore the first possibility, we decided to analyse the repertoire of Sub1 physical interactions using the TAP strategy (43). Accordingly, Sub1 was TAP-tagged and associated proteins were MS-analysed. We found among other proteins Spt5, consistent with previous work (38,47). More interestingly, we identified the Rpb7 subunit of RNAPII as a co-purifying protein. This discovery is extremely interesting since Rpb7, together with Rpb4, is near the CTD, and the heterodimer Rpb4/7 has been functionally related with CTD phosphorylation (12). To corroborate the specificity of Sub1-Rpb7 association, we purified Sub1–TAP complexes in a double tagged strain, Sub1–TAP Rpb7–HA, and analyzed the TAP purified complex by western blot using the corresponding antibodies to visualize Sub1 and Rpb7 (Figure 1A). We confirmed Sub1–TAP/Rpb7–HA association by co-immunoprecipitation (Co-IP), and demonstrated that Sub1 also associates with Rpb4, using a tripled tagged strain Sub1–TAP Rpb7–HA Rpb4–HA (Figure 1B). These data indicate that Sub1 associates with the Rpb4/7 heterodimer.

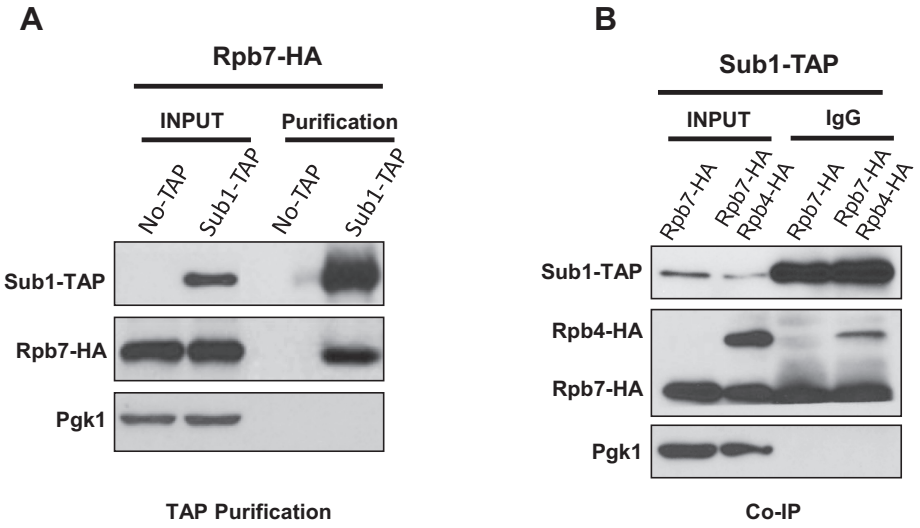

Figure 1. Sub1 interacts with the RNAPII through the Rpb4/7 heterodimer. (**A**) Sub1 tandem affinity purification from Sub1–TAP and Sub1–TAP Rpb7–HA whole cell extracts (WCE). Input and the purified proteins were precipitated with trichloroacetic acid and analyzed by western blotting using anti-TAP and anti-HA. Anti-Pgk1 was used as a loading control. (**B**) Co-IP performed using WCEs from Sub1–TAP (Rpb7–HA and Rpb7–HA/Rpb4–HA) with IgG Sepharose. Input and IPs were analyzed by western blotting with antibodies to the indicated proteins.

Allelic specific interaction between *SUB1* and *RPB4/7*

To gain more insight into the functional connection between Sub1 and the RNAPII stalk heterodimer, we analyzed genetic interaction between *SUB1* and *RPB4/7.* As Rpb7, but not Rpb4, is essential for cell viability we worked with the *rpb4Δ* mutant that displays slow growth at 28°C and thermosensitivity at 37°C (48,49). Deletion of *SUB1* partially suppresses the *rpb4Δ* growth defects at 28 and 34°C, but does not suppress *rpb4Δ* thermosensitivity at 37°C (Figure 2A). On the other hand, overexpression of *SUB1* using a strong constitutive or inducible promoter (*ADH1* and *GAL1*, respectively; Figure 2A and Supplementary Figure S1A), dramatically reduces *rpb4Δ* cell growth. In addition, we used an Rpb7-TAP strain where the C-terminal Rpb7-TAP tag causes growth defects (Supplementary Figure S1B). In this case, the growth defects at 28 and 34°C are also partially rescued by *SUB1* deletion (Supplementary Figure 1B). Therefore, *SUB1* genetically interacts with *RPB4* and *RPB7,* in agreement with the association of Sub1 with the Rpb4/7 heterodimer (Figure 1). However, this genetic result was unexpected, because deletion of *SUB1* mostly displays negative genetic interactions with mutations compromising transcription initiation and elongation, for instance mutations in TFIIB, TFIIE, TFIIH, Kin28, Ctk1, Bur1, Fcp1, Spt5/Spt4 (25,36,38,41,50). The positive genetic interaction between *SUB1* and *RPB4* and *RPB7* suggests that Sub1 may be gaining a function when the heterodimer function is altered.

To investigate the specificity of this interaction, we analyzed the genetic interaction between *SUB1* and *RPB1.* For that purpose we choose three *rpb1* alleles. Two of them are functionally and structurally related to Rpb4/7, *rpo21-4* and *rpb1-L1397S*, which are localized in the foot and clamp domains, respectively (51,52); and a third one, *rpb1-19,* is localized in the *jaw* domain (53), far from Rpb4/7 within the context of RNAPII complex (54).

The foot domain is a conserved region of RNAPII located at the surface of the complex, with poor or no conservation in their paralogs, Rpa190 (RNAPI) and Rpc160 (RNAPIII), or in their homologs in archaea and bacteria (55,56). The foot domain is crucial for the assembly and stability of RNAPII, by ensuring the correct association of Rpb1 with Rpb6 and Rpb4/Rpb7. In fact, assembly defects alter not only transcriptional activity but also RNAPII-DNA association and CTD phosphorylation (51). Thus, we used the *rpo21-4* (*rpb1-W954-LELE-955*) foot mutant (57,58) where the association of the Rpb4/7 heterodimer with RNAPII is reduced (51). This mutant displays reduced growth at 28°C and exhibits slow growth at 34°C and 37°C (Figure 2B). Interestingly, we observed that deletion of *SUB1* suppressed *rpo21-4* growth defects at 34 and 37°C, whereas *sub1Δ* does not affect growth at these temperatures in a *wt RPB1* background. Hence, *rpo21-4* suppression recapitulates the growth defects observed in the double mutant *rpb4Δsub1Δ.* These data indicate that *sub1Δ* suppresses mutations compromising the function of the Rpb4/7 heterodimer, either by deletion of *RPB4* or by a mutation on the Rpb1 foot domain.

The RNAPII clamp is a flexible structural element postulated to intervene in the regulation of DNA access to the cleft (59). An open clamp is observed in the absence of the Rpb4/7 heterodimer (56), while the complete enzyme presents a closed clamp conformation (54). Rpb4/7 plays a role in the mobility of this domain during transcription initiation (60,61). Therefore, we studied whether a *sub1Δ* deletion might genetically interact with the *rpb1-L1397S* clamp mutation. Specifically, this amino acid substitution occurs in the Rpb1 α-47b helix of the Switch 1 loop of the catalytic site within the structure of the RNAPII. In fact, the Switch 1 loop holds the DNA template strand at position +2/+3 downstream of the catalytic Mg^{2+} (62). The *rpb1-L1397S* mutant displays slow growth at 28°C, cold and thermosensitivity and reduced RNAPII associa-

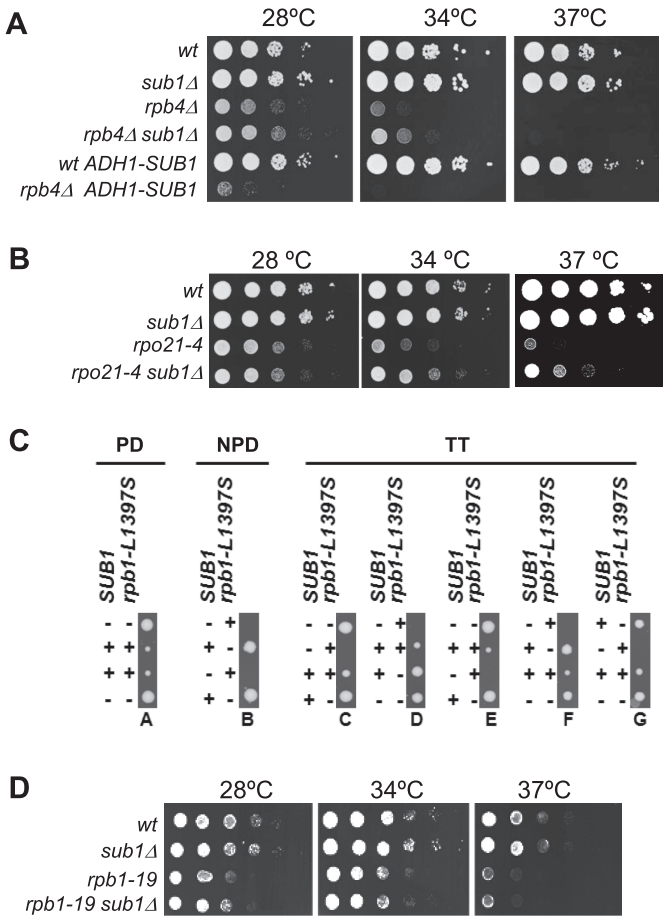

Figure 2. Allelic specific interaction between *SUB1* and *RPB4/7*. (**A**) Genetic interaction between *SUB1* and *RPB4*. *SUB1* deletion partially suppresses the slow growth phenotype of the *rpb4*Δ strain at 28 and 34°C, while overexpression of *SUB1* exacerbates it. *SUB1* was overexpressed from a strong constitutive *ADH1* promoter. Serial dilutions (1:10) of *wt* and mutant strains were spotted on selective SC media and grown for 2–3 days at the indicated temperatures. (**B**) Genetic interaction between *SUB1* and the *rpo21-4* mutation localized in the Rpb1 foot domain. Deletion of *SUB1* partially suppresses the slow growth phenotype of *rpo21-4* strain at 28, 34 and 37°C. Cells were assayed as in (A) in rich media. (**C**) *sub1Δ* and *rpb1-L1397S* are synthetically lethal. A diploid yeast strain heterozygous for both *SUB1* and *rpb1-L1397S* was sporulated and the meiotic progeny were separated by tetrad dissection, and allowed to grow for 3 days. Forty-six tetrads were dissected, with thirty showing tetratype segregation pattern (TT, five of which are shown), nine showing paternal ditype (PD) and 7 a nonpaternal ditype (NPD). The genotype of the resulting colonies was inferred by growth or no growth on selective medium and 37°C. The *sub1Δ* deletion is indicated as (−), and *wt* (+); and in the case of *RPB1*, (+) means *rpb1-L1397S* and (−) *wt*. Cells deleted for *rpb1-L1397S* alone (− −) show a slow growth phenotype, as reported previously. Double mutants cells (− +) were unviable. (**D**) There is not genetic interaction between *sub1Δ* and the *rpb1–19* mutation on the jaw domain of Rpb1. Serial dilution assay as in (B) showing that single and double mutants, *rpb1–19* and *rpb1-19sub1Δ* respectively, display similar growth.

tion with genes (52). Both, *rpb1-L1397S* and *sub1Δ* show upregulation of *IMD2* transcription (38,52), which is a hallmark of defects on TSS selection (63). Indeed, it has been shown that Sub1 participates in TSS selection (37). We then crossed the *rpb1-L1397S* mutant with the *sub1Δ* deletion mutant. The diploid cells were sporulated and the tetrads dissected. Interestingly, *sub1Δ* conferred synthetic lethality in combination with *rpb1-L1397S* (Figure 2C). This could be explained as a result of the additive effect of both mutations, *sub1Δ* and *rpb1-L1397S,* reducing RNAPII gene occupancy during transcription to levels that may be incompatible with cell viability (41,45,52). Also, synthetic lethality may be the result of the combined defect on elongation (38,64,65), and/or on the TSS selection (37,52). In any case and importantly, this result shows the essentiality of Sub1 in a context where the clamp function is impaired.

We also tested the effect of *SUB1* deletion on the *rpb1-19* jaw mutant. In this case no genetic interaction was observed (Figure 2D). As noted above, this domain is situated far from Rpb4/7 within the RNAPII structure (54). All of these data indicate an allele-specific interaction between *sub1Δ* and mutations localized within or proximal to the Rpb4/7 heterodimer in the context of the RNAPII 10 subunit complex. In addition, they corroborate the specificity of a functional relationship between Sub1 and the Rpb4/7 heterodimer consistent with their physical association (Figure 1).

A functional Rpb4/7 heterodimer is a requisite for Sub1 recruitment to gene promoters

We next asked if Sub1 association with promoter DNA was affected in *rpb4Δ* cells, because in these cells Rpb7 levels are significantly reduced, along with diminished association of RNAPII with DNA (66–68). Thus, we performed chromatin immunoprecipitation (ChIP) using the *wt* and *rpb4Δ* Sub1–HA tagged strains, where in addition to Sub1, we also analyzed Rpb1 and Rpb3 occupancy at the promoters of several constitutively transcribed genes. In agreement with published data (12), Rpb4 is required for proper RNAPII occupancy to gene promoters as inferred from reduced Rpb1 and Rpb3 association levels (Figure 3A, left and middle panel, respectively). Interestingly, Sub1 occupancy was also dramatically decreased in *rpb4Δ* cells. Thus, <20% of Sub1 was associated with chromatin in cells lacking Rpb4 (Figure 3A, right panel). To determine if this was simply a consequence of reduced RNAPII occupancy, we calculated Sub1/Rpb1 and Sub1/Rpb3 ratios, and observed that this was not the case, because the levels of Sub1 association with chromatin were even lower than those of Rpb1 and Rpb3 (Figure 3B). We observed that these effects on chromatin association are not due to diminished Sub1–HA protein levels, as they are unaltered in *rpb4Δ* cells (Figure 3C). Although we did not know whether Sub1 directly interacts with Rpb7 and/or Rpb4, it is possible that reduced Rpb7 levels in *rpb4Δ* cells (Figure 3D, and (66–68)) could account for the Sub1 defect on promoter association. We also tested Sub1–TAP association with chromatin in the clamp mutant, *rpb1-L1397S.* No effect was observed: Sub1 was properly detected at gene promoters in the mutant cells (Figure 3E, left panel) with no change in Sub1–TAP levels (Figure 3E, right panel).

In conclusion, our data indicate that a functional Rpb4/7 heterodimer is required for Sub1 association with gene promoters. It has been suggested that Sub1, together with TFIIB, functions in the recruitment of RNAPII to constitutively transcribed genes. Hence, the reduced level of Sub1 bound to promoters in *rpb4Δ* cells is likely not due to re-

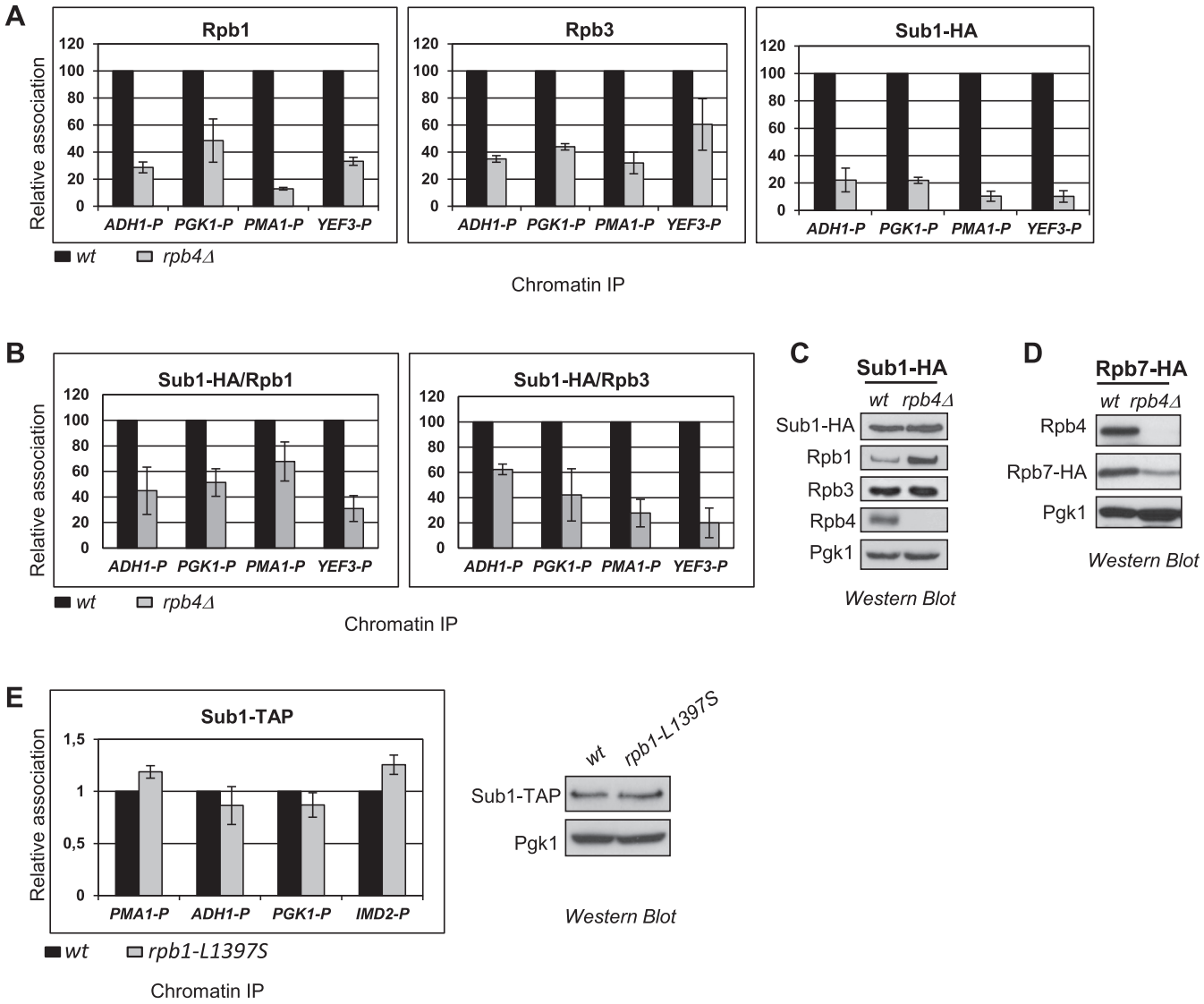

Figure 3. A functional Rpb4/7 heterodimer is a requisite for Sub1 recruitment to gene promoters. (**A**) Chromatin immunoprecipitation (ChIP) analyses were performed using *wt* and *rpb4*Δ strains. Left panel: Rpb1 binding to the promoter (P) of four constitutively expressed genes, *ADH1, PGK1, PMA1* and *YEF3* was examined by qPCR. Results were quantified (see Materials and Methods), and relative Rpb1 binding in *rpb4*Δ cells is plotted relative to that in *wt* cells (set equal to 100). The data plotted correspond to mean values from at least three independent experiments, and the error bars represent standard deviations. Middle and Right panels show relative Rpb3 and Sub1–HA binding, respectively, plotted as for Rpb1. (**B**) Plots of Sub1–HA/Rpb1, Sub1–HA /Rpb3 ratios in wild-type (*wt*) and *rpb4*Δ using data from Figure 1A. (**C**) Whole cell extracts (WCE) were prepared from wild-type (*wt*) and *rpb4*Δ strains expressing Sub1–HA and analyzed by western blotting using the following antibodies: anti-HA, anti-Rpb1 (8W16G), anti-Rpb3, anti-Rpb4 (2Y14) and anti-Pgk1 as a control for total protein. (**D**) Whole cell extracts (WCE) were prepared from wild-type (*wt*) and *rpb4*Δ strains expressing Rpb7–HA and analyzed by Western blotting using anti-HA, anti-Rpb4 and anti-Pgk1. (**E**) Left panel: ChIP analysis in *wt* and *rpb1-L1397S* cells as in (A) to analyze Sub1–TAP occupancy at the promoter (P) of three constitutively expressed genes (*PMA1*, *ADH1* and *PGK1*) and to the promoter of the *IMD2* inducible gene, whose expression is regulated by Sub1 (38,82), and is upregulated in the *rpb1-L1397S mutant* (52). Right panel: WCE were prepared from wild-type (*wt*) and *rpb1-L1397S* strains expressing Sub1–TAP and analyzed by Western blotting using anti-TAP and anti-Pgk1 as loading control.

duced RNAPII recruitment. Rather, it is more likely that Sub1 cannot be maintained stably associated to the DNA, and/or disassociates faster from the promoter, in the absence of the Rpb4/7 heterodimer.

Sub1 associates with the Rpb4/7 heterodimer via direct interaction with Rpb7

We next proceeded with *in vitro* assays to investigate whether the Rpb4/7-Sub1 interaction may be direct. For that purpose we carried out pull down assays using recombinant Sub1 and Rpb4/7 proteins. As shown in Figure 4A, Sub1 remains associated with Rpb4/7 after extensive washing, indicating that it directly interacts with the heterodimer. This result demonstrates for the first time that Sub1 interacts directly with the RNAPII complex. However, we did not know the direct target of Sub1: Rpb7, Rpb4 or both.

To identify the direct target of Sub1, we co-immunoprecipitated Sub1–TAP and Rpb7–HA using whole cell extracts from a *wt* strain expressing Rpb4–HA

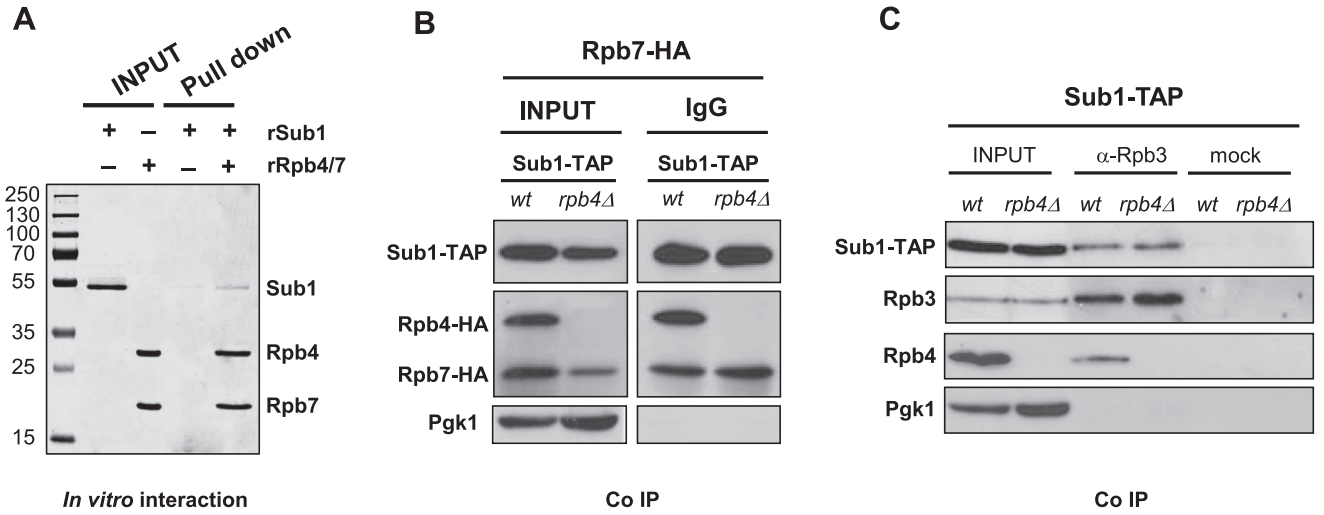

Figure 4. Sub1 associates with the Rpb4/7 heterodimer via direct interaction with Rpb7. (**A**) Pull down assay. 15% SDS-PAGE gel showing bands corresponding to recombinant proteins rSub1 and rRpb4/Rpb7-6His. (lanes 2 and 3, respectively) and pull down assays where rSub1 and a Co^{2+} resin have been incubated in the absence (lane 4) and in the presence of rRpb4/Rpb7-6His (lane 5). **(B)** Co-IP performed with IgG Sepharose using WCEs from *wt* strains expressing Sub1–TAP and Rpb7–HA/Rpb4–HA and from *rpb4Δ* cells expressing Sub1–TAP and Rpb7–HA. Input and IPs were analyzed by Western blotting with antibodies to the indicated proteins. (**C**) Co-IP performed on WCEs from Sub1–TAP cells (*wt* and *rpb4*Δ) using anti-Rpb3 antibody. As a control, the same amount of cell extracts was incubated only with Protein A sepharose. Inputs and IPs were analyzed by Western blotting with antibodies to the indicated proteins.

or from the *rpb4Δ* strain, and analyzed its association by western blot to determine the contribution of Rpb4 to Sub1 interaction with the heterodimer. Surprisingly, we observed that Sub1 still interacts with Rpb7 when lacking Rpb4 (Figure 4B). In fact, the interaction between Sub1 and Rpb7 is similar in isogenic *RPB4 wt* and *rpb4Δ* cells, though Rpb7–HA levels are reduced in the absence of Rpb4 as previously described (66–68). Therefore, from all these data we conclude that the target of Sub1 is Rpb7, in agreement with identifying only Rpb7 in the Sub1–TAP purifications (Figure 1A). This result also supports our conclusion that reduced association of Sub1 to chromatin in *rpb4Δ* cells is due to decreased Rpb7 levels (Figure 3A, D). In summary, our data clearly demonstrate that Sub1 interacts with Rpb4/7 and indicate that this interaction could explain how Sub1 is able to modulate CTD phosphorylation.

Additionally, we tested if Sub1 association with the core polymerase is influenced by Rpb4. For that purpose we immunoprecipitated Rpb3 from *wt* and *rpb4Δ* whole cell extracts expressing Sub1–TAP, and analyzed Sub1 association by western blot. As shown in Figure 4C, Sub1–TAP associates with RNAPII in the absence of Rpb4. Therefore, our results indicate that Sub1 targets Rpb7 to be associated with the RNAPII, though we cannot exclude that it can also interact with other RNAPII subunits. On the other hand, and consistent with *sub1Δ* suppression of *rpb4Δ* (Figure 2A and Supplementary Figure S1A), Sub1 may have a negative effect during transcription when interacting with Rpb7 in the absence of Rpb4.

Sub1 carboxy-terminal region is important for the functional interaction with Rpb4/7

Sub1 is a 292-residue polypeptide showing strong similarity to its human homolog PC4 (127 amino acids) over a 65-residue region (amino acids 41–105) that includes a single-stranded DNA binding domain (DBD) and sequences essential for co-activator function (33,34,69). Although Sub1 is highly related to PC4, yeast Sub1 is much larger than human PC4 (33 KDa versus 15 KDa (34,35,39). Specifically, Sub1 has an extra carboxy-terminal (CT) region of ∼190 amino acids with unknown function(s), suggesting that Sub1 might have functional differences from PC4 due to this additional domain. Like PC4, Sub1 has the capacity to tightly bind melted DNA and single-stranded DNA *in vitro* (34) and both have been implicated in DNA-dependent processes other than transcription, such as DNA repair and replication (69–72).

To explore which Sub1 region is involved in Rpb4/7 interaction, we investigated the importance of the Sub1 CT region and DNA-binding domain (DBD) relative to its interaction with Rpb4/7. For that purpose, two mutants were generated: (i) a strain where the chromosomal CT region of Sub1 (from amino acid 105 to stop) was substituted by a 6xHA epitope; and (ii) a triple-mutant, *sub1-FRN54-56AGG*, which encodes Phe54 → Ala, Arg55 → Gly and Asn56 → Gly replacements. Mutations in the corresponding residues in PC4 (Phe77, Lys78 and Lys79) severely affected the binding of PC4 to ssDNA *in vitro* (33). Protein expression of all the mutants was confirmed by western blot (Supplementary Figure S2A, upper panel). As shown, the levels of the Sub1ΔCT-HA protein are very low compared to that of Sub1 levels in the isogenic *wt* and *sub1-FRN54-56AGG* cells, and this effect is not due to defective transcription (Supplementary Figure S2A, lower panel). In *sub1ΔCT*

cells, where Sub1 contains only the DNA binding domain, the protein becomes very unstable.

We next analyzed the association of Sub1–HA with the promoters of several constitutively transcribed genes (*PMA1, PGK1, PYK1*), and with the promoter of the Sub1 target gene *IMD2*, in normal growth conditions (Figure 5A). For that purpose, we performed ChIP assays first in *wt*, and *sub1ΔCT* expressing Sub1–HA and Sub1ΔCT-HA, respectively (Figure 5A, left panel). As expected, Sub1–HA was efficiently recruited to the promoter of all of genes tested in *wt* cells. However, unexpectedly and very interestingly, Sub1 association was either unaffected or only slightly affected in *sub1ΔCT* cells (Figure 5A, left panel), although Sub1ΔCT-HA protein levels were extremely low (Supplementary Figure S2A). This result indicates that the C-terminal domain of Sub1 is not required for Sub1 occupancy of gene promoters, but can contribute to it. Thus, the CT domain, which is not present in PC4, might be involved in other functions, such as the regulation of Sub1 binding to the DNA and/or in the interaction with other factors. In contrast, and as expected, Sub1 association was almost abolished in *sub1-FRN54-56AGG* cells (Figure 5A, right panel), which is consistent with the importance of these residues for PC4 DNA binding capacity and with the clear homology between Sub1 and PC4 in the DBD (33,69,73).

We further tested the genetic interaction between *RPB4* and the Sub1 CT region and Sub1 DBD. Interestingly, deletion of the Sub1 CT region in *rpb4Δ* cells rescues their growth defects at 28 and 34°C (Figure 5B), recapitulating the effects of the *SUB1* complete deletion (see Figure 2A). In contrast, no effect was observed when the *SUB1* DBD mutation was expressed in the *rpb4Δ sub1Δ* cells (Figure 5C, Supplementary Figure S2B). Thus, it is likely that Sub1 may need the CT region to interact with Rpb4/7 upon its association to promoters through the DBD. This agrees with some decrease of Sub1 crosslinking at the promoters in the absence of the CT region (Figure 5A, left panel). If our hypothesis is correct, we should observe that association of Sub1ΔCT-HA with gene promoters decreases when lacking *RPB4*. Indeed, this is what we observed when we performed ChIP assays in *sub1ΔCT-HA* and *sub1ΔCT-HA rpb4Δ* cells (Figure 5D). Sub1ΔCT-HA association with the gene promoters is significantly reduced in *rpb4Δ* cells. Though this reduction (∼60–50%) is lower than the one observed for Sub1–HA *wt* in the absence of Rpb4 (∼80%, Figure 3A), it supports our idea that Sub1-CT region may interact with Rpb4/7 to be stably associated with the chromatin. Moreover, our data corroborate the genetic interaction between *SUB1* and *RPB4* and offer insight into the function(s) of Sub1 CT region, which has not been identified previously.

Sub1 and Fcp1 are components of the same complex

Several facts suggested that Sub1 and Fcp1 interact, at least as part of the same complex where the heterodimer Rpb4/7 is present. First, we reported that Sub1 is a regulator of Fcp1 and our data indicated that a function of Sub1 is to facilitate accumulation of Fcp1, likely by directly or indirectly increasing its stability. Additionally we showed that Fcp1 crosslinking to DNA was significantly reduced in the *sub1Δ* strain, with comparable reductions at both 5′ coding and terminator regions (41). Second, a number of findings suggested that Rpb4/7 might recruit Fcp1 to regulate CTD modifications, among them our more recent work showing that Fcp1 association to chromatin is impaired during the entire transcription cycle in the absence of Rpb4 (12). Furthermore, *in vitro* binding and yeast two-hybrid assays in *S. pombe* and *D. melanogaster*, respectively, showed that Rpb4 interacts with Fcp1 (74,75). Structural and biochemical studies also suggest that in *S. cerevisiae* Fcp1 might also interact with RNAPII through Rpb4/7 (76). Third, here we have clearly shown that Rpb4/7 interact with Sub1.

To investigate if Sub1 and Fcp1 interact, we performed CoIPs assays. We detected Fcp1-MYC by IP of Sub1–HA (Figure 6A). Furthermore, CoIPs assays showed that Sub1-Fcp1 interaction is resistant to treatment with either DNAse I or RNAse A, and only strong washing conditions, such as high salt (500 mM NaCl), weakened their association (Figure 6B). These data strengthen the conclusion that Sub1 is functionally related to RNAPII CTD phosphorylation and indicates that this function is likely to be executed via its interaction with Rpb4/7 and Fcp1. Moreover, our data uncover the existence of a physical and functional connection between Sub1, Rpb4/7 and Fcp1 which is important to maintain proper RNAPII phosphorylation levels, key in the biogenesis of mRNAs.

A full length Rpb1-CTD is required for efficient Sub1 association to gene promoters

Our data suggest that Sub1 may regulate CTD phosphorylation through interaction with Rpb4/7 and Fcp1, based on demonstration that Rpb4/7 is required for CTD dephosphorylation by CTD phosphatases (12). In addition, in an extensive genetic interaction network, *SUB1* and the CTD interact (77). This study revealed a number of significant genetic interactions as the CTD was progressively shortened. Indeed a negative genetic interaction between *rpb1-CTD11* (with a CTD containing only eleven heptad repeats) and the *SUB1* deletion was observed. Although we did not find a direct interaction between Sub1 and the CTD, we wondered if the CTD, as well as Rpb4/7, may contribute to Sub1 occupancy at promoters and to its association with RNAPII. To answer the question we took advantage of several CTD truncation mutants containing 11 and 20 repeats (*rpb1-CTD11* and *rpb1-CTD20*), respectively (77). First, we deleted *SUB1* in the *rpb1-CTD11* mutant to confirm the genetic interaction. As shown in Figure 7A, deletion of *SUB1* exacerbates the growth phenotype of the *rpb1-CTD11* cells. We next decided to investigate if Sub1 association to gene promoters was dependent on the length of the CTD. For that purpose, we TAP tagged Sub1 in the *RPB1-CTD wt*, *rpb1-CTD11* and *rpb1-CTD20* strains and we analyzed Sub1–TAP occupancy by ChIP in several gene promoters (Figure 7B and C). Interestingly, the occupancy of Sub1 in these regions gets worse as the number of CTD repeats decreases. Thus, in *rpb1-CTD11* cells, Sub1 occupancy of gene promoters is significantly diminished when compared to *wt* cells (Figure 7B, left panel) and this is not due to reduced Sub1 levels (Figure 7B, right panel). On the other hand, in *rpb1-CTD20* cells, Sub1 association with chromatin diminishes only slightly (Figure 7C) compared to

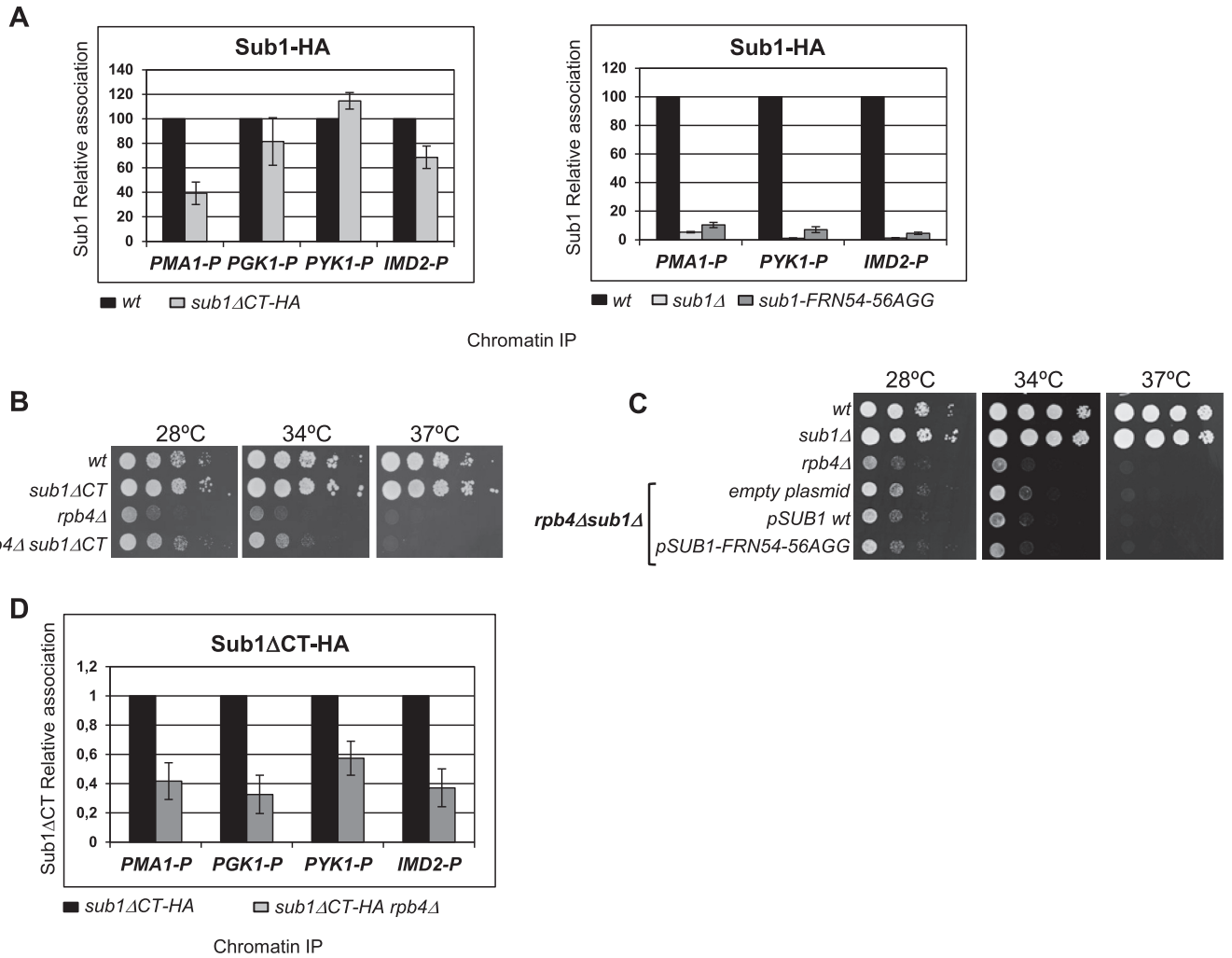

Figure 5. Sub1 carboxy-terminal region is important for the functional interaction with Rpb4/7. (**A**) Left panel: Chromatin immunoprecipitation (ChIP) analyses were performed using *wt* and *sub1ΔCT-HA* strains expressing Sub1–HA. Sub1 binding to the promoter (P) of three constitutively expressed genes, *PMA1, PGK1, PYK1* and the inducible gene *IMD2* was examined by qPCR. Results were quantitated (as in figure 3), and relative Sub1–HA binding in *sub1ΔCT-HA* cells is plotted relative to that in *wt* cells (set equal to 100). Right panel, Chromatin immunoprecipitation (ChIP) analyses were performed using *wt*, *SUB1* deletion mutant (*sub1*Δ), and the DNA binding mutant *sub1-FRN54-56AGG*. (**B**) Genetic interaction between *sub1ΔCT* and *rpb4Δ*. *SUB1* deletion partially suppresses the slow growth phenotype of *rpb4*Δ strain at 28 and 34°C in rich medium. Serial dilutions (1:10) of *wt* and mutant strains were spotted on rich medium and grown for 2–3 days at the indicated temperatures. (**C**) There is no genetic interaction between the ssDNA binding domain of Sub1 and *RPB4*. Serial dilutions (1:10) of the indicated strains were spotted on selective SC medium and grown for 2–3 days at the indicated temperatures. (**D**) ChIP analyses were performed in *wt* and *rpb4Δ* cells expressing Sub1ΔCT-HA protein.

wt cells. Moreover, the effect on Sub1 occupancy dependent on CTD length is specific for Sub1, as no effect is observed for Rpb4 and Rpb3 crosslinking in *rpb1-CTD11* cells (Figure 7B).

We next asked if Sub1–TAP association to the RNAPII was also affected when the CTD has only eleven repeats. In this case, we performed CoIP assays using *wt* and *rpb1-CTD11* whole cell extracts (Figure 7D). First, we analyzed levels of Rpb1 by western blot using whole cell extracts from *wt, rpb1-CTD11* and *rpb1-CTD20* cells. We used two different antibodies: 8WG16 directed against the CTD and Y-80 that recognizes the N-terminus of Rpb1 (Figure 7B, right panel and Figure 7C, right panel). We observed that levels of Rpb1 were significantly decreased in *rpb1-CTD11* when analyzed with the 8WG16 antibody, as previously shown (77) and in agreement with the number of CTD repetitions (11 versus 25–26 in an otherwise *wt* cell). However, Y-80 antibody showed increased Rpb1 levels. These effects were not observed in the *rpb1-CTD20* whole cell extracts, where Rpb1 levels are similar to the *wt* cells independently of the Rpb1 antibody used. We then performed Co-IP experiments using the anti-Rpb3 antibody, as no differences in Rpb3 levels were observed in the isogenic *wt* and *rpb1-CTD11* cells. Very interestingly, and in agreement with the ChIP data, there is less Sub1–TAP associated to the RNAPII in the *rpb1-CTD11* mutant than in the *wt* cells (Figure 7D). These results underscore the conclusion that Sub1 and the Rpb1-CTD are functionally related, which support the role of Sub1 in CTD phosphorylation (25). We conclude that Rpb4/7 and the Rpb1-CTD are important for Sub1 association with DNA. Furthermore, at least during transcription initiation, Sub1 appears to be localized to

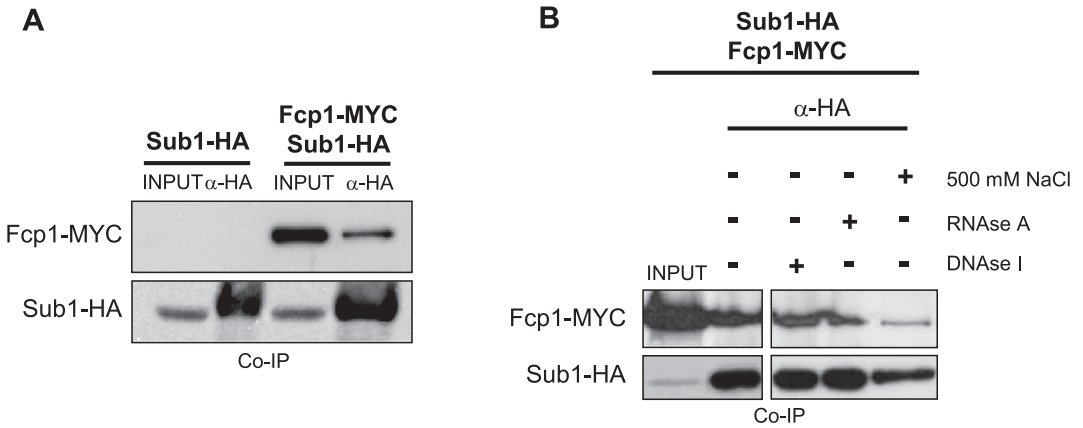

Figure 6. Sub1 and Fcp1 are components of the same complex. (**A**) Co-IP performed on WCEs from Sub1–HA and Sub1–HA Fcp1-MYC cells using an anti-HA antibody. Inputs and IPs were analyzed by Western blotting with antibodies to the indicated proteins (anti-MYC and anti-HA for Fcp1 and Sub1, respectively). (**B**) Same as in (**A**), except that in the fourth IP the beads were washed with lysis buffer containing 500 mM NaCl, and second and third IPs were treated with DNAse I and RNAse A, respectively.

a region near the CTD and Rpb4/7 of RNAPII. These results support the conclusion that Sub1 affects clamp function and modulates CTD phosphorylation throughout the transcription cycle.

DISCUSSION

The data presented here provide novel insight into how the transcriptional coactivator Sub1 modulates RNAPII transcription through interaction with Rpb4/7. Our findings that Sub1 directly interacts with the Rpb4/7 heterodimer, associates with Fcp1, and is genetically and functionally related to the CTD, explain how Sub1 modulates RNAPII phosphorylation, which crucially regulates the biogenesis of mRNAs. We also provide evidence indicating that Sub1 contributes to RNAPII clamp function elucidating Sub1 role during the transition from the open to the closed complex formation, thus facilitating transcription elongation.

Sub1, as a PIC component, directly interacts with Rpb4/7

We show that Sub1 interacts with Rpb4/7, most likely via Rpb7 because Sub1 binds RNAPII in the absence of Rpb4 (Figure 4). However, a fully functional heterodimer seems to be important for Sub1 association with chromatin, as Sub1 crosslinking to gene promoters is significantly reduced in the *rpb4* null mutant (Figure 3). It is likely that the reduced levels of Rpb7 in the *rpb4Δ* cells account for Sub1 chromatin association defects. The apparent discrepancy between the CoIP and ChIP experiments suggests that, while Rpb4 is not required for Sub1 interaction with RNAPII, a functional Rpb4/7 is necessary for Sub1 to stably associate with chromatin after RNAPII recruitment to the PIC. We propose a model where Sub1 is recruited to RNAPII through interaction with Rpb4/7, TFIIB (36,50,78) and DNA. Once at the PIC, Sub1 interacts with Rpb4/7 via its C-terminal region (Figures 5 and 8) to keep associated to RNAPII and chomatin.

SUB1 was originally isolated as an allele-specific suppressor of two TFIIB mutations (E62G and R78H) (35), located within the B-reader region (helix and loop, respectively) (79). Deletion of *SUB1* is synthetically lethal when combined with these two mutations and, in agreement, overexpression of *SUB1* is required for suppression of their cold sensitive phenotypes (35). Moreover, a specific interaction between Sub1 and TFIIB has also been described (35); and TFIIB is required for Sub1 recruitment to the promoters of constitutively transcribed genes (78).

Sub1 was subsequently identified as a PIC component (36). In this elegant study, ChIP data suggest that Sub1 mainly localizes to the promoter region of active genes in a manner dependent upon TBP. Additionally, FeBABE cleavage experiments indicate that Sub1 is located near the leading edge of the *HIS4* transcription bubble, which is also close to the TFIIB-linker helix (79,80). This is in agreement with a direct interaction between Sub1 and TFIIB (35). They proposed a model where Sub1 is first recruited to the PIC by protein interactions, likely with TFIIB, and at that point both factors would cooperate in promoter melting. Then, the interaction of Sub1 with DNA would stabilize the open complex, thus promoting transcription initiation and promoter clearance. 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. In fact, the residues mutated in TFIIB that specifically interact with *SUB1* are in the B-reader, which has been proposed to act in the capture of the template strand within the RNAPII active site (79).

Genetic data also suggest that Sub1, once bound to gene promoters, could help TFIIE and TFIIH to maintain the PIC in a stable but inactive conformation in the open complex (36). At this point, we propose that Sub1 may interact with Rpb4/7. One hypothesis is that the interaction is mediated by the carboxy-terminal region of Sub1. The intrinsically-disordered nature of the Sub1 C-terminal domain could allow the protein to span the distance between the bubble upstream junction and the RNAPII stalk (Figure 8). 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* (Figure 5B), and

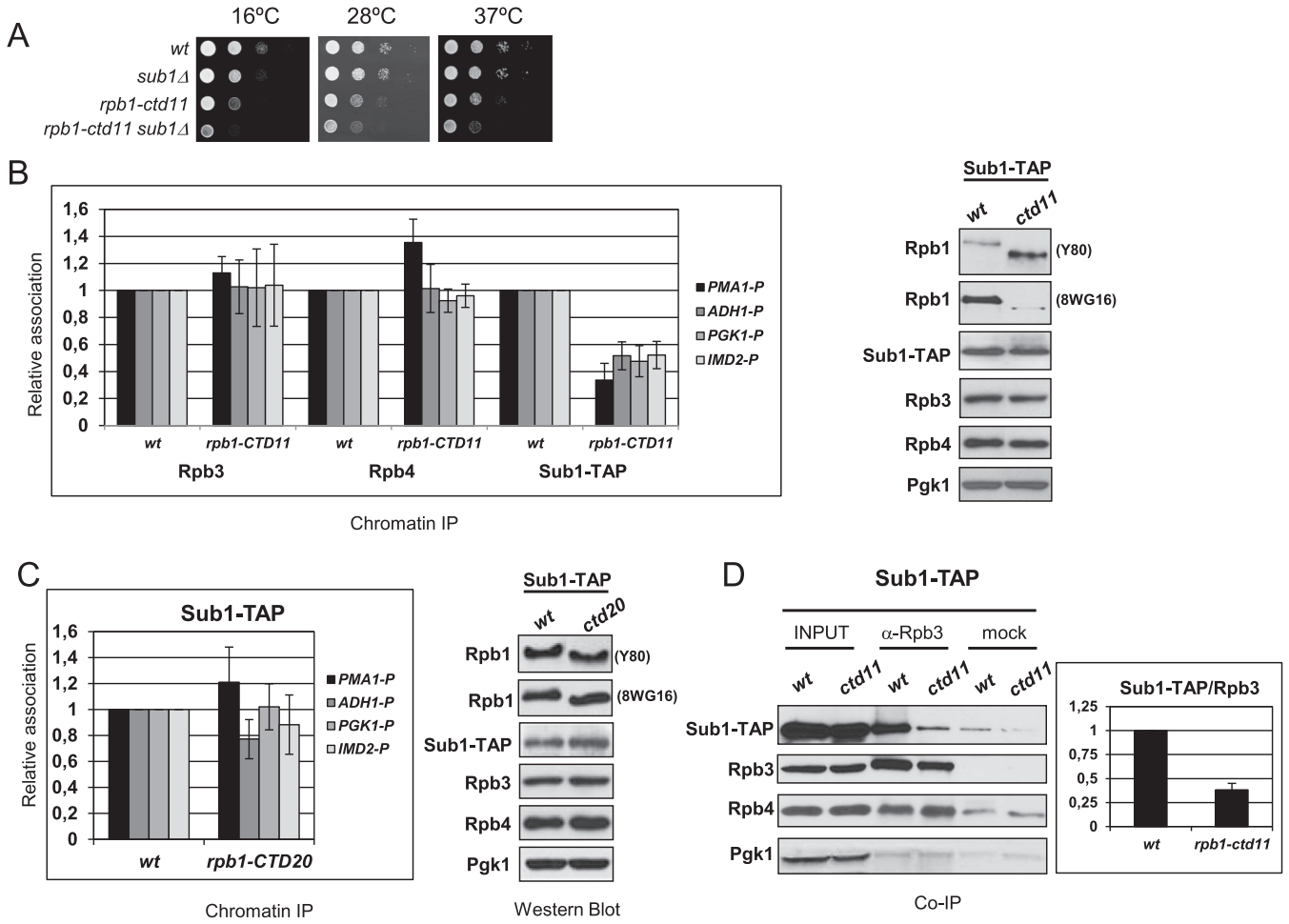

Figure 7. A full length Rpb1-CTD is required for efficient Sub1 association to gene promoters. (**A**) Genetic interaction between *sub1Δ and rpb1-CTD11*. The *sub1Δ* deletion increases the slow growth phenotype of *rpb1-CTD11* strain at 16, 28 and 37°C. Serial dilutions (1:10) of *wt* and mutant strains were spotted on rich medium and grown for 2–3 days at the indicated temperatures. (**B**) Chromatin immunoprecipitation (ChIP) analyses were performed using *wt* and *rpb1-CTD11* strains. Left panel: Rpb3, Rpb4 and Sub1–TAP binding to the promoter (P) of three constitutively expressed genes, *PMA1, ADH1* and *PGK1,* and to the promoter of the *IMD2* inducible gene, was examined by qPCR. Results were quantitated (see Material and Methods), and relative Rpb3, Rpb4 and Sub1–TAP binding in *rpb1-CTD11*cells is plotted relative to that in *wt* cells (set equal to 1). Right panel: WCE were prepared from wild-type (*wt*) and *rpb1-CTD11* strains expressing Sub1–TAP and analyzed by Western blotting using the indicated antibodies. (**C**) Same as in (B) for *wt* and *rpb1-CTD20* strains. (**D**) Left panel: Co-IP performed on WCEs from Sub1–TAP cells (*wt* and *rpb1-CTD11*) using an anti-Rpb3 antibody. As a control, the same amount of cell extract was incubated with Protein A Sepharose alone. Inputs and IPs were analyzed by Western blotting with antibodies to the indicated proteins. Right panel: Values from the quantification of Sub1-TAP and Rpb3 immunoreactive signals from three experiments were calcuated and Sub1-TAP/Rpb3 mean ratios were plotted (arbitrary units), where error bars represent standard deviations.

showed that the association of Sub1ΔCT with gene promoters is significantly reduced in the absence of *RPB4* (Figure 5D). In contrast, a mutation altering Sub1 DNA binding does not affect growth of *rpb4Δ* mutants (Figure 5D). This is the first evidence of a role for the CT region of Sub1, which is not conserved in human PC4, and that also provides insight into the mechanism used by Sub1 to function beyond the PIC, later on transcription. Furthermore, this is also the first demonstration that Sub1 directly interacts with RNAPII at least through the Rpb4/7 stalk.

Additionally, our data showing a genetic interaction between *SUB1*, *RPB4* and the *RPB1* foot domain (Figure 2A and B), suggest that the function of Sub1 is negative when the integrity of RNAPII is compromised, such as in the case of *rpb4Δ* and *rpo21-4* mutants (51). One likely hypothesis is that Sub1 has a negative role during transcription initiation, repressing transcription when PIC complexes are not well assembled and/or the integrity of the polymerase is altered. Therefore, in the *rpb4Δ sub1Δ* and *rpo21-4 sub1Δ* double mutants this repression would be abolished, enabling RNAPII to circumvent initiation defects due to *rpb4Δ* (81) and *rpo21-4* (51), allowing better cell growth (Figure 2A and B). On the other hand, and in agreement with this idea, overexpression of *SUB1* significantly aggravates *rpb4Δ* growth (Figure 2A). Our genetic data are also consistent with the proposed models for Sub1 function helping to maintain a stable PIC conformation (25,36), and its putative localization within the RNAPII-DNA complex at the initiation step (Figure 8).

In summary, our data and that of others (35,36,50) allow us to propose a model of Sub1 within the initiation complex (Figure 8). Sub1 binds DNA through its N-terminal

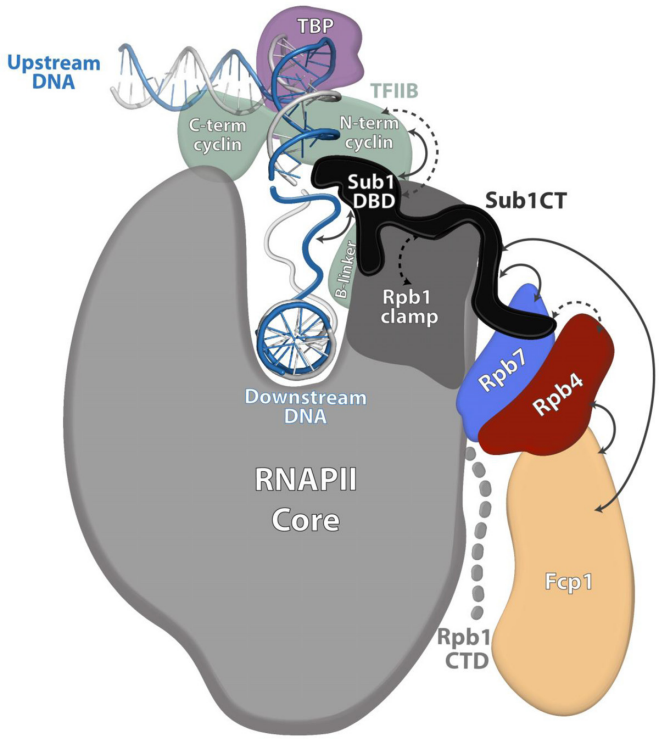

Figure 8. Schematic model showing the hypothetical localization of Sub1 during transcription initiation. Sub1 is bound to the promoter by interacting with upstream DNA at the junction between single- and double-stranded DNA (36) through its DNA Binding Domain (Sub1 DBD) (Figure 5). The proposed localization of Sub1 in this model explains the reported physical (solid arrow) and genetic (dashed arrow) interaction of Sub1 with TFIIB (35,50). The intrinsically-disordered C-terminal domain of Sub1 (Sub1 CT) may extend to directly interact with the Rpb4/7 heterodimer, as suggested by the specific genetic interaction between this domain and *RPB4* (Figure 5), and the physical direct interaction with Rpb7 (Figure 4). The genetic interaction between Sub1 and Rpb1 clamp (Figure 2C) is also illustrated. In addition, the model shows the connections of Fcp1 phosphatase with Rpb4 and Sub1 revealed by our genetic (Allepuz-Fuster *et al.*, Calvo & Manley, 2005) and CoIP assays (Figure 6), and consistent with the structural data (76). Our proposed model suggests that Sub1 may influence Rpb1-CTD phosphorylation (25) and Rpb1 clamp functionality through its interaction with Rpb4/7, which could also explain the already suggested role of Sub1 both during transcription initiation (36,37) and elongation (38).

DBD, while is stably maintained in the RNAPII-DNA complex by binding to Rpb4/7 via its CT region (Figure 8). Association of Sub1 with Fcp1 explains the role of Sub1 in the modulation of CTD phosphorylation during the entire transcription cycle (25). Our results here strongly suggest that Sub1 directly interacts with Rpb7, while Fcp1 interacts with Rpb4 to modulate RNAPII CTD phosphorylation (12). In addition, Sub1 interaction with Rpb4/7 and the Rpb1 clamp domain may help to explain how it may influence TSS selection (37) and transcription elongation rate together with Spt5 (38). This is supported by the genetic interactions between *SUB1* deletion and the *rpb1-L1397S* mutation localized within the clamp domain (Figure 2C, and (52), and by the fact that Spt5 associates with Sub1, and interacts with Rpb4/7 and the Rpb1 clamp domain (64,65).

Our study provides significant information about RNAPII transcription regulation by Sub1 and Rpb4/7, and suggests a model that could be very helpful for future structural and functional studies on RNAPII transcription machinery.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Carlson,M. (1997) Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD. *Annu. Rev. Cell Dev. Biol.*, **13**, 1–23.
2. Dahmus,M.E. (1996) Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J. Biol. Chem.*, **271**, 19009–19012.
3. Phatnani,H.P. and Greenleaf,A.L. (2006) Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev.*, **20**, 2922–2936.
4. Nonet,M., Sweetser,D. and Young,R.A. (1987) Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. *Cell*, **50**, 909–915.
5. Cho,E.J. (2007) RNA polymerase II carboxy-terminal domain with multiple connections. *Exp. Mol. Med.*, **39**, 247–254.
6. Hirose,Y. and Manley,J.L. (2000) RNA polymerase II and the integration of nuclear events. *Genes Dev.*, **14**, 1415–1429.
7. Meinhart,A., Kamenski,T., Hoeppner,S., Baumli,S. and Cramer,P. (2005) A structural perspective of CTD function. *Genes Dev.*, **19**, 1401–1415.
8. Buratowski,S. (2005) Connections between mRNA 3′ end processing and transcription termination. *Curr. Opin. Cell Biol.*, **17**, 257–261.
9. Murakami,K., Tsai,K.L., Kalisman,N., Bushnell,D.A., Asturias,F.J. and Kornberg,R.D. (2015) Structure of an RNA polymerase II preinitiation complex. *Proc. Natl. Acad. Sci. U.S.A.*, **112**, 13543–13548.
10. Plaschka,C., Lariviere,L., Wenzeck,L., Seizl,M., Hemann,M., Tegunov,D., Petrotchenko,E.V., Borchers,C.H., Baumeister,W., Herzog,F. *et al.* (2015) Architecture of the RNA polymerase II-Mediator core initiation complex. *Nature*, **518**, 376–380.
11. Robinson,P.J., Trnka,M.J., Bushnell,D.A., Davis,R.E., Mattei,P.J., Burlingame,A.L. and Kornberg,R.D. (2016) Structure of a complete mediator-RNA polymerase II pre-initiation complex. *Cell*, **166**, 1411–1422.
12. Allepuz-Fuster,P., Martinez-Fernandez,V., Garrido-Godino,A.I., Alonso-Aguado,S., Hanes,S.D., Navarro,F. and Calvo,O. (2014) Rpb4/7 facilitates RNA polymerase II CTD dephosphorylation. *Nucleic Acids Res.*, **42**, 13674–13688.
13. Eick,D. and Geyer,M. (2013) The RNA polymerase II carboxy-terminal domain (CTD) code. *Chem. Rev.*, **113**, 8456–8490.

14. Heidemann,M., Hintermair,C., Voss,K. and Eick,D. (2013) Dynamic phosphorylation patterns of RNA polymerase II CTD during transcription. *Biochim. Biophys. Acta*, **1829**, 55–62.
15. Chapman,R.D., Heidemann,M., Hintermair,C. and Eick,D. (2008) Molecular evolution of the RNA polymerase II CTD. *Trends Genet.*, **24**, 289–296.
16. Schroder,S., Herker,E., Itzen,F., He,D., Thomas,S., Gilchrist,D.A., Kaehlcke,K., Cho,S., Pollard,K.S., Capra,J.A. *et al.* (2013) Acetylation of RNA polymerase II regulates growth-factor-induced gene transcription in mammalian cells. *Mol. Cell*, **52**, 314–324.
17. Kelly,W.G., Dahmus,M.E. and Hart,G.W. (1993) RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc. *J. Biol. Chem.*, **268**, 10416–10424.
18. Ranuncolo,S.M., Ghosh,S., Hanover,J.A., Hart,G.W. and Lewis,B.A. (2012) Evidence of the involvement of O-GlcNAc-modified human RNA polymerase II CTD in transcription in vitro and in vivo. *J. Biol. Chem.*, **287**, 23549–23561.
19. Sims,R.J., Rojas,L.A., Beck,D., Bonasio,R., Schuller,R., Drury,W.J., Eick,D. and Reinberg,D. (2011) The C-terminal domain of RNA polymerase II is modified by site-specific methylation. *Science*, **332**, 99–103.
20. Buratowski,S. (2003) The CTD code. *Nat. Struct. Biol.*, **10**, 679–680.
21. Buratowski,S. (2009) Progression through the RNA polymerase II CTD cycle. *Mol. Cell*, **36**, 541–546.
22. Hsin,J.P. and Manley,J.L. (2012) The RNA polymerase II CTD coordinates transcription and RNA processing. *Genes Dev.*, **26**, 2119–2137.
23. Jeronimo,C., Bataille,A.R. and Robert,F. (2013) The writers, readers, and functions of the RNA polymerase II C-terminal domain code. *Chem. Rev.*, **113**, 8491–8522.
24. Schreieck,A., Easter,A.D., Etzold,S., Wiederhold,K., Lidschreiber,M., Cramer,P. and Passmore,L.A. (2014) RNA polymerase II termination involves C-terminal-domain tyrosine dephosphorylation by CPF subunit Glc7. *Nat. Struct. Mol. Biol.*, **21**, 175–179.
25. Garcia,A., Rosonina,E., Manley,J.L. and Calvo,O. (2010) Sub1 globally regulates RNA polymerase II C-terminal domain phosphorylation. *Mol. Cell. Biol.*, **30**, 5180–5193.
26. Hanes,S.D. (2014) The Ess1 prolyl isomerase: Traffic cop of the RNA polymerase II transcription cycle. *Biochim. Biophys. Acta.*, **1839**, 316–333.
27. Verdecia,M.A., Bowman,M.E., Lu,K.P., Hunter,T. and Noel,J.P. (2000) Structural basis for phosphoserine-proline recognition by group IV WW domains. *Nat. Struct. Biol.*, **7**, 639–643.
28. Wu,X., Wilcox,C.B., Devasahayam,G., Hackett,R.L., Arevalo-Rodriguez,M., Cardenas,M.E., Heitman,J. and Hanes,S.D. (2000) The Ess1 prolyl isomerase is linked to chromatin remodeling complexes and the general transcription machinery. *EMBO J.*, **19**, 3727–3738.
29. Ge,H. and Roeder,R.G. (1994) Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell*, **78**, 513–523.
30. Kaiser,K., Stelzer,G. and Meisterernst,M. (1995) The coactivator p15 (PC4) initiates transcriptional activation during TFIIA-TFIID-promoter complex formation. *EMBO J.*, **14**, 3520–3527.
31. Kretzschmar,M., Kaiser,K., Lottspeich,F. and Meisterernst,M. (1994) A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. *Cell*, **78**, 525–534.
32. Malik,S., Guermah,M. and Roeder,R.G. (1998) A dynamic model for PC4 coactivator function in RNA polymerase II transcription. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 2192–2197.
33. Werten,S., Stelzer,G., Goppelt,A., Langen,F.M., Gros,P., Timmers,H.T., Van der Vliet,P.C. and Meisterernst,M. (1998) Interaction of PC4 with melted DNA inhibits transcription. *EMBO J.*, **17**, 5103–5111.
34. Henry,N.L., Bushnell,D.A. and Kornberg,R.D. (1996) A yeast transcriptional stimulatory protein similar to human PC4. *J. Biol. Chem.*, **271**, 21842–21847.
35. Knaus,R., Pollock,R. and Guarente,L. (1996) Yeast SUB1 is a suppressor of TFIIB mutations and has homology to the human co-activator PC4. *EMBO J.*, **15**, 1933–1940.
36. Sikorski,T.W., Ficarro,S.B., Holik,J., Kim,T., Rando,O.J., Marto,J.A. and Buratowski,S. (2011) Sub1 and RPA associate with RNA polymerase II at different stages of transcription. *Mol. Cell*, **44**, 397–409.
37. Braberg,H., Jin,H., Moehle,E.A., Chan,Y.A., Wang,S., Shales,M., Benschop,J.J., Morris,J.H., Qiu,C., Hu,F. *et al.* (2013) From structure to systems: high-resolution, quantitative genetic analysis of RNA polymerase II. *Cell*, **154**, 775–788.
38. Garcia,A., Collin,A. and Calvo,O. (2012) Sub1 associates with Spt5 and influences RNA polymerase II transcription elongation rate. *Mol. Biol. Cell*, **23**, 4297–4312.
39. Calvo,O. and Manley,J.L. (2001) Evolutionarily conserved interaction between CstF-64 and PC4 links transcription, polyadenylation, and termination. *Mol. Cell*, **7**, 1013–1023.
40. He,X., Khan,A.U., Cheng,H., Pappas,D.L. Jr, Hampsey,M. and Moore,C.L. (2003) Functional interactions between the transcription and mRNA 3' end processing machineries mediated by Ssu72 and Sub1. *Genes Dev.*, **17**, 1030–1042.
41. Calvo,O. and Manley,J.L. (2005) The transcriptional coactivator PC4/Sub1 has multiple functions in RNA polymerase II transcription. *EMBO J.*, **24**, 1009–1020.
42. Burke,D., Dawson,D. and Stearns,T. (2000) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, NY.
43. Pascual-Garcia,P., Govind,C.K., Queralt,E., Cuenca-Bono,B., Llopis,A., Chavez,S., Hinnebusch,A.G. and Rodriguez-Navarro,S. (2008) Sus1 is recruited to coding regions and functions during transcription elongation in association with SAGA and TREX2. *Genes Dev.*, **22**, 2811–2822.
44. Sakurai,H., Mitsuzawa,H., Kimura,M. and Ishihama,A. (1999) The Rpb4 subunit of fission yeast Schizosaccharomyces pombe RNA polymerase II is essential for cell viability and similar in structure to the corresponding subunits of higher eukaryotes. *Mol. Cell. Biol.*, **19**, 7511–7518.
45. Calvo,O.G. and García,A. (2012) RNA polymerase II phosphorylation and gene expression regulation. *Protein Phosphorylation in Human Health*. InTech, Rijeka, pp. 151–194.
46. Schmitt,M.E., Brown,T.A. and Trumpower,B.L. (1990) A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. *Nucleic Acids Res.*, **18**, 3091–3092.
47. Lindstrom,D.L., Squazzo,S.L., Muster,N., Burckin,T.A., Wachter,K.C., Emigh,C.A., McCleery,J.A., Yates,J.R. and Hartzog,G.A. (2003) Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. *Mol. Cell. Biol.*, **23**, 1368–1378.
48. McKune,K., Richards,K.L., Edwards,A.M., Young,R.A. and Woychik,N.A. (1993) RPB7, one of two dissociable subunits of yeast RNA polymerase II, is essential for cell viability. *Yeast*, **9**, 295–299.
49. Woychik,N.A. and Young,R.A. (1989) RNA polymerase II subunit RPB4 is essential for high- and low-temperature yeast cell growth. *Mol. Cell. Biol.*, **9**, 2854–2859.
50. Wu,W.H., Pinto,I., Chen,B.S. and Hampsey,M. (1999) Mutational analysis of yeast TFIIB. A functional relationship between Ssu72 and Sub1/Tsp1 defined by allele-specific interactions with TFIIB. *Genetics*, **153**, 643–652.
51. Garrido-Godino,A.I., Garcia-Lopez,M.C. and Navarro,F. (2013) Correct assembly of RNA polymerase II depends on the foot domain and is required for multiple steps of transcription in Saccharomyces cerevisiae. *Mol. Cell. Biol.*, **33**, 3611–3626.
52. Kwapisz,M., Wery,M., Despres,D., Ghavi-Helm,Y., Soutourina,J., Thuriaux,P. and Lacroute,F. (2008) Mutations of RNA polymerase II activate key genes of the nucleoside triphosphate biosynthetic pathways. *EMBO J.*, **27**, 2411–2421.
53. Scafe,C., Martin,C., Nonet,M., Podos,S., Okamura,S. and Young,R.A. (1990) Conditional mutations occur predominantly in highly conserved residues of RNA polymerase II subunits. *Mol. Cell. Biol.*, **10**, 1270–1275.
54. Armache,K.J., Mitterweger,S., Meinhart,A. and Cramer,P. (2005) Structures of complete RNA polymerase II and its subcomplex, Rpb4/7. *J. Biol. Chem.*, **280**, 7131–7134.
55. Garcia-Lopez,M.C., Pelechano,V., Miron-Garcia,M.C., Garrido-Godino,A.I., Garcia,A., Calvo,O., Werner,M., Perez-Ortin,J.E. and Navarro,F. (2011) The conserved foot domain of RNA pol II associates with proteins involved in transcriptional initiation and/or early elongation. *Genetics*, **189**, 1235–1248.

56. Cramer,P., Bushnell,D.A. and Kornberg,R.D. (2001) Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. *Science*, **292**, 1863–1876.
57. Archambault,J., Schappert,K.T. and Friesen,J.D. (1990) A suppressor of an RNA polymerase II mutation of Saccharomyces cerevisiae encodes a subunit common to RNA polymerases I, II, and III. *Mol. Cell. Biol.*, **10**, 6123–6131.
58. Archambault,J., Drebot,M.A., Stone,J.C. and Friesen,J.D. (1992) Isolation and phenotypic analysis of conditional-lethal, linker-insertion mutations in the gene encoding the largest subunit of RNA polymerase II in Saccharomyces cerevisiae. *Mol. Gen. Genet.*, **232**, 408–414.
59. Armache,K.J., Kettenberger,H. and Cramer,P. (2003) Architecture of initiation-competent 12-subunit RNA polymerase II. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 6964–6968.
60. Grohmann,D. and Werner,F. (2010) Hold on!: RNA polymerase interactions with the nascent RNA modulate transcription elongation and termination. *RNA Biol.*, **7**, 310–315.
61. Grohmann,D. and Werner,F. (2011) Cycling through transcription with the RNA polymerase F/E (RPB4/7) complex: structure, function and evolution of archaeal RNA polymerase. *Res. Microbiol.*, **162**, 10–18.
62. Gnatt,A.L., Cramer,P., Fu,J., Bushnell,D.A. and Kornberg,R.D. (2001) Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 A resolution. *Science*, **292**, 1876–1882.
63. Jenks,M.H., O'Rourke,T.W. and Reines,D. (2008) Properties of an intergenic terminator and start site switch that regulate IMD2 transcription in yeast. *Mol. Cell. Biol.*, **28**, 3883–3893.
64. Li,W., Giles,C. and Li,S. (2014) Insights into how Spt5 functions in transcription elongation and repressing transcription coupled DNA repair. *Nucleic Acids Res.*, **42**, 7069–7083.
65. Martinez-Rucobo,F.W., Sainsbury,S., Cheung,A.C. and Cramer,P. (2011) Architecture of the RNA polymerase-Spt4/5 complex and basis of universal transcription processivity. *EMBO J.*, **30**, 1302–1310.
66. Edwards,A.M., Kane,C.M., Young,R.A. and Kornberg,R.D. (1991) Two dissociable subunits of yeast RNA polymerase II stimulate the initiation of transcription at a promoter in vitro. *J. Biol. Chem.*, **266**, 71–75.
67. Sheffer,A., Varon,M. and Choder,M. (1999) Rpb7 can interact with RNA polymerase II and support transcription during some stresses independently of Rpb4. *Mol. Cell. Biol.*, **19**, 2672–2680.
68. Runner,V.M., Podolny,V. and Buratowski,S. (2008) The Rpb4 subunit of RNA polymerase II contributes to cotranscriptional recruitment of 3' processing factors. *Mol. Cell. Biol.*, **28**, 1883–1891.
69. Brandsen,J., Werten,S., van der Vliet,P.C., Meisterernst,M., Kroon,J. and Gros,P. (1997) C-terminal domain of transcription cofactor PC4 reveals dimeric ssDNA binding site. *Nat. Struct. Biol.*, **4**, 900–903.
70. Wang,Z. and Roeder,R.G. (1998) DNA topoisomerase I and PC4 can interact with human TFIIIC to promote both accurate termination and transcription reinitiation by RNA polymerase III. *Mol. Cell*, **1**, 749–757.
71. Mortusewicz,O., Roth,W., Li,N., Cardoso,M.C., Meisterernst,M. and Leonhardt,H. (2008) Recruitment of RNA polymerase II cofactor PC4 to DNA damage sites. *J. Cell Biol.*, **183**, 769–776.
72. Pan,Z.Q., Ge,H., Amin,A.A. and Hurwitz,J. (1996) Transcription-positive cofactor 4 forms complexes with HSSB (RPA) on single-stranded DNA and influences HSSB-dependent enzymatic synthesis of simian virus 40 DNA. *J. Biol. Chem.*, **271**, 22111–22116.
73. Werten,S., Wechselberger,R., Boelens,R., van der Vliet,P.C. and Kaptein,R. (1999) Identification of the single-stranded DNA binding surface of the transcriptional coactivator PC4 by NMR. *J. Biol. Chem.*, **274**, 3693–3699.
74. Kimura,M., Suzuki,H. and Ishihama,A. (2002) Formation of a carboxy-terminal domain phosphatase (Fcp1)/TFIIF/RNA polymerase II (pol II) complex in Schizosaccharomyces pombe involves direct interaction between Fcp1 and the Rpb4 subunit of pol II. *Mol. Cell. Biol.*, **22**, 1577–1588.
75. Tombacz,I., Schauer,T., Juhasz,I., Komonyi,O. and Boros,I. (2009) The RNA Pol II CTD phosphatase Fcp1 is essential for normal development in Drosophila melanogaster. *Gene*, **446**, 58–67.
76. Kamenski,T., Heilmeier,S., Meinhart,A. and Cramer,P. (2004) Structure and mechanism of RNA polymerase II CTD phosphatases. *Mol. Cell*, **15**, 399–407.
77. Aristizabal,M.J., Negri,G.L., Benschop,J.J., Holstege,F.C., Krogan,N.J. and Kobor,M.S. (2013) High-throughput genetic and gene expression analysis of the RNAPII-CTD reveals unexpected connections to SRB10/CDK8. *PLoS Genet.*, **9**, e1003758.
78. Rosonina,E., Willis,I.M. and Manley,J.L. (2009) Sub1 functions in osmoregulation and in transcription by both RNA polymerases II and III. *Mol. Cell. Biol.*, **29**, 2308–2321.
79. Kostrewa,D., Zeller,M.E., Armache,K.J., Seizl,M., Leike,K., Thomm,M. and Cramer,P. (2009) RNA polymerase II-TFIIB structure and mechanism of transcription initiation. *Nature*, **462**, 323–330.
80. Liu,X., Bushnell,D.A., Wang,D., Calero,G. and Kornberg,R.D. (2010) Structure of an RNA polymerase II-TFIIB complex and the transcription initiation mechanism. *Science*, **327**, 206–209.
81. Sharma,N. and Kumari,R. (2013) Rpb4 and Rpb7: multifunctional subunits of RNA polymerase II. *Crit. Rev. Microbiol.*, **39**, 362–372.
82. Koyama,H., Sumiya,E., Nagata,M., Ito,T. and Sekimizu,K. (2008) Transcriptional repression of the IMD2 gene mediated by the transcriptional co-activator Sub1. *Genes Cells*, **13**, 1113–1126.