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Identificación y caracterización de elementos reguladores de la expresión de *SUS1* y nuevas funciones celulares para la proteína Sus1 en *Saccharomyces cerevisiae*.

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CERTIFICAN que el Ingeniero Agrónomo BERNARDO CUENCA BONO ha realizado bajo su dirección en el CIPF y bajo su tutela en la UPV respectivamente, el trabajo que lleva por título "Identificación y caracterización de elementos reguladores de la expresión de *SUS1* y nuevas funciones celulares para la proteína Sus1 en *Saccharomyces cerevisiae*", y autorizan su presentación para optar al grado de Doctor en Biotecnología,

Y para que así conste, expiden y firman el presente certificado en Valencia, enero de 2016.

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"Nunca te das cuenta de lo que ya has hecho, sólo puedes ver lo que te queda por hacer" ***Marie Curie***

"Todo parece imposible hasta que se hace" ***Nelson Mandela***

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Resumen

Una de las características que definen a una célula eucariota es la presencia de una envoltura nuclear. Aunque este hecho permite la separación física entre núcleo y citoplasma, la presencia de un número variable de aberturas, denominadas complejos del poro nuclear (NPCs), permiten un flujo constante de moléculas e información entre ambos compartimentos. Ciertas moléculas difunden de forma pasiva, pero otras necesitan energía e interacciones específicas a través de transportadores y componentes del NPC para transitar entre ambos compartimentos. Entre las moléculas selectivamente exportadas del núcleo al citoplasma se encuentran los RNAs mensajeros (mRNAs). La separación física del núcleo y del citoplasma en eucariotas, aísla los procesos de transcripción y traducción, permitiendo a la célula seleccionar en el núcleo los transcritos competentes para ser exportados y que darán lugar a una proteína funcional en el citoplasma.

Adecuar los niveles de transcritos en una célula, en función de las necesidades nutritivas, reproductivas o de relación con el entorno, es esencial para la vida. Para ello, los mecanismos encargados de regular la transcripción, el procesamiento, la estabilidad, la degradación, el exporte o la traducción de los transcritos, se encuentran altamente

acoplados física y espacialmente con el fin de regular finamente los niveles de mensajeros en la célula.

En el núcleo de las células de la levadura *Saccharomyces cerevisiae* se encuentra Sus1, una proteína de 11 kDa altamente conservada en eucariotas. Sus1 forma parte del co-activador transcripcional SAGA, siendo componente de un submódulo implicado en la desubicuitinación de la histona H2B. Además, Sus1 es uno de los componentes del complejo TREX2, que interacciona con el poro nuclear en la periferia del nucleo y está implicado en el exporte de RNAs mensajeros y en estabilidad genómica. La presencia de Sus1 en ambos complejos permite el acoplamiento físico y espacial de los fenómenos de transcripción y exporte de mRNAs. Además, el gen *SUS1* posee dos intrones, siendo este un evento muy inusual en el genoma de *S. cerevisiae*. A diferencia de otros hongos o metazoos, el porcentaje de genes con intrones en *S. cerevisiae* es muy reducido (5%) y solo 10 genes poseen más de un intrón interrumpiendo su secuencia codificante.

Las características peculiares del gen *SUS1*, el papel de la proteína que codifica coordinando procesos durante la biogénesis del mRNA y su conservación funcional en eucariotas superiores, motivó las investigaciones llevadas a cabo en esta Tesis doctoral.

En este trabajo hemos estudiado en detalle la biogénesis de los transcritos de *SUS1*. Se han identificado diferentes factores, tanto en *cis* como en *trans*, implicados en la regulación de la expresión de *SUS1* y en la función de la proteína que codifica. Por otro lado, hemos estudiado la relación genética de *SUS1* con componentes de la maquinaria de degradación citoplasmática 5'→3' y hemos ampliado los conocimientos respecto al papel de Sus1 durante la biogénesis de los mRNAs, no solo en el núcleo sino también en el citoplasma.

Resum

Una de les característiques que definixen a una cèl·lula eucariota és la presència d'un embolcall nuclear. Este fet permet la separació física entre nucli i citoplasma, encara que la presència d'un nombre variable d'obertures, denominades complexos del porus nuclear (NPCs), permet un flux constant de molècules i informació entre ambdós compartiments. Certes molècules difonen de forma passiva, però altres necessiten energia i interaccions específiques amb transportadors i components del NPC per a transitar entre ambdós compartiments. Entre les molècules selectivament exportades del nucli al citoplasma es troben els RNAs missatgers (mRNAs). La separació física del nucli i del citoplasma aïlla els processos de transcripció i traducció en eucariòtes, permetent a la cèl·lula seleccionar en el nucli els transcrits competents per a ser exportats i que donaran lloc a una proteïna funcional en el citoplasma.

Adequar els nivells de transcrits en una cèl·lula, en funció de les necessitats nutritives, reproductives o de relació amb l'entorn, és essencial per a la vida. Per a això, els mecanismes encarregats de regular la transcripció, el processament, l'estabilitat, la degradació, l'exportació o la traducció dels transcrits, es troben altament acoblats física i espacialment amb el fi de regular finament els nivells de missatgers en la cèl·lula.

En el nucli de les cèl·lules del rent *Saccharomyces cerevisiae* es troba Sus1, una xicoteta proteïna d'11 kDa altament conservada en eucariotes. Sus1 forma part del coactivador transcripcional SAGA, sent component d'un submòdul implicat en la modificació de histones. A més, Sus1 és un dels components del complex TREX2, que interacciona amb l'embolall nuclear a la perriferia del nucli i està implicat en l'export de RNAs missatgers. La presència de Sus1 en ambdós complexos permet l'adaptament físic i espacial dels fenòmens de transcripció i exportació de mRNAs. A més, el gen *SUS1* posseïx dos introns i este fet és inusual en el genoma de *S. cerevisiae*. A diferència d'altres fongs o metazous, el percentatge de gens amb introns en *S. cerevisiae* és molt reduït (5%) i només 10 gens posseïxen més d'un intró interrompent la seua seqüència codificant.

Les característiques peculiars del gen *SUS1*, el paper de la proteïna que codifica coordinant processos durant la biogènesi del mRNA i la seua conservació funcional en eucariotes superiors, va motivar les investigacions dutes a terme en esta tesi doctoral.

En este treball hem estudiat en detall la biogènesi dels transcrits de *SUS1*. S'han identificat diferents factors, tant en *cis* com en *trans*, implicats en la regulació de l'expressió de *SUS1* i en la funció de la proteïna que codifica. D'altra banda, hem estudiat la relació genètica de

SUS1 amb components de la maquinària de degradació citoplasmática 5'→3' i hem ampliat els coneixements respecte al paper de Sus1 durant la biogènesi dels mRNAs, no sols en el nucli sinó també al citoplasma.

Abstract

One of the defining features of a eukaryotic cell is the presence of a nuclear envelope. This allowed the physical separation between nucleus and cytoplasm, although the presence of a variable number of openings called nuclear pore complexes (NPCs) allowed a constant flow of molecules and information between the two compartments. Certain molecules passively diffuse, but others need energy and specific interactions with transporters and components of the NPC to travel between through both compartments. The messenger RNAs (mRNAs) are among the molecules selectively exported from the nucleus to the cytoplasm. The physical separation between nucleus and cytoplasm isolates the processes of transcription and translation in eukaryotic cells, allowing the cell to select core transcripts competent for export and that will lead to a functional protein in the cytoplasm.

The right transcript levels in a cell, depending on the nutritional requirements, reproductive or relationship with the environment is essential for life. To this end, mechanisms regulating transcription, processing, stability, degradation, export or translation of the transcripts, are physically and spatially highly coupled in order to finely regulate the transcript levels in the cell.

In Saccharomyces cerevisiae, *SUS1* codes for a small protein of 11 kDa highly conserved in all eukaryotes. *SUS1* is part of the SAGA transcriptional co-activator, being a submodule component involved in chromatin remodeling. In addition, *SUS1* is one of the components of the TREX2 complex, wich interacts with the nuclear pore in the periphery of the nucleus and it is involved in the export of messenger RNAs. The presence of *SUS1* in both complexes allows the physical and spatial coupling phenomena of transcription and export of mRNAs. In addition, *SUS1* has two introns which is an unusual fact for the *S. cerevisiae* genome. Unlike other fungi or metazoans, the percentage of genes with introns in *S. cerevisiae* is very low (5%) and only 10 genes have more than one intron interrupting its coding sequence.

The unusual characteristics of *SUS1*, the role of Sus1 coordinating processes during mRNA biogenesis and its functional conservation in higher eukaryotes, led the research conducted in this dissertation.

In this work we studied in detail the biogenesis of *SUS1* transcripts. We have identified different factors, acting in *cis* and *trans* that are involved in regulating the expression of *SUS1* and function of the protein it encodes. On the other hand, we have studied the genetic relationship of *SUS1* with components of the 5 '→ 3' cytoplasmic degradation machinery and expanded the

knowledge about the role of *SUS1* during the biogenesis of mRNAs, not only in the nucleus but also in the cytoplasm.

Abreviaturas

Acetil-CoA	Acetil Coenzima A
ATP	Adenosín trifosfato*
BP	*Branch Point*
CBC	*Cap Binding Complex*
CCR4-NOT	*Not1p to Not5p, Caf1p, Caf40p, Caf130p and Ccr4p complex*
CTD	Dominio carboxilo-terminal de Rpb1*
DNA	Ácido desoxirribonucleico*
DUB	Complejo desubicuitinasa*
GTF	Factor general de la transcripción*
E1	Exón 1 de *SUS1*
E2	Exón 2 de *SUS1*
E3	Exón 3 de *SUS1*
GFP	*Green Fluorescent Protein.*
HAT	Histona acetil transferasa*
HDAC	Histona deacetil transferasa*
hnRNPs	*Heterogeneus ribonucleoprotein particle*
I1	Intrón1 de *SUS1*
I2	Intrón 2 de *SUS1*
MDa	Mega Daltons
mRNA	RNA mensajero*
mRNP	Ribonucleopartícula de RNA mensajero*
NGD	*No-Go Decay*
NMD	*Nonsense Mediated Decay*
NPC	Complejo del poro nuclear*

NSD	*Non-Stop Decay*
nt	Nucleótidos
OD	Densidad óptica*
ORF	Región abierta de lectura*
pb	Pares de bases
PBs	*P-bodies*
PCR	Reacción en cadena de la polimerasa*
PIC	Complejo de preiniciación*
Poli (A)	Poliadeninas
Poli (T)	Politiminas
Pre-mRNA	Precursor del mRNA*
PTC	Codones de parada de traducción prematuros*
RT-qPCR	RT-PCR cuantitativa en tiempo real*
RNA	Ácido ribonucleico*
RNA/Pol II	RNA polimerasa II*
RT-PCR	Reacción de la Cadena de Polimerasa en Transcripción Reversa*
SAGA	*Spt/Ada/Gcn5 acetyltransferase*
SGD	*Saccharomyces Genome Database*
SGs	Gránulos de estrés*
snRNAs	RNA pequeño nuclear*
SR	Ricas en Serinas*
TAP	Purificación de afinidad en tandem*
TBP	Proteína de unión a la caja TATA*
TF	Factor de transcripción *

TREX	*Transcription export complex*
TREX2	*Transcription export complex 2*
TRAMP	*Trf4/Air2/Mtr4p Polyadenylation complex*
UAS	Secuencia de activación aguas arriba*
Ub	Ubicuitina
UTR	Región no traducible*
WB	Western blot
3'ss	Sitio de splicing en 3'*
3'UTR	Región no traducible en 3'*
5'ss	Sitio de splicing en 5'*
5'UTR	Región no traducible en 5'*
7mG	*7-methylguanosine*

* traducido del inglés

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I. INTRODUCCIÓN

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I.I. Organización del DNA en el núcleo.

En el núcleo de las células eucariotas, el ácido desoxirribonucleico (DNA) se encuentra enrollado alrededor unas proteínas denominadas histonas. 146 pares de bases (pb) de la doble cadena de DNA, se enrollan alrededor de un octámero en forma de disco, formado por 2 subunidades de las histonas H2A, H2B, H3 y H4, que se denomina nucleosoma (Luger *et al*., 1997). Hasta 80 pb actúan de espaciador entre cada nucleosoma, siendo la histona H1 la que se encarga de unir nucleosomas entre sí, cerrando los espacios libres de histonas y facilitando el empaquetamiento del DNA en estructuras más complejas (Thoma *et al*., 1979). De este modo el material genético se almacena en una estructura formada por DNA, histonas y proteínas no histónicas, que se denomina cromatina y que permite la condensación del DNA en estructuras de orden superior denominados cromosomas (Figura I.1).

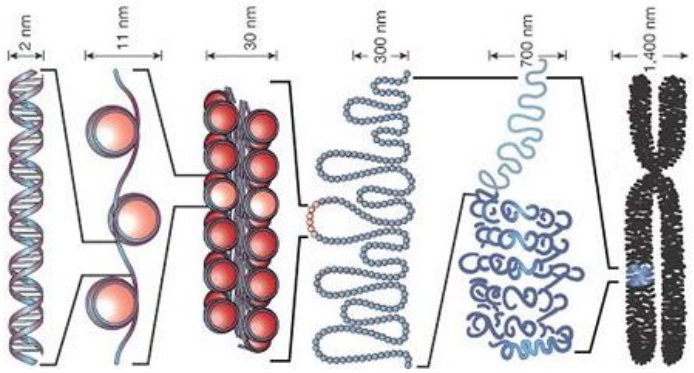

Figura I.1. Niveles de empaquetamiento del DNA en eucariotas. Tamaño en nanómetros (nm). De izquierda a derecha: molécula de DNA (2 nm), nucleosomas en "collar de cuentas" (11 nm), fibra de cromatina (30 nm), cromosoma extendido (300 nm), sección condesada de cromosoma (700nm) y cromosoma en metafase (1.400 nm). Adaptado de Felsenfeld & Groudine, 2003.

La íntima interacción entre el DNA y las histonas permite que estas proteínas participen en diferentes procesos que afectan al ácido desoxirribonucleico, como son la regulación de la transcripción, la replicación del DNA o su reparación (Millar & Grunstein, 2006). Los mecanismos por los cuales las histonas ejercen su acción sobre el material genético, son principalmente a través de modificaciones postraduccionales en las histonas, incorporación de variantes de histonas en los nucleosomas o por la acción de complejos remodeladores de la cromatina dependientes de adenosín trifosfato (ATP); (Ho & Crabtree, 2010). De este modo, las histonas actúan como estructuras dinámicas capaces de influir sobre el nivel de empaquetamiento y accesibilidad al DNA.

I.II. Modificaciones postraduccionales de las histonas.

Las 4 histonas principales que forman parte del nucleosoma (H2A, H2B, H3 y H4), poseen extremos aminoterminales de entre 15 y 30 aminoácidos que están sujetos a diferentes tipos de modificaciones postraduccionales. Además, un extremo carboxilo terminal en H2A sobresale del nucleosoma y está sujeto a modificaciones postraduccionales (Davey *et al.*, 2002). Acetilación, ubicuitinación, metilación y fosforilación son las principales en levadura, existiendo distintos complejos conservados a lo largo de la evolución con actividad modificadora de la cromatina (Kouzarydes, 2007). Cada uno de estos cambios sobre las histonas es reversible y desencadena cascadas de señales en las mismas. De este modo la cromatina remodela su estructura, por ejemplo facilitando la activación o la represión de la expresión génica (Suganuma & Workman, 2008). Debido a que nuestro interés se centra en un complejo relacionado con la acetilación y la ubicuitinación, estas son las modificaciones que son mencionadas brevemente en esta introducción.

Asociadas a complejos multiproteicos, las histonas acetiltransferasas (HATs) actúan transfiriendo a dichas proteinas un grupo acetilo, desde la molécula de Acetil-CoA al residuo ε-amino de una lisina diana. Las HATs son

capaces de desestabilizar la cromatina y facilitar el acceso a factores transcripcionales, mientras que por otro lado, existen deacetiltransferasas de histonas (HDACs) capaces de ejercer el efecto contrario. En algunos casos se ha demostrado la acción de ciertas HDACs como activadores de la expresión génica (De Nadal *et al*., 2004; Wang *et al*., 2004).

La ubicuitinación consiste en una modificación postraduccional mediante la cual se incorporan a una proteína uno o más monómeros de ubicuitina (Ub). La Ub es una proteína de 8,5 kDa, con una estructura característica y altamente conservada en la evolución, que se une covalentemente a otras proteínas a través de una serie de reacciones enzimáticas (Scheffner *et al*., 1995). Cuando una proteína es ubicuitinada, actúa como una marca implicada en modificar la función de la proteína diana (monoubicuitinación) o en conducirla a degradación vía proteasoma (mayoritariamente por poliubicuitinación). La ubicuitinación, al ser un proceso reversible, permite reciclar esta proteína. Un conjunto de enzimas denominadas desubicuitinasas (DUBs) son las encargadas de eliminar la Ub de las proteínas monoubicuitinadas o de desensamblar cadenas de Ub tras la degradación de la proteína diana a través del proteasoma. El proteasoma es un macrocomplejo catalítico que se encarga de reconocer las proteínas

poliubicuitinadas, de eliminar la cadena de Ubs y degradar la proteína diana en pequeños péptidos. En los últimos años se ha descrito el papel de la ubicuitinación y del proteasoma en el control de la expresión génica a través del papel que ejerce la Ub en la modificación de la función de ciertas proteínas y en la degradación de factores proteicos asociados a las mRNPs (Brooks, 2010; Kwak *et al*., 2010).

Entre los complejos capaces de remodelar las histonas a través de modificaciones postranscripcionales encontramos un macrocomplejo de 1,8 MDa denominado SAGA (Spt–Ada–Gcn5 acetyltransferase). SAGA está implicado en la regulación de la transcripción de un 10% de los genes de levadura, especialmente de genes relacionados con la respuesta a diferentes tipos de estrés (Huisinga & Pugh, 2004), si bien recientemente se ha propuesto un papel más general (Bonnet G&D 2014). El papel de SAGA en la regulación de la expresión génica, viene determinado por la actividad de Gcn5 y Ubp8 como modificadores postraduccionales de las histonas (Baker & Grant, 2007; Rodriguez-Navarro, 2009). Gcn5 posee actividad HAT y se encarga de acetilar lisinas en las histonas H3 y H2B (Trievel *et al*.,1999), mientras que Ubp8 se encarga de desubicuitinar la lisina 123 de la histona H2B (Henry *et al*., 2003). Ubp8 está implicada en activación transcripcional, siendo parte del denominado

submódulo de desubicuitinación (DUB) junto a Sgf73, Sgf11 y Sus1 (Kohler *et al*., 2006; Kohler *et al*., 2008; Pascual *et al*., 2008). Estudios estructurales del submódulo DUB, han desvelado que interacciones entre estas cuatro proteínas son necesarias para reconstituir su actividad in vitro (Kohler *et al*., 2010; Samara *et al*., 2010). Las subunidades y funciones de SAGA desde levadura hasta eucariotas superiores (Figura I.2), se encuentran altamente conservadas (Rodriguez-Navarro 2009) y han desvelado la implicación de este complejo en el desarrollo celular y en la base molecular de distintas enfermedades (Koutelou *et al*., 2010).

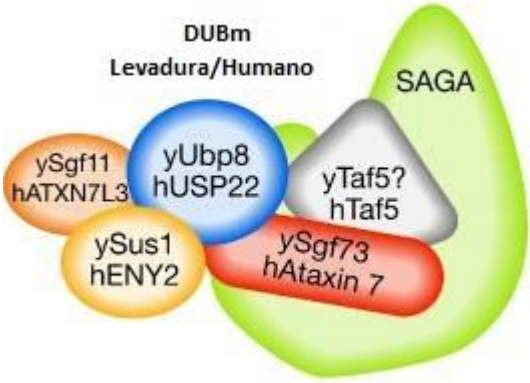

Figura I.2. Las subunidades que conforman SAGA se encuentran consevadas desde levadura hasta humanos. Sus1 forma parte del complejo de deubiquitinación (DUBm) junto a Sgf11, Ubp8 y Sgf73. Adaptado de la revisión de Rodríguez-Navarro, 2009.

I.III. La biogénesis del mRNA.

Una vez se ha remodelado la cromatina, diferentes factores implicados en la transcripción pueden acceder al DNA para promover la síntesis de los transcritos. El inicio de la transcripción viene determinado por el reconocimiento previo de secuencias en el promotor de los genes. El reclutamiento de los factores generales de la transcripción (GTFs) y del mediador, permitirán el asentamiento de la RNA/Pol II en la región de inicio de la transcripción y la formación del denominado complejo de preiniciación (PIC). La actividad helicasa de uno de los GTFs (TFIIH), separa la doble cadena de DNA molde para que se inicie la síntesis de ácido ribonucleico (RNA). Modificaciones en una de las subunidades de la RNA/Pol II, así como en reclutamiento de diferentes coactivadores de la transcripción se encargarán de permitir el acceso al DNA y al nuevo transcrito generado a lo largo de las diferentes etapas del proceso de síntesis del mRNA.

La RNA/Pol II está formada por doce subunidades que conforman un complejo de 0,5 MDa. Una de ellas es Rpb1 y posee un extremo carboxilo terminal (CTD) que consiste en la repetición en tándem de un péptido de siete aminoácidos (YSPTSPS). En levaduras encontramos 26 repeticiones de este heptapéptido en Rpb1, mientras que en otros eucariotas la longitud es variable (hasta 52). En

el PIC, Rpb1 se encuentra hipofosforilada. La fosforilación de las Serinas en la 2ª, 5ª o 7ª posición, determinarán el inicio y la elongación de la transcripción, así como el reciclaje de la polimerasa una vez terminado el transcrito. Estas modificaciones promueven la remodelación de la cola CTD de la polimerasa y el reclutamiento cotranscripcional de factores a la mRNP (Perales & Bentley, 2009). De este modo, la cola CTD tiene un papel esencial en el reclutamiento cotranscripcional de los factores implicados en multiples etapas de la biosíntesis de los mRNAs (Hahn & Young, 2011).

Como habíamos mencionado anteriormente, SAGA es un regulador de la expresión génica que participa en la remodelación de la cromatina, pero también actúa como un co-activador de la transcripción reclutando factores generales de la transcripción (Timmers & Tora, 2005).

Tras el inicio de la transcripción, un conjunto de modificaciones en el transcrito darán lugar a una molécula de RNA madura. La adición de la caperuza de trimetilguanosina (7mG) en el extremo 5', el splicing o el corte y poliadenilación del extremo 3' del transcrito, son algunas de las etapas clave en la formación y maduración de un mRNA. La visión respecto a estos procesos como pasos consecutivos e independientes ha cambiado en los últimos años, puesto que muchas evidencias han ido mostrando que cada uno de estos pasos influencia a los

demás y hay una comunicación/acoplamiento entre los factores que determinan la biogénesis del RNA mensajero (Figura I.3); (Maniatis & Reed, 2002; Dieppois & Stutz, 2010; Rodríguez-Navarro & Hurt, 2011).

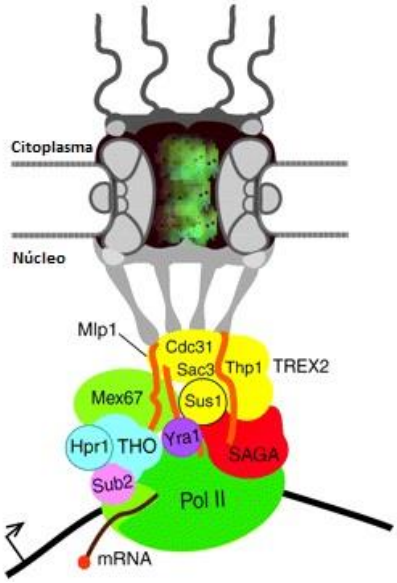

Figura I.3. Acoplamiento físico, mediado por SAGA y TREX2, de los procesos de elongación de la transcripción y exporte de mRNAs a través del poro nuclear. Adaptado de Dieppois & Stutz, 2010.

La molécula de mRNA, a medida que se va sintetizando, es reconocida por multitud de RNAs y factores proteicos que se van ensamblando para modificar y vigilar el procesamiento del transcrito. De este modo se forma una ribonucleopartícula (mRNP) competente para ser exportada a través del complejo del poro nuclear (NPC) hasta el citoplasma, donde el mRNA será traducido

a proteína (Luna *et al*., 2008, Kohler & Hurt, 2007). La mRNP es una macromolécula heterogénea muy dinámica, sobre la cual se reclutan y liberan multitud de factores durante su síntesis, splicing, degradación o traducción.

Actualmente conocemos que los diferentes procesos durante la biogénesis de los mRNAs están íntimamente coordinados (Kuersten & Goodwin, 2005; Maciag *et al*., 2006; Maniatis & Reed, 2002; Orphanides & Reinberg, 2002; Moore & Proudfoot, 2009). Después de que la RNA/Pol II inicie la transcripción y una vez que el transcrito posee entre 22-25 nucleótidos, se añade al extremo 5' una caperuza de protección (7mG). Esta estructura es reconocida por el complejo de unión a la caperuza (CBC), compuesto por Cbp20 y Cbp80, que estabiliza y protege al transcrito de su degradación por parte de las exonucleasas 5'→3'. Además el complejo CBC estimula el ensamblaje del spliceosoma a los pre-mRNAs (Görnemann *et al*., 2005). Durante la transcripción, un complejo dinámico formado por los snRNAs (small nuclear RNAs; U1, U2, U4, U5 y U6)) y sus proteínas asociadas, se une de forma secuencial a la molecula precursora del mRNA (pre-mRNA), procesando el transcrito para eliminar los intrones. El spliceosoma se encarga además de reclutar factores de exporte del mRNA que formaran una mRNP competente para ser exportada al citoplasma. Por último, el extremo 3' del

transcrito es reconocido y cortado endonucleolíticamente y entre 70 y 100 adenosinas son incorporadas (más de 200 en mamíferos). La cola de poliadedinas (poli(A)) se encargará de proteger a los transcritos de las exonucleasas que podrían degradarlo en dirección 3'→5', facilita el exporte del núcleo al citoplasma y forma una estructura en bucle que permitirá la traducción en el citoplasma. Durante todos estos procesos existen multitud de puntos de control en los que se verificará la calidad del transcrito para que pueda llegar a traducción o bien ser degradado (Doma & Parker, 2007; Fasken & Corbett, 2009; Moore & Proudfoot, 2009). Diversas son las maquinarias encargadas de reconocer y degradar transcritos que contengan errores tanto en el núcleo como en el citoplasma. Al menos todos los pasos nucleares durante la biogénesis del mRNA están acoplados, pero etapas que están separadas físicamente por la membrana nuclear, también están coordinadas mediante diferentes factores proteicos (Shen *et al*., 2010; Trcek & Singer, 2010). Muchos de los factores implicados en procesos postranscripcionales de la mRNP, son reclutados a través de la cola CTD de la polimerasa II. Además, hay que tener en cuenta que muchos factores proteicos encargados de los procesos de estabilización y degradación de transcritos, tanto en el núcleo como en el citoplasma, se incorporan a la mRNP cotranscripcionalmente y se

encargan de regular finamente la expresión génica (Garneau *et al*., 2007).

Debido a la temática de esta Tesis doctoral, profundizaremos en esta introducción en los procesos de splicing y degradación de RNAs mensajeros en levadura.

I.IV. El splicing en *S. cerevisiae*.

Los transcritos primarios o pre-mRNAs pueden contener dentro de su secuencia unas regiones denominadas intrones. Los intrones vienen definidos generalmente, en sus extremos y en el interior de su secuencia, por regiones altamente conservadas (secuencias de splicing). Al inicio de un intrón encontramos la secuencia del sitio de splicing en 5' (5'ss), siendo GUAUGU la secuencia más común en levadura (secuencia consenso). El final del intrón se define por una secuencia YAG (siendo Y cualquier pirimidina) que se denomina sitio de splicing en 3' (3'ss). Dentro del intrón encontramos un residuo de adenosina (A) o *branchpoint* (BP), que se encuentra flanqueado por una secuencia consenso llamada *branchsite* (BS) cuya secuencia más habitual en levadura es UACUAAC. El BP y el 3'ss necesitan un espacio óptimo entre sí para que el intrón pueda procesarse (Cellini *et al*., 1986). Entre 9 y 138 nt separan el BP del 3'ss en intrones de levadura (Fouser &

Friesen, 1987). Entre el BP y el 3'ss podemos encontrar regiones ricas en pirimidinas (poli(T)) implicadas en su reconocimiento. Cabe destacar que en *S. cerevisiae* y aguas arriba del BS es frecuente encontrar otras regiones ricas en timinas (T) (Bon *et al*., 2003). Además, nucleótidos en las regiones exónicas adyacentes a los 5'ss y 3'ss son importantes para su reconocimiento y se encuentran altamente conservadas.

La maquinaria encargada del procesamiento de los intrones (spliceosoma), es un complejo ribonucleoproteico muy dinámico formado por diferentes subunidades (U1, U2, U4, U5, U6 snRNPs), que junto a otras proteínas, se asocian secuencialmente a los intrones a través del reconocimiento de las secuencias de splicing.

El splicing se define como el proceso mediado por el spliceosoma, que se encarga de eliminar los intrones de los pre-mRNAs en dos reacciones secuenciales de transesterificación, tras las cuales las regiones codificantes o exones quedan unidas para formar un mRNA (Figura I.4). Este proceso está altamente conservado en todos los organismos eucariotas y es esencial para la regulación de la expresión génica (Hoskins & Moore, 2012).

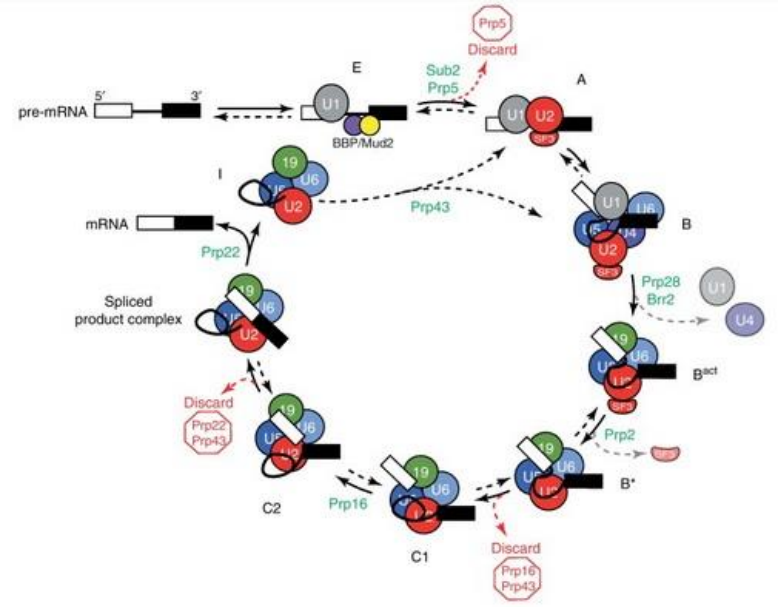

Figura I.4. El proceso de splicing y las proteinas que conforman el spliceosoma en levadura. Adaptado de Hoskins & Moore, 2012.

I.IV.I. Genes con intrones en levadura.

Desde que en 1996 se publicó el genoma completo de *S. cerevisiae*, actualmente se conocen más de 6.600 pautas abiertas de lectura (ORFs), de las cuales se ha verificado que alrededor de 4.900 codifican para proteínas. Solamente 284 intrones interrumpen la ORF de 274 genes en levadura, de los cuales 10 poseen más de un intrón. Por otro lado, la región no traducible en 5' (5'UTR) de algunos genes (en concreto 24) contiene intrones y de estos tan solo uno posee otro intrón interrumpiendo la ORF (*RPS22B*). Estos datos son

ampliados constantemente con el descubrimiento de nuevos intrones y regiones abiertas de lectura a través de múltiples análisis globales en levadura (SGD Project. http://www.yeastgenome.org).

Alrededor del 5% de los genes de la levadura *S. cerevisiae* contienen intrones y estos suelen localizarse dentro de la ORF cerca del extremo 5' y con un tamaño medio de 300 nucleótidos (nt); (Bon *et al*., 2003). Este porcentaje es muy bajo si lo comparamos con otras levaduras como *Schizosaccharomyces pombe*, donde el 43% de los genes nucleares contienen intrones (hasta 15 intrones en un mismo gen), normalmente de pequeño tamaño (81 nt de media) y con secuencias de reconocimiento de splicing poco conservadas (Wood *et al*., 2002). En el caso de eucariotas superiores, hasta el 80% de los genes de Drosophila contienen intrones (Sakharkar & Kangueane, 2004) y lo excepcional son los genes que no los poseen.

A pesar de la poca representación, en cuanto a número de genes con intrones en levadura, 10.000 de las 38.000 moléculas de mRNA que existen en una célula provienen de genes con intrones (Ares *et al.*, 1999). Los intrones no se distribuyen al azar en el genoma de levadura, sino que la mayoría se encuentran en genes que codifican para proteínas ribosomales, donde alrededor de 100 genes producen el 90% de los

transcritos de genes con intrones (Meyer & Vilardell, 2009). Otros grupos funcionales como las proteínas implicadas en secreción o meiosis también se encuentran sobrerrepresentados entre los que más genes con intrones poseen. Cabe destacar que aunque la mayoría de los intrones no son funcionalmente relevantes para el crecimiento celular (Parenteau *et al*., 2008), un buen número de genes implicados en el metabolismo del mRNA necesitan de ellos para la funcionalidad de la proteína que codifican. Además, las secuencias de splicing en intrones de este grupo funcional suelen no respetar la secuencia consenso (Tabla I.1).

Nombre sistemático	**Nombre estándar**	**Número de nucleótidos**	**5'ss**	**BP**	**3'ss**
YDR381W	YRA1	766	GUAUGU	**GUCUAAC**	UAG
YML034W	SCR1	126	**GUGAGU**	UACUAAC	UAG
YKL186C	MTR2	154	**GUACGU**	**AACUAAC**	CAG
YJL041W	NSP1	117	GUAUGU	UACUAAC	UAG
YNL004w	HRB1	341	GUAUGU	**UACUAAU**	UAG
YBR11W-A	SUS1 I1	80	**GUAUGA**	**UACUGAC**	UAG
YBR11W-A	SUS1 I2	70	GUAUGU	UACUAAC	UAG

Tabla I.1. Secuencias de splicing en genes implicados en el procesamiento del mRNA en levadura. En negrita secuencias distintas a la consenso en *S. cerevisiae*. Información obtenida de SGD Project.

I.IV.II. El splicing alternativo en levadura.

Debido a que en levadura la mayor parte de los genes que contienen intrones no posee más que un intrón, se reduce la posibilidad del splicing alternativo. Este mecanismo es muy frecuente en eucariotas superiores, siendo uno de los más extendidos en la regulación en la expresión génica. El splicing alternativo puede consistir en: a) el alargamiento o acortamiento de un exón por elección de sitios alternativos de splicing, b) la pérdida o inclusión de un exón a través de la acción de factores intensificadores o silenciadores de splicing y c) la eliminación o retención de un intrón que dará lugar a proteínas de distintos tamaños dependiendo de la posición del primer codón de parada de traducción en el transcrito (Keren *et al*., 2010). En metazoos, este hecho da lugar a una gran diversidad de transcritos y proteínas funcionales a partir de un mismo gen. En general, los exones alternativos poseen una longitud o señales no óptimas para su reconocimiento y son modulados por factores en *trans* que reconocen intensificadores o silenciadores del splicing en las secuencias de los intrones. Algunos de estos factores serían las proteínas SR (ricas en repeticiones de los aminoácidos, serina (S) y arginina (R)), SR-like o las hnRNP (complejos heterogéneos de RNA y proteínas), pero existen escasas

evidencias de homólogos funcionales en levadura (Lund *et al*., 2008; Wahl *et al.*, 2009). Aunque en humanos al menos el 74% de los genes está sujeto al splicing alternativo, pocos son los ejemplos descritos en *S. cerevisiae* y consisten principalmente en la retención del intrón asociado a mecanismos de regulación de la expresión y degradación del transcrito (Grund *et al*., 2008; Juneau *et al*., 2009).

I.IV.III. Algunos ejemplos de genes de levadura cuya expresión se encuentra regulada por sus intrones.

En *S. cerevisiae* y dentro del grupo de las proteínas ribosomales, existen algunos ejemplos como la proteína S14, cuya expresión está regulada por la presencia de intrones. Es codificada por dos genes distintos *RPS14A* y *RPS14B* y sus RNAs se encuentran en la célula en un ratio de 10:1. Ante un exceso de S14 existe una autorregulación a través de la unión de S14 al pre-mRNA de *RPS14B*, inhibiendo su splicing y conduciendo los transcritos a degradación (Fewell & Woolford, 1999). Otro caso estudiado en profundidad es la proteína L30, cuyo transcrito *RPL30* se procesa eficientemente en condiciones normales, pero ante un exceso de proteína, L30 se une a sus propios transcritos y el pre-mRNA se acumula en la célula (Vilardell & Warner, 1994).

Otros ejemplos en los cuáles se ha estudiado profundamente el papel de los intrones en la regulación de la expresión son *YRA1*, *MER1*, *SRC1* o *PTC7*.

YRA1 codifica para un componente del complejo de transcripción/exportación (TREX), implicado en el exporte de mRNA. Cuando hay exceso de proteína Yra1, esta se une a sus propios transcritos e inhibe su splicing (Preker *et al*., 2002). *YRA1* posee un intrón largo y un BS inusual que permiten la formación de una estructura secundaria esencial para esta autorregulación. Mediante este mecanismo se favorece el exporte frente al splicing de los transcritos, promoviendo su degradación en el citoplasma (Preker & Guthrie, 2006).

Mer1 es una proteína, asociada a U1snRNP, que se expresa solamente durante la meiosis y activa el splicing de al menos tres pre-mRNAs (*AMA1*, *HFM1/MER2* y *REC107/MER103*) a través de su unión a un "Mer1 enhancer element" presente en estos transcritos (Spingola & Ares, 2000).

Por último, *SRC1* y *PTC7*, son capaces de procesar sus intrones de forma alternativa y dar lugar a dos proteínas funcionales a partir de un mismo gen (Grund *et al*., 2008; Juneau *et al*., 2009). En el caso de *SRC1* existen dos 5'ss que pueden ser utilizados, mientras que en el caso de *PTC7* es la retención o no del intrón (sin codones de parada prematuros en su

secuencia), la que determina la formación de dos transcritos funcionales.

I.V. La degradación de mRNAs en *S. cerevisiae*.

I.V.I. Elementos que protegen al transcrito de su degradación.

Los mRNAs de eucariotas son protegidos cotranscripcionalmente en sus extremos con estructuras que impiden su degradación y que además reclutan factores de inicio de traducción como eIF4G y Pab1 (Garneau *et al*., 2007). En el núcleo, una vez que los transcritos poseen entre 20 y 30 nt de longitud, se incorpora una modificación en el extremo 5' a través de la acción secuencial de tres enzimas (Shuman 1995; Shatkin & Manley, 2000). En este proceso denominado capping, el extremo 5' se protege con la denominada caperuza 7mG. A ella se unen Cbp20 y Cbp80 que conformaran el complejo de la caperuza (complejo CBC), implicado en la protección del extremo 5' frente a degradación, así como en diferentes procesos durante la biogénesis del transcrito en el núcleo y el reclutamiento de factores implicados en el inicio de la traducción (eIF4G). Una vez en el citoplasma, las proteínas del CBC serán reemplazadas por eIF4E promoviendo la unión de los ribosomas

(Barabino & Keller, 1999). Cuando termina la transcripción de los mRNAs en eucariotas, el extremo 3' es cortado y se añade una cola poli(A). Diferentes proteínas capaces de unirse a la cola poli(A), como por ejemplo Pbp1 o Pab1, terminarán de procesar este extremo y protegerlo de la acción de las exonucleasas. Además, Pab1 se encargará de permitir la interacción física entre las estructuras en 5' y 3', e incluso de facilitar el reclutamiento de factores de inicio de traducción como eIF4G.

I.V.II. Degradación de los transcritos en el núcleo.

La degradación de los transcritos comienza cuando alguna de las estructuras que protegen sus extremos no se forma adecuadamente (Jiao *et al*., 2010), cuando estas son desplazadas para promover la degradación del mRNA o bien si se producen errores en la secuencia del transcrito durante su síntesis. Otro modo por el que puede comenzar la degradación de un transcrito, es a través de un ataque endonucleolítico, que lo partiría y generaría fragmentos con extremos desprotegidos. De este modo, cuando un extremo 5' o 3' del transcrito queda accesible, diferentes enzimas pueden iniciar la degradación. En células eucariotas, para evitar la acumulación en la célula de transcritos truncados, con errores o que pueden dar lugar a la traducción de proteínas no funcionales, existen

numerosos mecanismos de control de calidad (Doma & Parker, 2007; Fasken & Corbett, 2009). En el núcleo, diferentes factores proteicos se unen cotranscripcionalmente a la mRNP para asegurar su calidad, reconociendo los transcritos deletéreos y conduciéndolos a degradación a través un complejo denominado exosoma (Schmid & Jensen, 2008). El exosoma está formado por nueve subunidades y diferentes factores auxiliares como las riboexonucleasas Dis3/Rrp44 o Rrp6 (Vanacova & Stefl, 2007). Este complejo se encarga de degradar multitud de RNAs en la célula en dirección 3'→5'. Existe un exosoma citoplasmático que, a diferencia del nuclear, no posee la subunidad catalítica Rrp6 ni la subunidad Rrp47. Uno de los cofactores que se unen al exosoma nuclear es el complejo TRAMP, el cual detecta defectos en la terminación del transcrito y los conduce a degradación vía exosoma nuclear (Saguez *et al*., 2008).

I.V.III. Degradación de los transcritos en el citoplasma.

En el citoplasma actúan diferentes mecanismos de control para asegurar la calidad de los transcritos. Los principales son *Non-Stop Decay* (NSD), *No-Go Decay* (NGD) y *Nonsense Mediated Decay* (NMD) (Garneau *et*

al., 2007). Todas estas maquinarias conducen transcritos a degradación por una de las dos rutas principales en el citoplasma que describiremos más adelante.

El proceso de NSD se encarga de reconocer y conducir a degradación transcritos que no poseen un codón de parada, mientras que NGD se encarga de degradar a través de un ataque endonucleolítico a transcritos donde los ribosomas encuentran dificultades para avanzar. Por último, NMD detecta y degrada transcritos que contienen codones de parada de traducción prematuros (PTCs). Transcritos con mutaciones, pre-mRNAs que no han sufrido un splicing eficiente o pre-mRNAs productos de splicing alternativo, son potenciales blancos para NMD. Los componentes esenciales del complejo NMD son Upf1, Upf2 y Upf3 (Johansson *et al.*, 2007). Estos componentes básicos se encuentran conservados a lo largo de la evolución y aunque la función del complejo es similar, el mecanismo de acción es distinto en levadura y eucariotas superiores.

Para que se inicien las principales rutas de degradación citoplasmática, los mRNAs han de ser depoliadenilados previamente como paso común y necesario en levadura. Pan2-Pan3 se encargan de recortar la cola poli(A) hasta 60-80 nt (Brown *et al*., 1996), pero la principal deadenilasa en levadura es el complejo

CCR4-NOT (Tucker *et al*., 2001). Tras la depoliadenilación, existen dos rutas irreversibles encargadas de degradar el transcrito en dirección 5'→3' o 3'→5'.

La degradación en dirección 5'→3' se produce cuando los enzimas Dcp1/2 eliminan la caperuza y desprotegen el transcrito en 5' mediante un proceso denominado decapping. En este momento comienza una degradación en dirección 5'→3' por parte de la exoribonucleasa Xrn1 (Parker & Song, 2004, Garneau et al., 2007, Franks & Lykke-Andersen, 2008; Balagopal & Parker, 2009a). Esta maquinaria de degradación lleva asociada un grupo de activadores del decapping, que asociados al transcrito se encargan de promover la acción de Dcp1/2. Entre los factores que promueven el decapping, cabe destacar a Edc3, Pbp1 Lsm1-7, Pat1 o Dhh1 (Garneau *et al*. 2007).

Cuando el extremo 3' de un transcrito queda desprotegido, son las exonucleasas del exosoma citoplasmático las que atacan el transcrito en dirección 3'→5' (Parker & Sheth, 2007).

Los dos mecanismos presentados (Figura I.5) no son excluyentes y ambos pueden participar en la degradación de un mismo transcrito.

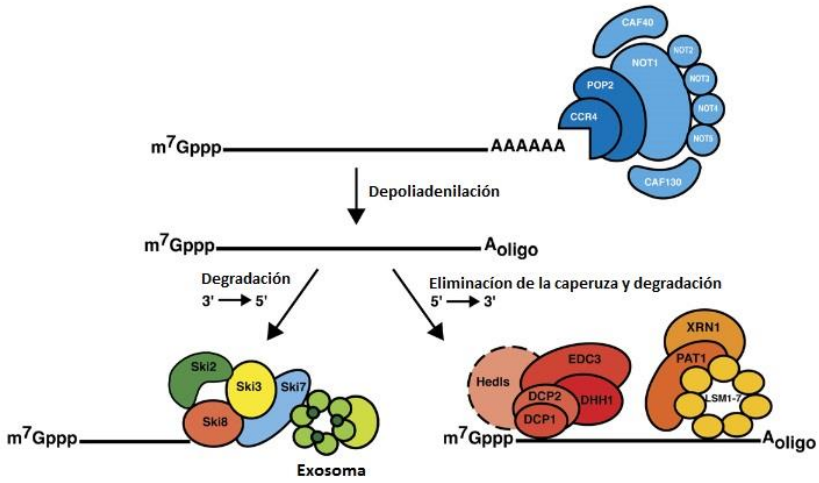

Figura I.5. Principales rutas de degradación citoplasmática de mRNAs. Degradación en dirección 3'→5' (abajo, izquierda) y degradación en dirección 5'→3' (abajo, derecha). Adaptado de Parker & Sheth, 2007.

I.V.IV. P-bodies y gránulos de estrés.

Se ha sugerido que, en el citoplasma, los mRNAs están sujetos a un movimiento dinámico entre traducción, almacenamiento y degradación (Parker & Sheth, 2007). La mRNP incorpora y substituye componentes para regular si un transcrito ha de ser utilizado en rondas sucesivas de traducción, almacenamiento o bien si ha de ser degradado, siendo la composición de la mRNP la que determina su localización en el citoplasma. Los transcritos se distribuyen en el citosol asociados a diferentes tipos de complejos que se encargan de procesarlos. Entre ellos

encontramos los P-bodies (PBs) o los gránulos de estrés (SGs) (Balagopal & Parker, 2009a; Buchan & Parker, 2009).

Los P-bodies están implicados en la represión de la traducción, almacenamiento de mRNAs y degradación de los mismos (Buchan *et al*., 2008). Los componentes de la maquinaria de degradación 5'→3' forman parte de estos agregados (Sheth & Parker, 2003), pero otras proteínas han sido identificadas interaccionando con los PBs en algunos mutantes o condiciones específicas de crecimiento. Entre ellas encontramos componentes de la maquinaria de NMD (Sheth & Parker, 2006) o de la maquinaria de inicio de traducción como eIF4E, eIF4G y Pab1 (Hoyle *et al*., 2007; Brengues & Parker, 2007).

Un segundo agregado citoplasmático bien estudiado, son los gránulos de estrés (Balagopal & Parker, 2009a). Los SGs aparecen cuando el inicio de traducción está alterado y están compuestos por agregados de mRNPs asociadas a factores de inicio de traducción y proteínas de unión a la cola poli(A). En levadura, los SGs contienen entre otros componentes a Pab1 (poli(A) binding protein) y las subunidades ribosomales eIF4E, eIF4G, eIF3.

Aunque en levadura existen otros tipos de agregados citoplasmáticos, PBs y SGs son los mejor estudiados y los procesos mayoritarios encargados de mantener un balance entre mRNAs que son traducidos,

almacenados o degradados. Además, como mostramos en la Figura I.6, tanto PBs como SGs están interconectados entre sí (Buchan & Parker, 2009).

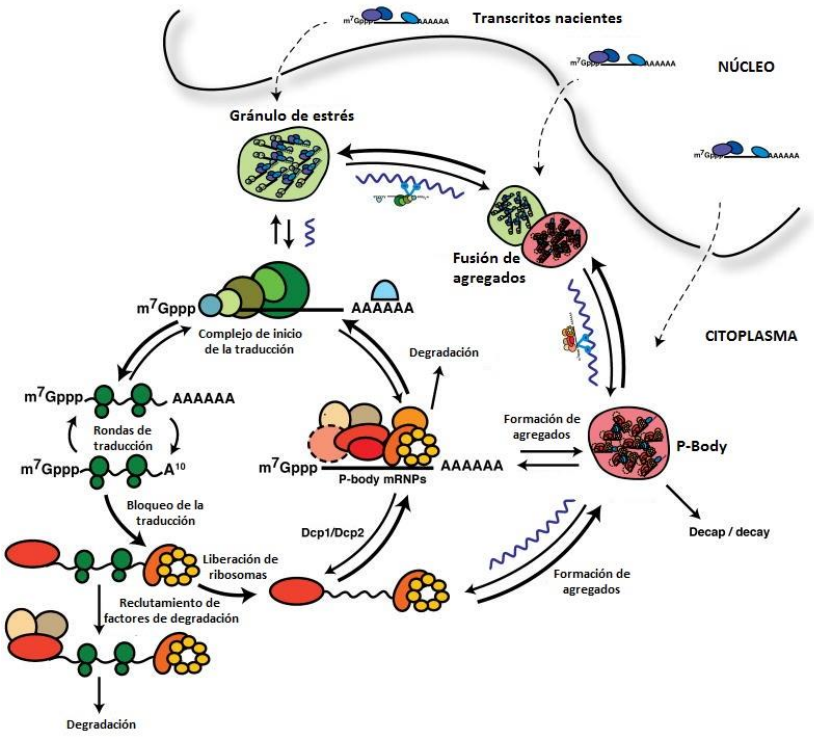

Figura I.6. Interconexión de P-bodies y gránulos de estrés alrrededor de los procesos de traducción, almacenamiento y degradación de mRNPs. Adaptado de Buchan & Parker, 2009.

I.VI. El gen *SUS1* y las funciones de la proteína que codifica.

La secuencia de nucleótidos de *SUS1* (Sl gene Upstream of ySa1) presenta algunas características que hacen de este gen un caso muy particular en *S. cerevisiae*. Su pequeño tamaño y la presencia de dos intrones interrumpiendo su secuencia codificante, permitieron que este gen permaneciera oculto en los rastreos del genoma de *S. cerevisiae* anteriores a su descubrimiento (Rodríguez-Navarro *et al*., 2004; Galán y Rodríguez-Navarro; 2012). Como hemos mencionado anteriormente, en levadura, solamente 10 de los más de 6200 genes anotados poseen dos intrones, siendo *SUS1* uno de ellos.

La proteína que codifica, Sus1, se encuentra conservada a través de la evolución no solo en su secuencia de aminoácidos sino en su función (Rodriguez-Navarro, 2009). Esta proteína de 11kb forma parte del co-activador transcripcional SAGA (complejo acetiltransferasa Spt/Ada/Gcn5) y del complejo de exporte de mRNAs del poro nuclear TREX2 (Figura I.7).

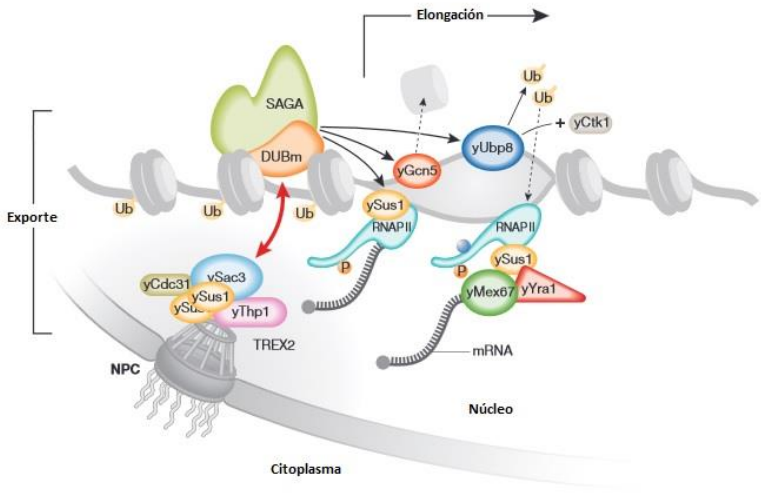

Figura I.7. Sus1 forma parte de complejos asociados a la elongación de la transcripción y al exporte de mRNAs. Adaptado de la revisión de Rodríguez-Navarro 2009.

Sus1, junto a Sgf73, Ubp8 y Sgf11, pertenece a un submódulo de deubicuitinación (DUB) capaz de eliminar la ubicuitina de histonas H2B monoubicuitinadas y de este modo promover la metilación de la histona H3 (Kohler et al., 2006; Kohler et al. 2008). En el poro nuclear, Sus1, Thp1, Sac3, Cdc31 y Sem1 forman parte del complejo TREX2 (Rodríguez-Navarro *et al*., 2004; Faza *et al*., 2009; Luna *et al*., 2009; García-Oliver *et al.*, 2012) y participa en el exporte de mRNAs. La cristalización de Sus1-Sac3-Cdc31 ha permitido desvelar las interactiones en las que participa Sus1, Figura I.8, y que permiten el acoplamiento de la maquinaria de traducción y de exporte de mRNAs (Jani *et al*. 2009; Wilmes & Guthrie, 2009).

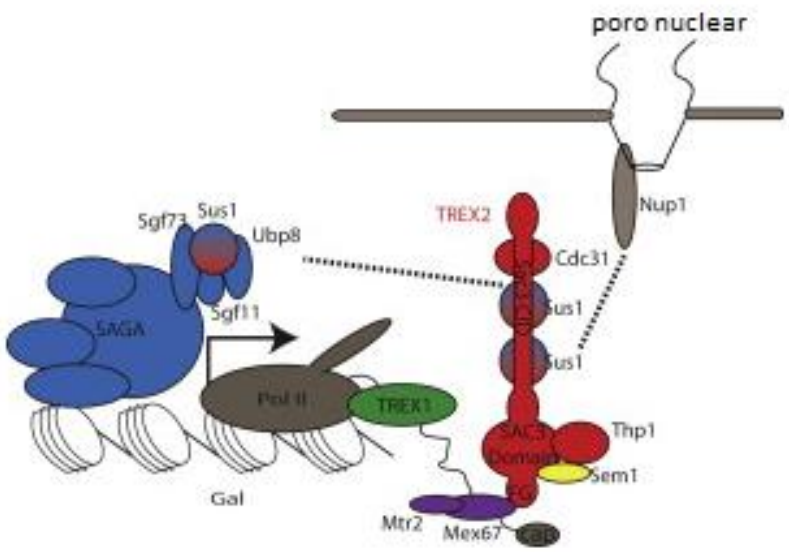

Figura I.8. Iteracciones protéicas en las que participa Sus1 durante el acoplamiento de transcripción y exporte de mRNAs a través del poro nuclear. Adaptado de la revisión de Wilmes & Guthrie, 2009.

La presencia de Sus1 en SAGA y en TREX2, se encarga de coordinar los procesos de transcripción y exporte de mRNAs en células eucariotas. Se ha demostrado que Sus1 es esencial para el posicionamiento del gen *GAL1* en la periferia nuclear cuando está transcripcionalmente activo (Cabal *et al*. 2006). Por otro lado, Sus1 es reclutado tanto a los promotores como a lo largo de la ORF de ciertos genes, participando en la elongación transcripcional a través de su interacción con la RNA/PolII y los factores de exportación Yra1 y Mex67 (Pascual-García *et al*., 2008). Sus1 es necesario para la

consecución de eventos postranscripcionales en el núcleo, afectando la morfología y localización de la mRNP (Chekanova *et al*., 2008). Tanto en levadura como en *Drosophila*, Sus1 ha sido localizado en el citoplasma, indicando que esta proteína puede poseer funciones en este compartimento celular (Pascual-García *et al*., 2008; Kopytova *et al*. 2010). Por tanto Sus1 se ha mostrado a lo largo de los años como una proteína clave, implicada en distintas etapas del proceso de expresión génica.

II. OBJETIVOS

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Durante el desarrollo de esta Tesis doctoral hemos profundizado en el estudio de la secuencia de *SUS1*, para determinar que regiones influyen en la regulación de la expresión de sus transcritos, de la proteína que codifica y de las implicaciones funcionales en las células de levadura. Por otro lado, la identificación de Sus1 en el citoplasma, motivó la investigación de una posible implicación funcional de la proteína Sus1 más allá de núcleo.

Los objetivos fundamentales de esta Tesis son:

- Estudiar los mecanismos encargados de regular la expresión del gen *SUS1*.

- Investigar la relación funcional de la proteína *Sus1* con factores citoplasmáticos implicados en la degradación del mRNA.

III. RESULTADOS

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III.I. CAPÍTULO I

Los resultados de este capítulo fueron publicados en la revista científica Nucleic Acids Research, en Julio de 2011. Se adjunta el texto íntegro de esta publicación, que representa el contenido del capítulo I en el apartado de resultados.

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SUS1 introns are required for efficient mRNA nuclear export in yeast

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Abstract

Efficient coupling between mRNA synthesis and export is essential for gene expression. Sus1/ENY2, a component of the SAGA and TREX-2 complexes, is involved in both transcription and mRNA export. While most yeast genes lack introns, we previously reported that yeast *SUS1* bears two. Here we show that this feature is evolutionarily conserved and critical for Sus1 function. We determine that while *SUS1* splicing is inefficient, it responds to cellular conditions, and intronic mutations either promoting or blocking splicing lead to defects in mRNA export and cell growth. Consistent with this, we find that an intron-less *SUS1* only partially rescues *sus1Δ* phenotypes. Remarkably, splicing of each *SUS1* intron is also affected by the presence of the other and by *SUS1* exonic sequences. Moreover, by following *SUS1* RNA and protein levels we establish that nonsense-mediated decay (NMD) pathway and the splicing factor Mud2 both play a role in *SUS1* expression. Our data (and those of the accompanying work by Hossain *et al.*) provide evidence of the involvement of splicing, translation, and decay in the regulation of early events in mRNP biogenesis; and imply the additional requirement for a balance in splicing isoforms from a single gene.

Introduction

A significant part of mRNP biogenesis takes place co-transcriptionally, and functional links between chromatin modifications, transcriptional elongation, splicing and mRNA export have been shown (1,2). Sus1 (ENY2 in metazoans) is a small, evolutionarily conserved 11-kDa protein that is a component of two molecular assemblies with roles in both transcription and mRNA export (3,4). Sus1 can be found in the SAGA complex, involved in chromatin function, where is part of the histone H2B deubiquitinating (DUB) module together with Ubp8,

Sgf11 and Sgf73 (5,6). Interactions between these four proteins are necessary to reconstitute full DUB activity *in vitro*, as revealed recently by structural studies (7,8). In addition, during transcription Sus1 can be recruited to promoters and along coding regions, where it interacts with RNA Pol II and the mRNA export factors Yra1 and Mex67 (9). At the nuclear pore, Sus1 is a component of the TREX2 complex formed by Sac3, Thp1, Sus1, Cdc31 and Sem1 (10–12). Sus1 plays a role in mRNA export from the nucleus and it is implicated in anchoring active genes at the nuclear periphery through gene gating (12,13). Moreover, in specific circumstances Sus1 can be observed at cytoplasmic structures (14). Thus, Sus1 participates in multiple stages of gene expression, with roles in chromatin function and mRNA biogenesis.

In addition, the *SUS1* gene structure is of particular interest. Unlike most yeast genes, which are typically intronless, *SUS1* bears two introns; and while most yeast introns display conserved splicing signals (15), both the 5' splice site and branch site of the first intron of *SUS1* are non-canonical (3,16). Interestingly, *Saccharomyces cerevisiae* introns are not randomly distributed throughout the genome, but most are found in highly expressed genes (17).

Notably, introns are found as well in a number of genes functionally relevant to RNA metabolism, such as *YRA1*, *MTR2*, *SRC1*, *NSP1* and *HRB1* (18). Interestingly, *SRC1* is a rare example of alternative splicing in yeast (19,20), and both *YRA1* and *SRC1* are functionally and genetically linked to *SUS1* (10,20). In fact, extensive work on *YRA1* points to the intricate circuitry of an mRNP life, encompassing splicing, export, and degradation (21–25).

Here we assessed a link between Sus1 function and *SUS1* unusual genomic structure. We find that *SUS1* introns are evolutionarily conserved, and their deletion leads to defects in RNA export and to slow growth. The unconventional splicing signals in *SUS1* are also important for *SUS1* function, which is affected by non-essential splicing factors such as Mud2 and modulated by the non-

sense mediated decay (NMD) pathway. Thus, proper *SUS1* expression requires a delicate balance of splicing, translation and decay.

Materials and methods

Identification of S. cerevisiae *SUS1* orthologs and phylogenetic trees

Putative orthologues of *S. cerevisiae SUS1* gene were identified by BLASTn (26) and WU-BLAST2 (27) searches on public databases and default search parameters were used. A list of species used in our analysis can be found at Supplementary Table S1. The evolutionary transformation of three structural characters of *SUS1* (intron number, 5'ss and BS) were optimized onto the known phylogeny of 25 eukaryotes species (28,29). Ancestral and derived multistate transformations were achieved considering equal weighted unordered characters in MacClade program (30).

Yeast cultures and microbiological techniques

Growth assays to test the intron functionality were done by growing cells at 30ºC on synthetic selective medium [SC: glucose 2%, ammonium sulfate 0.5%, yeast nitrogen base 0.17% and supplements (Dropout)] Lacking Tryptophan (Trp) to 0.3–0.4 OD600. Subsequently, 10-fold serial dilutions of an equal number of cells were made and drops spotted onto SC-trp plates. Growth was recorded after 2–3 days of incubation at 30 and 37ºC. Yeast cell transformations were done by the LiAc/SS carrier DNA/PEG method (31). For wild-type (WT), *mud2*Δ, *upf1*Δ and *mud2*Δ*upf1*Δ splicing pattern comparison, cells were grown at 30ºC in YPD (2% glucose, 1% yeast extract and 2% peptone) and collected when 0.6 OD_{600}. To test different stress conditions influencing *SUS1* splicing pattern, cells were grown in YPD at 30ºC until 0.4 OD_{600} and then divided in three

flask, in order to incubate the cell cultures under different treatments: (i) cells were grown 2 h more at 30ºC in YPD; (ii) cells were incubated 20 min at 42ºC; or (iii) carbon source was shifted to galactose and the cells were grown 2 h at 30ºC. After each treatment, cells were collected, frozen in liquid nitrogen and stored at −80ºC until RNA extraction. For copper assays, cultures at 30ºC were grown to 0.4 OD_{600} in SC medium lacking leucine and equal volumes were dropped onto SC-Leu plates containing $CuSO_4$ ranging from 0 to 1.0 mM (32). Plates were photographed after 3 days at 30 ºC.

RNA analysis

Total RNA was harvested from yeast cultures by the Hot/Acid-phenol method (33). RNA was quantified using Nanodrop and quality was checked by agarose gels with ethidium bromide (etBr). Northern blot analyses were carried out as in (34). *SUS1* and U1 snRNA detection was done using T7-transcribed riboprobes.

Reverse transcription PCRs and qRT–PCRs

RT–PCR and qRT–PCR analysis were performed using 1 µg of total RNA. After DNase-I treatment (Promega), RNAs were purified by Phenol/Chloroform extraction. Reverse transcription was performed using standard procedures, with random hexamers and M-MLV reverse transcriptase (Invitrogen). Specific pairs of primers were used to amplify *SCR1* or *SUS1* transcripts containing exons 1 and 3; using 3 µl of cDNA as template (previously diluted 1/20). Amplified products were run in a 2% agarose-EtBr gel and visualised. Specific primers for each *SUS1* transcript or *SCR1* ncRNAs were used to amplify qRT–PCR products from 3 µl of cDNA (previously diluted 1/10) and using SYBR® GreenER™ qPCR SuperMix (Invitrogen) in a 10 µl final volume. Each sample was run in duplicate with the standard curve. Real-Time PCR was performed using a LightCycler® (Roche). An activation

step of 10 min at 95ºC followed by 45 cycles of 10 s at 94ºC, 15 sat 50ºC and 20 s at 72ºC were used for mRNA, intron 1 (I1) and intron 2 (I2) primer pairs. For I1 and I2 transcripts, the 72ºC step was extended to 30 s. For *SCR1* RNA, 38 cycles of 10s at 94ºC, 15s at 60ºC and 20 s at 72ºC were used. See the 'Materials and Methods' in Supplementary Data for a list of primers. The amount of each *SUS1* transcripts was represented in arbitrary units (AU). One arbitrary unit is equivalent to 10□2 fmol/mg of total RNA.

Protein purification, western blot and inmunoprecipitation analysis

For crude protein extracts, 1.5 OD_{600} from cultures on exponential growth at 30ºC were collected. Cells were lysed in 150 µl of NaOH, 2 M plus 7.5% (v/v) ß-mercaptoethanol and proteins were precipitated for 10 min on ice. After centrifugation, the pellet was resuspended in 50 µl of denaturing loading buffer. Samples were heated at 95ºC before loading and separation in 16% SDS–PAGE gels. Western blot analysis was performed using anti-LexA and anti-GFP according to standard procedures.

For GFP-Sus1 immunoprecipitations 50 ml of WT and *mud2*Δ *upf1*Δ strains containing plasmids bearing GFP or GFP-SUS1g were grown on selective medium to OD_{600} 0.5. Cells were harvested, washed, and resuspended in 200 µl of ice-cold lysis buffer (50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM PMSF and protease inhibitors). The same volume of glass beads was added and cells were broken by four pulses of vortexing Turing 1 min at 4ºC. The supernatant were immunoprecipitated during 1h at 4ºC in a turning wheel and using anti-GFP antibody (Roche) coupled to dynabeads (Invitrogen). The immunoprecipitates were washed three times for 10 min with lysis buffer and subsequently resuspended in 50 µl of SDS–PAGE sample buffer. Western blot analysis of

samples was made using anti-GFP and anti-Sus1 antibodies.

In Situ Hybridization (FISH)

Fluorescent in situ hybridization (FISH) against poly(A)+RNA was done growing yeast cells in 100 ml of YPD medium at 30ºC to an 0.5 OD_{600}. Then, cultures were divided and a half of each culture were rapidly shifted to 37ºC incubator for 3h, and the other half was incubated at 30ºC during that time. Cells were immediately fixed adding 10% of formaldehyde, 1h at room temperature. The fixative was removed by two rounds of centrifugation and washed with 0.1 M potassium phospate (pH 6.4). Cells were resuspended in ice-cold washing buffer (1.2 M sorbitol and 0.1 M potassium phosphate, pH 6.4), and subsequently, cell wall was digested with 0.5mg/ml of Zymolyase 100T and samples were applied on poly-L-lysine-coated slide wells. Non-adhering cells were removed by aspiration, cells were rehydrated with 2X SSC (0.15M NaCl and 0.015M sodium citrate) and hybridized overnight at 37ºC in 20 µl of prehybridization buffer (formamide 50%, dextran sulfate 10%, 125 µg/ml of *Escherichia coli* tRNA, 4X SSC, 1X Denhardt's solution and 500 µg/ml herring sperm DNA) with 0.8 pmol of Cy3-end-labeled oligo(dT) in a humid chamber. After hybridization, slides were washed with 1X SSC at room temperature, air-dried and mounted using VECTASHIELD® Mounting Medium with DAPI. Detection of Cy3-oligo(dT) was performed using a Leica DM600B fluorescence microscope.

Results

The *SUS1* gene

Two features of *SUS1* genomic structure are of particular interest. As we previously showed, *SUS1* (ENY2 in metazoans) bears two introns, a rare feature in the *S. cerevisiae* genome (3). Only 5% of the genes in *S. cerevisiae* have introns and of those just ten carry two (18, 35–37). In addition, while most yeast introns display conserved splicing signals, both the 5' splice site and branch site of the first intron of *SUS1* are non-canonical. Interestingly, other yeast introns with regulated splicing also have non-canonical splice sites (21). Thus, we decided to explore the degree of evolutionary conservation of these features, which may indicate their biological relevance.

We identified *SUS1* homologs in different species (Supplementary Tables S1 and S2), and performed an evolutionary analysis. Our results (Figure 1) reveal the following: first, *SUS1* is likely to have evolved from an ancestral gene containing four introns (Figure 1A). While in fungi genes containing one, three or four introns are equally probable; after the branching to *C. neoformans* (lane 3 in Figure 1) a single-intron gene (*SUS1* I1) is unequivocally the most parsimonious ancestral reconstruction. Subsequent evolution leads to the addition of a second intron in most of the Saccharomycotina group of species. Second, having two introns in fungi correlates with carrying a non-canonical 5'ss in the first intron (Figure 1B and Table 1). Third, non-consensus BS in I1 is only present in *S. bayanus*, *S. mikatae*, *S. paradoxus* and *S. cerevisiae* (Figure 1C). Strikingly, in fungi, *SUS1* genes having I1 with the non-consensus BS UACUGAC correlates with the presence of a highly conserved 3'UTR of 300 nt (Supplementary Figure S1). Altogether these data suggest a biological relevance for *SUS1* introns in its expression and we decided to further address this question.

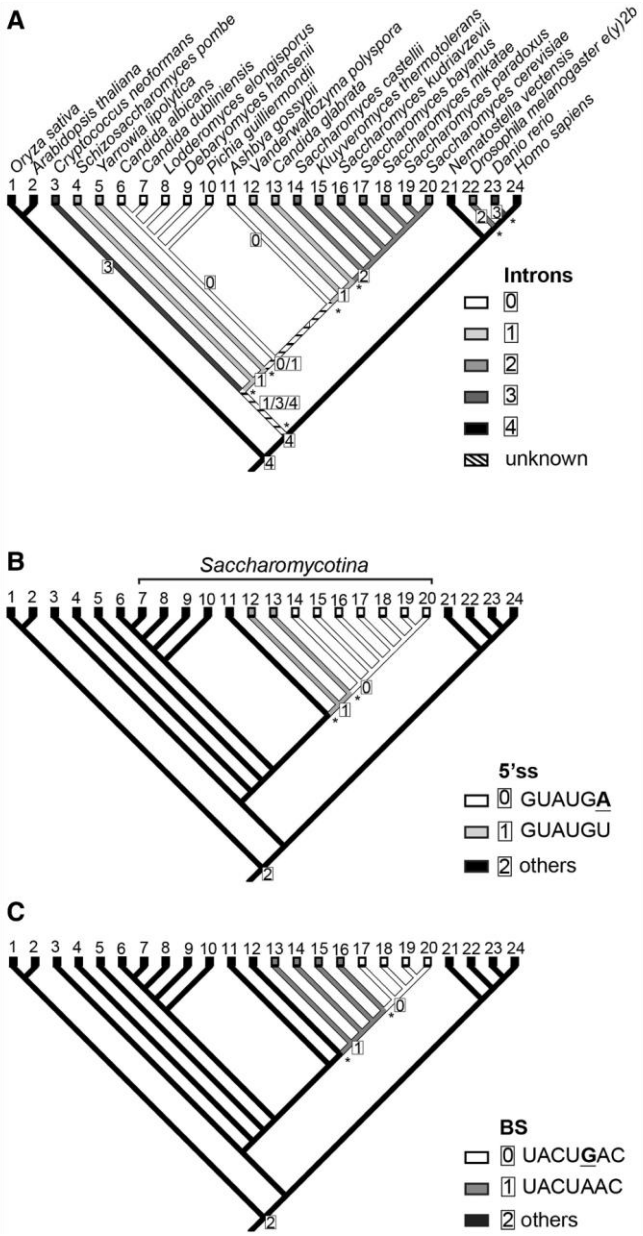


Figure 1. Evolution of three *SUS1* structural characteristics. (A) Evolution of the number of introns in *SUS1*. Intron number (boxes) in *SUS1* orthologues for 24 eukaryotic species is represented based on a known topology (28). According to the distribution of ancestral and derived states, seven evolutionary changes (in asterisks) are required

in the tree to explain intron number diversity of *SUS1*. (B) The Saccharomycotina subphylum incorporates a second intron in *SUS1* orthologues when a second intron appeared. Numbers in boxes represent non-consensus sequence (0), consensus sequence (1) or other sequences for 5'ss in *S. cerevisiae* and related fungi. (C) A non-consensus BS appears in the I1 of *SUS1* for the most related species to *S. cerevisiase*. Numbers in boxes represent non-consensus sequence (0), consensus sequence (1) or other sequences for BS in *S. cerevisiae* and related fungi.

Species	Introns	I1			I2		
		5′ss	BS	3′ss	5′ss	BS	3′ss
S. cerevisiae	2	GUAUG**A**	UACU**G**AC	UAG	GUAUGU	UACUAAC	UAG
S. mikatae	2	GUAUG**A**	UACU**G**AC	UAG	GUAUG**A**	UACUAAC	UAG
S. paradoxus	2	GUAUG**A**	UACU**G**AC	UAG	GUAUG**A**	UACUAAC	UAG
S. bayanus	2	GUAUG**A**	UACU**G**AC	CAG	GUAUG**C**	UACUAAC	UAG
S. castellii	2	GUAUG**A**	UACU**A**AC	AAG	GUAUG**A**	UACUAAC	CAG
S. kudriavzevii	2	GUAUG**A**	UACUAAC	UAG	GUAUG**A**	UACUAAC	UAG
K. thermotolerans	2	GUAUG**A**	UACUAAC	CAG	GUAUGU	UACUAAC	UAG
C. glabrata	1	GUAUGU	UACUAAC	CAG			
V. polyspora	1	GUAUGU	**C**ACUAAC	AAG			
Y. lipolytica	1	GU**GA**GU	**C**ACUAAC	CAG			
S. pombe	1	GUAU**AA**	U**UA**UAAC	UAG			

Table 1. Splicing signals in *SUS1* and *SUS1* ortholog introns. Sequence of 5' splicing site (5'ss), Branch Site (BS) and 3' splice site (3'ss) were described for most evolutionary related species. 5'ss, BS, and 3'ss consensus sequences in *S. cerevisiae* are GUAUGU, UACUAAC and YAG (Y:pyrimidine) respectively (45). In bold and underlined, nucleotides different from consensus splicing sequences in *S. cerevisiae*.

SUS1 splicing

We used quantitative and semi-quantitative RT–PCR to determine the relative splicing efficiencies of *SUS1* introns. Bands corresponding to the fully spliced (mRNA) and *SUS1* transcripts retaining I1, were consistently detected in WT cells (Figure 2A). This is in agreement with both data from Hossain *et al*. (16) and genome-wide studies (38,39), reporting detectable amounts of *SUS1* transcripts retaining I1. We hypothesize that splicing of *SUS1* I1 is either inefficient or subject to regulation, leading to cellular accumulation of unprocessed *SUS1* transcripts.

To test this idea, we analyzed *SUS1* splicing under different conditions of temperature and carbon source, where *SUS1* function is more determinant (10).

Quantitative RT–PCRs of RNA collected from WT cells incubated 20 min at 42ºC revealed that *SUS1* expression is reduced by the temperature shift, with accumulation of unspliced *SUS1* transcripts and reduction of the corresponding mRNA (Figure 2B). Notably, *SUS1* expression is induced by growth in galactose, while its splicing pattern remains unchanged (Figure 2B). Altogether, our results indicate changes in expression and splicing efficiency dependent on growth conditions and suggest that *SUS1* splicing is a regulated event.

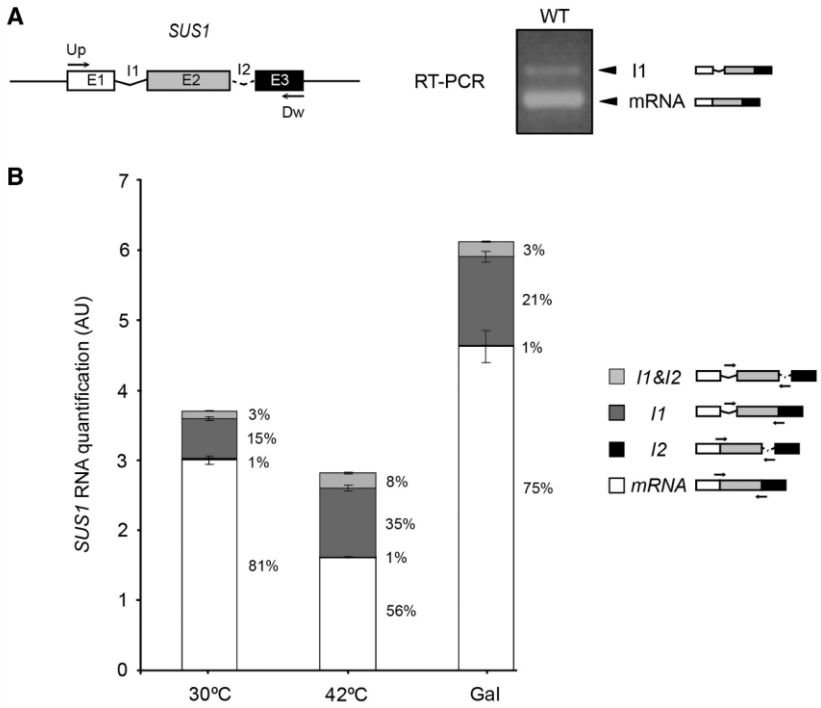


Figure 2. *SUS1* splicing is regulated. (A) Analysis of *SUS1* transcripts by semi-quantitative RT–PCR. Left, *SUS1* primer positions. Right, *SUS1* transcripts at 30ºC. Bands corresponding to mature RNA (mRNAs) and pre-mRNA retaining I1 are indicated. (B) Quantitative analysis of *SUS1* splicing pattern under different growth conditions, as indicated (Gal: growth at 30ºC with galactose as carbon source). The percentage of each *SUS1* transcript is indicated. Primer pairs to specifically detect each *SUS1* transcript by qRT–PCR are depicted on the right.

To analyze *SUS1* splicing we generated constructs based on the *CUP1* reporter system, widely used to follow splicing efficiency *in vivo* (32). *cup1*Δ cells were transformed with one of the following reporters (Figure 3A): pACT1-CUP1, pSUS1g-CUP1 (with both *SUS1* introns), pSUS1-*I1*Δ-CUP1 (I1 removed) and pSUS1-BS-CUP1 (I1 with UACU**G**AC mutated to UACU**A**AC, rendering its BS consensus). Splicing was monitored by both assessing copper tolerance and primer extension analyses. As illustrated in Figure 3A deletion of I1 improves growth compared to the genomic construct (compare lanes 2 and 4), likely due to a better splicing efficiency of *SUS1* transcripts lacking I1, which suggests that I1 splicing is limiting for *SUS1* expression. Accordingly, the construct SUS1-BS-CUP1 displays a similar copper tolerance to the construct SUS1-*I1*Δ-CUP1 (Figure 3A, lanes 3 and 4). These results were further confirmed by primer extension (data not shown) and are consistent with those presented in the accompanying paper (Hossain *et al*. this issue). Thereby we conclude that *SUS1* splicing efficiency likely is limited by the suboptimal BS in I1.

We further explored this possibility by assessing the role of Mud2 in *SUS1* expression. Mud2, the yeast homolog of U2AF, interacts with BBP, an essential factor that identifies the BS and its required for splicing of a variety of transcripts (40,41). Mud2 is not essential, so we analyzed *SUS1* splicing in *mud2*Δ cells. Northern blot analysis of RNA from *mud2*Δ cells shows accumulation of SUS1 pre-mRNA species with a concomitant decrease of mature RNA, compared to WT (see below in Figure 4B and Hossain *et al*.). Thus, we decided to further analyze *SUS1* splicing in *cup1*Δ*mud2*Δ cells, using our *CUP1* reporters. Consistent with the northern blot analysis, deletion of *MUD2* leads to a marked reduction in copper tolerance for cells carrying SUS1g-CUP1 (Figure 3A, compare lanes 2 and 6). Interestingly, while mutation of the I1 BS to consensus (SUS1-BS-CUP1) dramatically

improves copper tolerance in *mud2*Δ cells, this tolerance is further increased by deleting I1 (Figure 3A, lanes 7 and 8). This is consistent with a requirement for Mud2 in I1 splicing in part but not exclusively due to the non-consensus BS, and the non-consensus 5'ss of I1 is likely to be involved as well (Hossain *et al.* this issue).

To further assess the dependency of *SUS1* splicing on Mud2 *in vivo*, we have quantified by qRT–PCR the different splicing forms in *mud2*Δ cells. Absence of Mud2 correlates with a substantial enrichment of pre-mRNA retaining either both introns or I1, rising from 16% of total transcripts to 68% (a 4-fold increase, Figure 3B). However, the largest change in absolute terms is the dramatic decrease in the mature mRNA species (a 10-fold change). In summary, our data support a prominent role for Mud2 in *SUS1* expression. It is likely that other factors involved in the early recognition of introns are relevant to *SUS1* expression as well (Hossain et al., this issue).

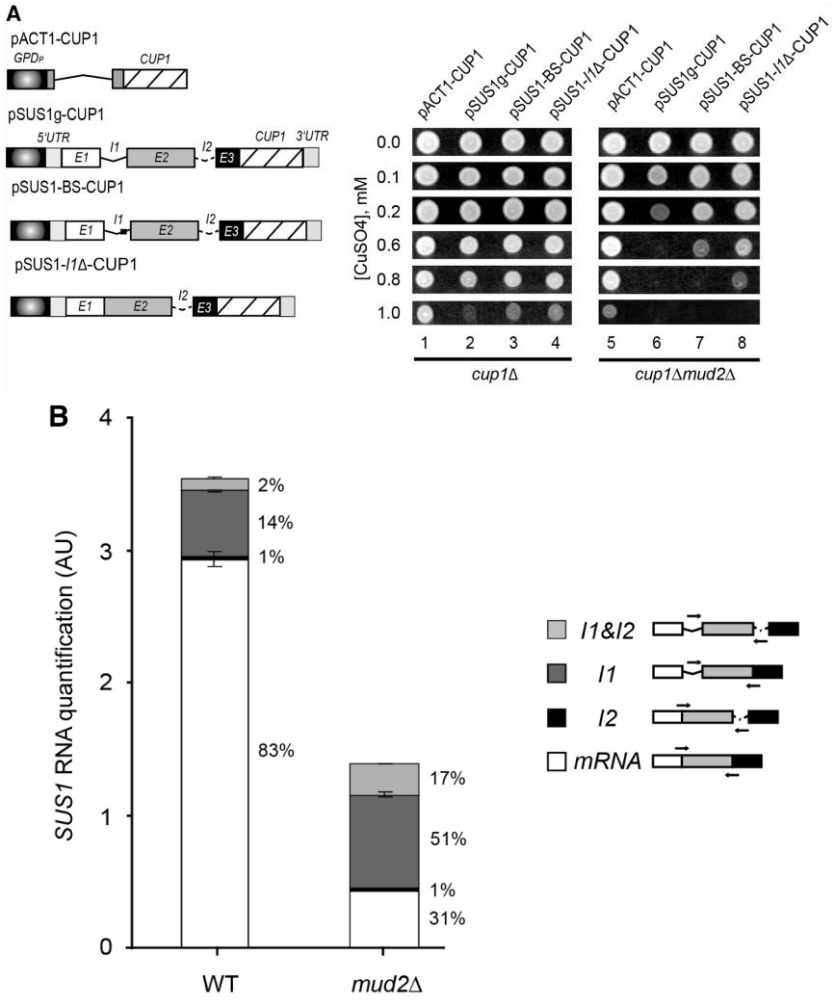


Figure 3. Mud2 is required for efficient *SUS1* splicing. (A) On the right, copper assays of *cup1*Δ and *cup1*Δ*mud2*Δ cells transformed with different SUS1-CUP1 versions (shown at the left). Comparable number of cells was spotted onto plates containing $CuSO_4$, as shown. (B) Quantification of *SUS1* transcripts by qRT–PCR, from RNA extracted from WT or *mud2*Δ cells. The percentage of each *SUS1* transcript is indicated. Primers used to specifically amplify each transcript are depicted on the right.

SUS1 unspliced transcripts are targeted by NMD

The reduced *SUS1* expression in *mud2*Δ cells (Figure 3B) might be a consequence of the appearance of premature stop codons (PTC) in transcripts containing I1, which would trigger NMD (42). Consistent with this hypothesis, qRT–PCR and northern analyses of RNA from both WT and *upf1*Δ strains revealed a 1.5-fold increase in accumulation of intron-containing *SUS1* transcripts in *upf1*Δ cells (Figure 4A and B, lane 3).

To test whether products of *SUS1* splicing (or mis-splicing) in *mud2*Δ cells were targeted for degradation by the NMD pathway, we determined the levels of *SUS1* transcripts in *mud2*Δ*upf1*Δ cells (Figure 3). Northern blot and semi-quantitative RT–PCR analyses (Figure 4B) show accumulation of unspliced *SUS1* transcripts in *mud2*Δ*upf1*Δ cells, compared to those of WT. qPCR results (Figure 4C) indicate that this accumulation corresponds to a 6-fold increase. Thus we conclude that *SUS1* splicing depends on Mud2 and that *SUS1* unspliced transcripts are targeted by NMD.

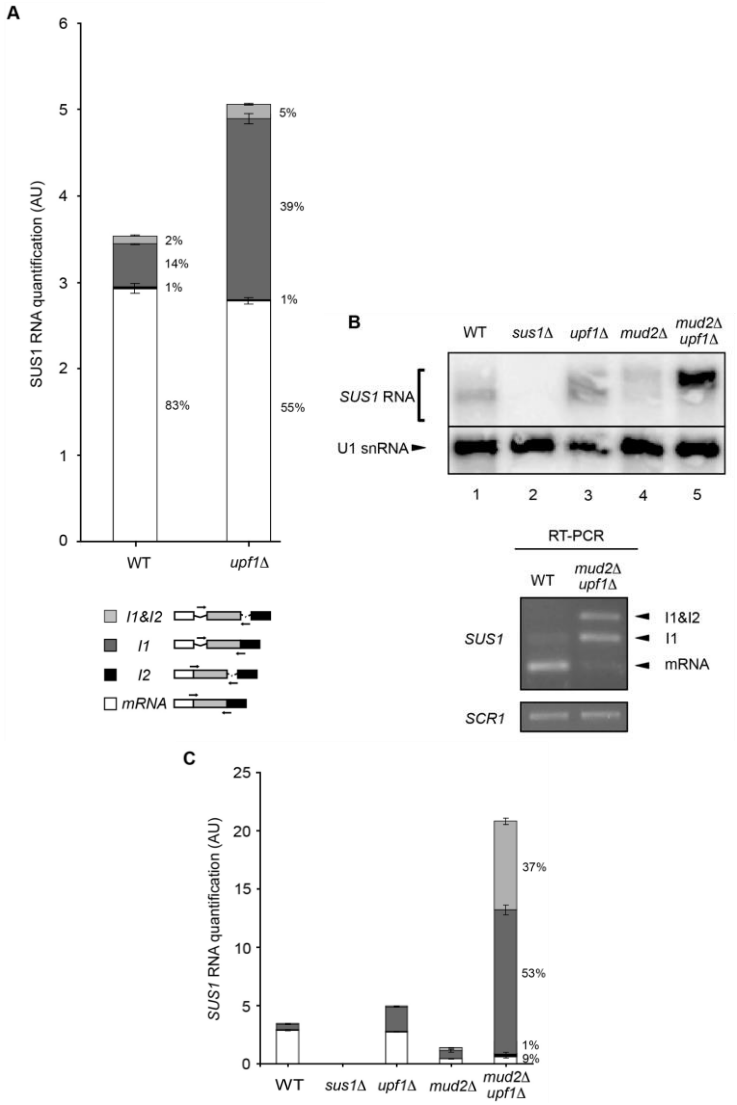


Figure 4. *SUS1* transcripts are targeted by the Non-sense Mediated Decay pathway (NMD). (A) qRT–PCR quantification of *SUS1* transcripts from RNA extracted from WT or *upf1*Δ cells growing at 30ºC. (B) Northern blot (upper panel) and semi-quantitative RT–PCR (lower panel) showing the *SUS1* RNA accumulation in mutant strains affecting *SUS1* splicing. U1 snRNA and *SCR1* are used as loading controls in northern and RT–PCR analysis respectively. (C) qRT–PCR quantification of *SUS1* transcripts from WT, *sus1*Δ *upf1*Δ *mud2*Δ and *mud2*Δ*upf1*Δ. The Percentage of each *SUS1* transcript is indicated for *mud2*Δ*upf1*Δ.

Sus1 protein expression is regulated by its introns

Many introns regulate gene expression by a variety of mechanisms, and in yeast there are several instances of introns involved in autoregulatory loops (*YRA1* or *RPL30*) (17). Here we show that *SUS1* introns have a critical role on mRNA levels, and in order to assess whether they are necessary to maintain appropriate Sus1 amounts, we determined the quantity of Sus1 protein expressed from several constructs including those carrying modified splicing signals. Plasmids bearing SUS1cDNA or the genomic version of *SUS1* (SUS1g), N-terminally fused to LexA or GFP tags, were introduced into a mutant strain lacking endogenous *SUS1*. Levels of LexA (Figure 5A) or GFP (Supplementary Figure S2) were monitored by western blot analyses. Our results indicate that elimination of *SUS1* introns causes accumulation of Sus1 protein when compared to SUS1g (Figure 5A compare upper band in lanes 2 and 3).

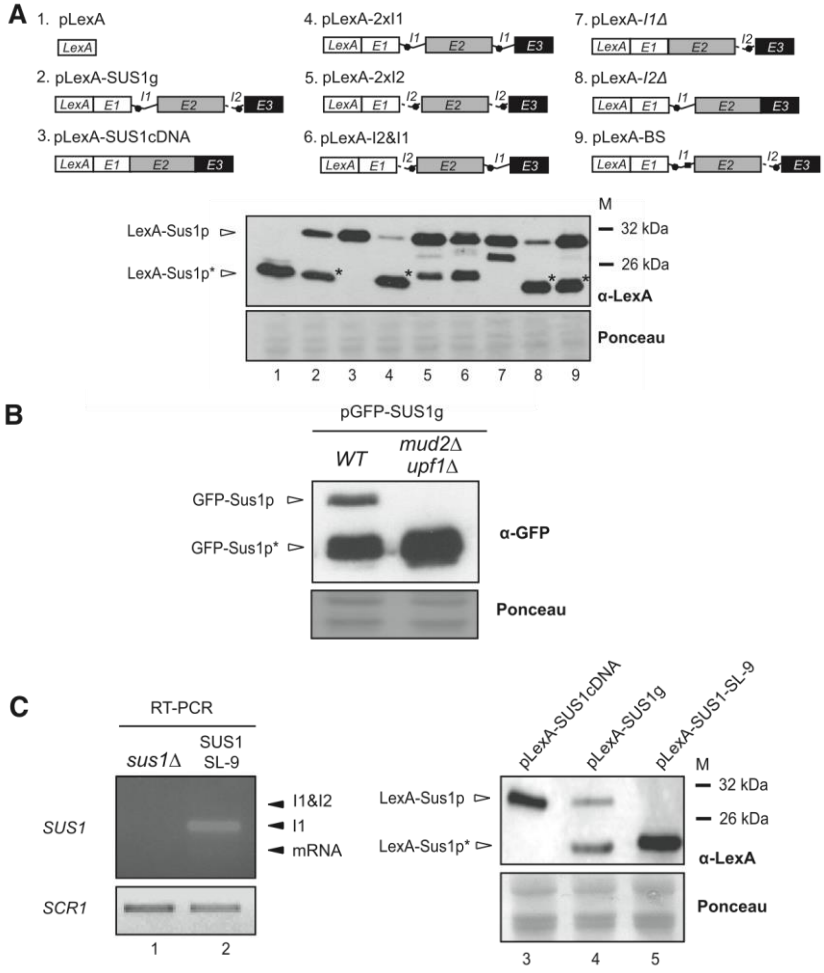
To study how the presence and position of each intron contribute to Sus1 protein levels, we cloned different SUS1 constructs in pBTM-LexA (43) under the *ADH1* promoter. Expression of the LexA-Sus1 fusion proteins was monitored by western blotting using anti-LexA antibodies (Figure 5A). Notably, the presence of I1 at both locations (2xI1, Figure 5A construct 4) provoked the strongest reduction in Sus1 protein levels (Figure 5A, lane 4). As expected from our splicing analysis, I1 elimination (lane 7), substitution by I2 (lane 5) or mutation of its BS signal to the consensus (lane 9), stimulated Sus1 expression to levels comparable to the SUS1cDNA version. Of note, swapping introns (lane 6) or removing I2 (lane 8) also affects Sus1 protein expression. We conclude that both the presence and the position of both introns are key determinants of Sus1 protein expression levels.

Although production of protein isoforms by alternative splicing in yeast is rare, *SUS1* potentially could be alternatively spliced to produce different proteins. N-terminal

tagging of *SUS1* with both introns enables the detection of alternate forms of Sus1 protein if they exist. Strikingly, a smaller intense band similar in size to the tag alone was observed when using both antibodies (marked band with an asterisk in Figure 5A). This band is consistent with a smaller version of Sus1 protein translated from pre-mRNAs retaining either I1 or both introns. Intron 1 retention introduces a PTC, and the predicted protein translated from this sequence will include exon 1 and 6 additional residues. To assess whether this protein is not a degradation product of full-length Sus1, we hypothesized that its abundance will correlate with an increase in *SUS1* transcripts containing I1. Therefore, we determined Sus1 protein relative abundance in two different situations. First, we followed the ratio small/full-length Sus1 protein (pLexA-SUS1g) in *mud2*Δ*upf1*Δ cells, where unspliced versions of *SUS1* are enriched compared to WT (Figure 4B and C). Consistent with our hypothesis, we observe an accumulation of the small Sus1 in the double mutants (Figure 5B). Second, we cloned into pBTM-LexA a version of the SUS1 gene [SL-9 mut, (10)] that has a mutated BS sequence (**U**ACUGAC mutated to **C**ACUGAC), which blocks splicing of I1, driving accumulation of *SUS1* transcripts retaining I1 (Figure 5C, left panel). As expected a prominent band consistent with the size of the small Sus1 protein, is recognized using anti-LexA antibodies, while no full-length Sus1 protein is observed (Figure 5C right panel). Altogether, we conclude that a small version of Sus1 is translated from *SUS1* transcripts containing I1.

To further verify that the small band detected in our western analyses contains Sus1 residues, we transfected pGFP-SUS1g in WT and *mud2*Δ*upf1*Δ cells and subsequently we immunoprecipitated whole cell extracts using anti-GFP antibodies. Western blotting reveals two GFP forms (Figure 5D), one (~38kDa) consistent with full-length Sus1-GFP; and a second (~28kDa) consistent with the translation of Sus1 exon 1 plus 6 residues, fused to GFP (GFP-Sus1p*). Consistent with our model, this form is enriched in extracts from *mud2*Δ*upf1*Δ cells bearing

pGFP-SUS1g (which leads to accumulation of *SUS1* transcripts retaining I1, lane 3). Additional confirmation that the 28 kDa band includes a truncated version of Sus1 was obtained by probing the same immunoprecipitated with anti-Sus1 antibodies, producing the same result (Figure 5D, right panel).



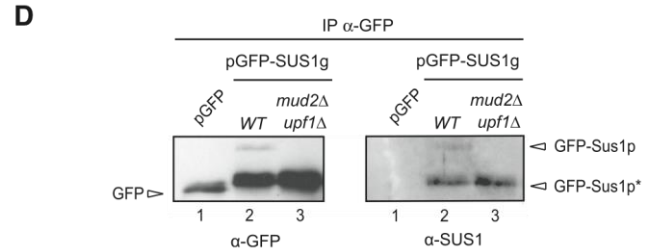


Figure 5. Sus1 levels are regulated by the presence and position of *SUS1* introns. (A) Whole cell extracts from *sus1*Δ cells transformed with the indicated plasmids (top) were used to follow Sus1 protein levels by western blot using anti-LexA (bottom). Black hexagons in *SUS1* introns scheme indicate position of PTCs if the introns are not removed. The ponceau staining was used as a loading control. Asterisk indicates the putative protein translated from *SUS1* transcripts retaining I1. (B) Sus1 levels of whole cell extracts from WT and *mud2*Δ*upf1*Δ strains containing pGFP-SUS1g were analyzed by western blot using anti-GFP. The ponceau was used as loading control. (C) Left, *SUS1* transcripts detected by RT–PCR from and SL-9 cells. Right, Sus1 levels in whole cell extracts, from *sus1*Δ cells transformed with the indicated plasmids, measured as in (A). (D) Anti-GFP immunoprecipitated proteins from WT and *mud2*Δ*upf1*Δ strains bearing pGFP or pGFP-SUS1g plasmid were analyzed by western blot using anti-GFP (left panel) or anti-Sus1 (right panel) antibodies respectively.

Exonic sequences enhance *SUS1* splicing

To further decipher the molecular mechanisms controlling *SUS1* splicing, we have investigated whether exonic sequences flanking *SUS1* introns might contribute to their splicing. To do this, we have analyzed the splicing efficiency of *SUS1* introns in another exonic context, using the *TAF14* gene. *TAF14* has one canonical intron (position 10–114nt, from the start codon). We have followed the expression of this construct by western analysis of LexA fusions (Figure 6A). As anticipated given its reduced splicing, replacement of the *TAF14* native intron by *SUS1* I1 decreases the amount of expressed LexA-Taf14 protein (Figure 6A, lanes 1 and 2). In an attempt to recapitulate *SUS1* gene organization, we cloned I2 at the same distance from I1 that it is located in *SUS1* ORF, creating a

Taf14 exon2* (see construct design in Figure 6A). Surprisingly, incorporation of I2 leads to a further splicing block, causing a strong reduction in Taf14 protein levels (Figure 6A, lanes 2 and 3). This is consistent with the observed importance of both introns in *SUS1* expression (Figure 5). Next we decided to explore the role of exon 2 sequences in *SUS1* splicing using our LexA-TAF14 construct containing *SUS1* introns (TAF14-I1&I2SUS1). Intriguingly, replacing the *TAF14* sequence between *SUS1* introns (TAF14 exon 2*) by *SUS1* exon 2, restores splicing to almost native Taf14 levels (Figure 6A, compare lanes 1 and 4).

To better substantiate this observation, we have monitored splicing efficiency by growth in copper using a *CUP1* construct in which the original *SUS1* exon 2 was replaced by a sequence corresponding to TAF14 exon 2* (see construct design in Figure 6B). In agreement with our western results, replacement of *SUS1* exon 2 by *TAF14* sequence results in reduced copper tolerance consistent with a reduced splicing (Figure 6B, lane 3). Thus we conclude that exon 2 sequences on *SUS1* gene support proper *SUS1* splicing.

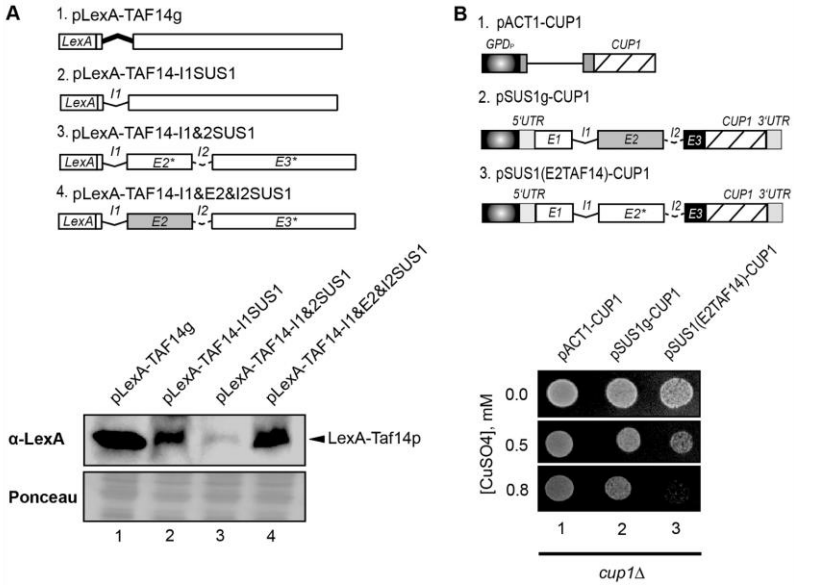


Figure 6. Exonic regions induce *SUS1* splicing. (A) LexA-Taf14 protein levels were monitored by western analysis of whole cell extracts, from cells transformed with the indicated plasmids and using anti LexA antibodies. The ponceau staining was used as a loading control. (B) Top, scheme showing the *CUP1* constructs used to monitor the role of *SUS1* exon 2 in *SUS1* splicing. In construct 3 the exon 2 of *SUS1* is replaced by *TAF14* sequences. Splicing was monitored by a copper assay, shown at the bottom.

SUS1 introns are required for Sus1 cellular function

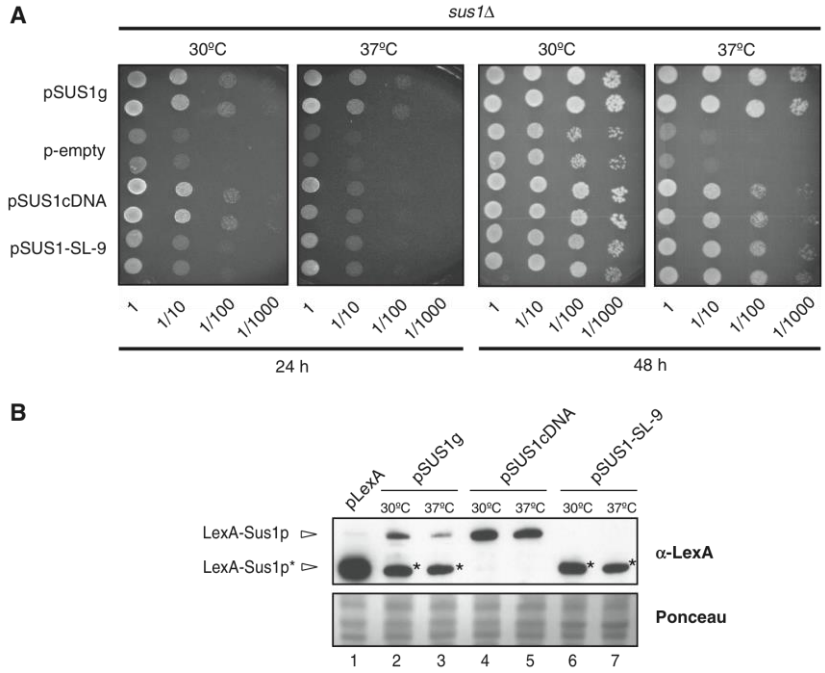
To address the biological relevance of *SUS1* introns we have examined the phenotype of *sus1*Δ cells transformed with plasmids containing either *SUS1* with its introns (pSUS1g) or without (pSUS1cDNA). *sus1*Δ cells are sensitive to high temperature (ts) and their growth can be affected by the carbon source [data not shown and (10)]. Notably, while sus1D cells transformed with pSUS1g lose the ts phenotype, there is less suppression of the phenotype with the pSUS1cDNA (Figure 7A, see growth at 37ºC). This is consistent with the differential expression profile observed for *SUS1* transcripts at high temperature (Figure 2B), and we speculate that this profile is relevant to the role of Sus1 at 37ºC.

An intriguing explanation could be that *SUS1* transcripts containing I1 have functionality at higher temperatures. To assess this hypothesis, we tested the growth at 37ºC of ***sus1***Δ cells transformed with pSUS1-SL-9 (with an I1 containing a non-consensus BS, which renders mostly *SUS1*-I1 retained transcripts, as described in Figure 5C). As expected from its deficient splicing, cells transformed with this construct grow similar to an empty plasmid at 30ºC (Figure 7A, compare growth of p-empty and pSUS-SL-9 at 30ºC after 24 h). Surprisingly, this construct is able to complement the ts phenotype exhibited at 37ºC to a larger extent (Figure 7A, compare growth of p-empty and pSUS-SL-9 at 37ºC after 48h) than the cDNA, albeit less so than the gDNA. These data indicate that *SUS1* introns are required for optimal growth and have a biological relevance.

To further address whether changes in Sus1 protein expression could account for the differences observed in our complementation assay, we followed the levels of Sus1 isoforms at different temperatures when expressed from the genomic version (p*SUS1*g), the cDNA (p*SUS1*cDNA), or the SL-9 BS mutant (p*SUS1*-SL-9) (Figure 7B). As expected from the results of the RNA analyses, a down-regulation of full-length Sus1p is observed at 37ºC (compare upper band in lanes 2 and 3). Although a reduction in Sus1p levels is also appreciable in cells containing the cDNA version, Sus1p levels at 37ºC are still elevated when compared to those of wt both at 30 or 37ºC (lanes 4 and 5 versus 2 and 3). A slight downregulation of Sus1p* also occurs at 37ºC (lanes 2 and 3) and this is more apparent when *SUS1* is expressed form the SL-9 BS mutant version (lanes 6 and 7). These data support our hypothesis that the changes in the relative levels of Sus1 isoforms account for the differences observed in our complementation assays.

Sus1 is necessary for mRNA export, and in ***sus1***Δ cells nuclear accumulation of poly(A)+ RNA is readily detected at 30 and 37ºC [Figure 7C and (10)]. To assess

the effect of *SUS1* introns on this phenotype, we performed RNA in situ hybridization (FISH) in exponentially growing ***sus1***Δ cells, transformed with p*SUS1*g, p*SUS*1cDNA, or p*SUS1-I1*Δ (Figure 7C). Consistent with the growth complementation results shown in Figure 7B, both SUS1g and SUS1cDNA were able to complement to a large extend the mRNA export defect of sus1D cells at 30ºC (Figure 7C upper panel). In contrast, FISH signal from ***sus1***Δ cells transformed with p*SUS1*cDNA and grown at 37ºC, indicates that the *SUS1* gene lacking both introns is not able to efficiently complement the ***sus1***Δ mRNA export defect. Moreover, this is replicated by a construct lacking I1. Thus we conclude that *SUS1* introns are required for the *SUS1* role in optimal mRNA export in *S. cerevisiae.*



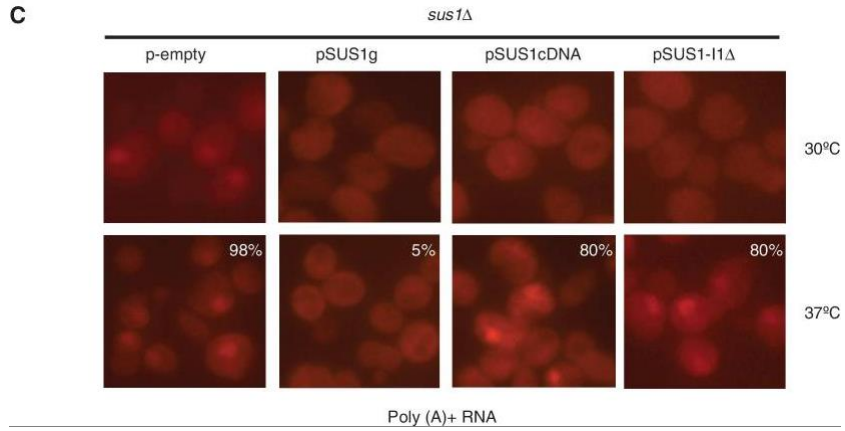


Figure 7. *SUS1* introns are required for optimal growth and mRNA export. (A) *sus1*Δ strains containing an empty plasmid (p-empty), the same plasmid bearing *SUS1* with its introns (pSUS1g), without introns (pSUS1cDNA) or with a suboptimal BS at I1 (pSUS1-SL-9), were spotted applying serial dilutions of an equal number of cells onto SC-trp plates and incubated at 30 or 37ºC. (B) Whole-cell extracts from *sus1*Δ cells transformed with the indicated plasmids and grown at 30 or 37ºC for 4h, were used to follow Sus1 isoforms proteins levels by western blot using anti-LexA. The ponceau staining was used as a loading control. Asterisk indicates the putative protein translated from *SUS1* transcripts retaining I1. (C) FISH analysis of nuclear mRNA export in *sus1*Δ cells. Localization of poly(A)+RNA at 30ºC and after shifting to 37ºC for 3h, in *sus1*Δ strains transformed with empty, SUS1g, SUS1cDNA or SUS1-I1 containing plasmids, was performed using Cy3-labeled oligo(dT) probe. The percentage of *sus1*Δ cells with nuclear export defect is indicated for each condition.

Discussion

The study presented here provides insights into the intricate mechanism that regulates *SUS1* expression, a crucial factor linking transcription and mRNA export. We found that (i) *SUS1* introns are required for its functionality, (ii) protein levels of Sus1 are controlled by *SUS1* introns, (iii) *SUS1* expression is regulated by splicing and NMD and (iv) intronic and exonic sequences participate in *SUS1* regulation. The accompanying manuscript by Hossain *et al.* in this issue exposes the relevance of *SUS1* expression as a model for understanding the role of core spliceosomal components in alternative splicing.

Discovery of the *SUS1* gene in a genetic screening revealed an intriguing genomic organization (10). *SUS1* gene (438nt) consists of three exons (of 71, 140, and 77 nt) and two introns (80 and 70 nt). Notably, their size and position are widely conserved (Supplementary Table S2). Sus1 plays important roles during transcription elongation and mRNA biogenesis, which likely explains its high degree of evolutionary conservation, from yeast to human. Why does this small gene, with key functions at different stages in gene expression, contains two introns in yeast, where most genes contain none? A provocative hypothesis is that this allows the *SUS1* gene to be a sensor of these processes, acting in a yet unknown feedback control mechanism. We demonstrate here that splicing and decay of *SUS1* transcripts regulate expression of Sus1 protein. This strategy is reminiscent of that of *YRA1*, another factor involved in the coupling of mRNA export and transcription. Intriguingly, *YRA1* shares with *SUS1* having both an atypical intron and its expression regulated at the level of splicing, degradation and export of its transcripts, although likely by different mechanisms (22,24,25). Moreover, mutations in *SUS1* and *YRA1* are synthetic lethal, and Sus1 and Yra1 interact physically. A possible scenario is that these factors are finely tuned to work together to sense or modulate correct

mRNP biogenesis, as they are sensitive to alterations in several aspects of this pathway.

Our findings reveal that I1 is the major determinant of *SUS1* splicing efficiency. Elimination of I1 or mutation of its BS leads to more efficient *SUS1* splicing. These data are consistent with the observed weak splicing efficiency of I1, largely due to its non-consensus BS and imply that I1 and its BS are crucial for *SUS1* expression. We also show that splicing of *SUS1* largely depends on the BS recognition factor Mud2. However, we see that deletion of I1 has a stronger effect than mutation of the BS on *mud2*Δ cells. These results imply that other features of I1 are dependent on Mud2, likely the non-consensus 5' splice site of I1 (GUAUGA). In agreement with this, Hossain *et al.*, in the accompanying study have addressed the relevance of non-canonical splicing signals for I1 and I2, revealing that the 5' splice site of I1 is in fact an important determinant of its processing.

We find an intriguing co-evolution between the presence of a non-consensus BS in I1 and the existence of a conserved long sequence at the 3'-end of the gene. In this context, transcriptome analyses showed that *SUS1* transcripts retaining I1 carry a longer 3'UTR than the fully spliced RNAs (38). We hypothesize that efficient splicing and proper 3'-end formation in *SUS1* could be linked, as it has been shown for other transcripts (44). Current work in our lab tries to address how important is this 3'-end sequence in *SUS1* regulation.

Intron 2 of *SUS1* also appears to play an important role in splicing, despite its apparent lack of unusual features. We find that removing I2 can be detrimental for protein expression for LexA-SUS1 (Figure 5A, lane 8), and intriguingly, the sole presence of I2 provokes a splicing block when placed into the *TAF14* gene (Figure 6A), which is consistent with the data from Hossain et al. addressing the splicing efficiency of I2. In addition, swapping *SUS1* introns also affects Sus1 protein levels, strongly arguing for a co-dependence in splicing of both *SUS1* introns. We also have found that splicing of *SUS1* introns is also

affected by exonic sequences, as evidenced by the low expression of *TAF14-SUS1* chimerical constructs (Figure 6). Remarkably this can be suppressed by replacing the *TAF14* newly-created middle-exon with that of *SUS1*. Consistent with this, substituting *SUS1* exon 2 by this *TAF14* 'exon 2' reduces Cup1 expression in *SUS1-CUP1* constructs. Thus our data suggest a positive role for exon 2 in *SUS1* splicing. Work is currently under way to address whether exonic splicing enhancers are involved.

We have verified the biological relevance of *SUS1* introns, by showing that a cDNA version of *SUS1* is not able to fully complement *sus1*Δ phenotypes in mRNA export and growth [Figure 7; we also assess that this is not due to a lack of protein production from an intron-less construct (lanes 2 and 3 versus 4 and 5, Figure 7B)]. Similar behavior was observed for *YRA1* cDNA (21). Observations that *SUS1* splicing is inefficient under optimal growth (Figure 2A) suggest that *SUS1* splicing could be regulated. Consistently, we find that the ratios between the different species of *SUS1* RNAs are influenced by growth conditions. In fact, at higher temperatures in which *SUS1* function is more critical, pre-mRNA forms retaining the I1 or both introns (I1 and I2) are more abundant (Figure 2B). This change in the transcript ratios could account for the lack of fully complementation by SUS1-cDNA at 37ºC (Figure 7B and C). In this context a striking observation is the existence of a small protein containing part of Sus1, likely translated form a transcript containing I1 (Figure 5). Western analyses are consistent with this peptide being produced, in agreement with the functional requirement of transcripts containing I1, suggesting a role of the I1 *SUS1* transcripts. Our results are consistent with the notion that the small Sus1 peptide is not a proteolytic product of the full-length protein. First, it accumulates in *mud2*Δ*upf1*Δ cells, where full-length Sus1 production is reduced (Figure 5); and second, the small Sus1 product is absent in cells bearing an intron-less *SUS1*, where full-length Sus1 is enhanced (Figure 7). An alternate explanation, invoking a selective degradation of

full-length Sus1 when encoded by an intron-containing *SUS1* gene, is difficult to conceptualize and not consistent with the abundance of the small peptide in cells bearing the *SUS1* SL-9 BS mutant, which cannot be spliced and thus cannot encode full-length Sus1. Notably, the functional requirement of transcripts containing I1 at high temperature is suggestive of a biological role for this unprocessed *SUS1* transcript. More work is needed to assign this functionality to the pre-mRNA, its product, or both.

Our results, together with those from the accompanying report of Hossain *et al.*, provide compelling evidence indicating that expression of *SUS1* hinges on a balance of several factors including limited splicing and degradation pathways, modulated by intronic and exonic sequences. The question now emerges as to how this complex strategy gives *SUS1* its relevant role in multiple aspects of mRNP biogenesis.

Supplementary data

Supplementary Data are available at NAR Online.

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Supplementary Table 1

Arabidopsis thaliana
Ashbya gossypii
Candida albicans
Candida dubliniensis
Candida glabrata
Cryptococcus neoformans
Danio rerio
Debaryomyces hansenii
Drosophila melanogaster e(y)2b
Homo sapiens
Kluyveromyces lactis
Kluyveromyces thermotolerans
Lodderomyces elongisporus
Nematostella vectensis
Oryza sativa
Pichia guilliermondii
Saccharomyces bayanus
Saccharomyces castellii
Saccharomyces cerevisiae
Saccharomyces kudriavzevii
Saccharomyces mikatae
Saccharomyces paradoxus
Schizosaccharomyces pombe
Vanderwaltozyma polyspora
Yarrowia lipolytica

Suppl. Table 1. List of species used to analyze *SUS1* genomic evolution.

Supplementary Table 2

		Number of nucleotides								
Species	**Introns**	**ORF**	**CDS**	**Exon 1**	**Intron 1**	**Exon 2**	**Intron 2**	**Exon 3**	**Intron 3**	**Exon 4**
S. cerevisiae	2	441	291	71	80	140	70	80		
S. bayanus	2	449	291	71	84	140	74	80		
S. paradoxus	2	441	291	71	77	140	73	80		
S. kudriavzevii	2	446	291	71	78	140	77	80		
S. mikatae	2	440	285	65	81	140	74	80		
K. thermotolerans	2	464	300	80	93	140	71	80		
S. castellii	2	450	288	68	71	140	91	80		
C. glabrata	1	447	303	223	144	80				
V. polyspora	1	421	303	83	119	220				
A. gossypii	0	300	300	300						

		Number of nucleotides								
Species	**Introns**	**ORF**	**CDS**	**Exon 1**	**Intron 1**	**Exon 2**	**Intron 2**	**Exon 3**	**Intron 3**	**Exon 4**
K. lactis	0	291	291	291						
P. guillermondii	0	294	294	294						
D. hansenii	0	306	306	306						
L. elongisporus	0	336	336	336						
C. dubliniensis	0	342	342	342						
C. albicans	0	360	360	360						
Y. lipolytica	1	354	276	62	78	214				
S. pombe	1	398	330	80	68	250				
C. neoformans	3	873	303	83	209	71	227	69	134	80

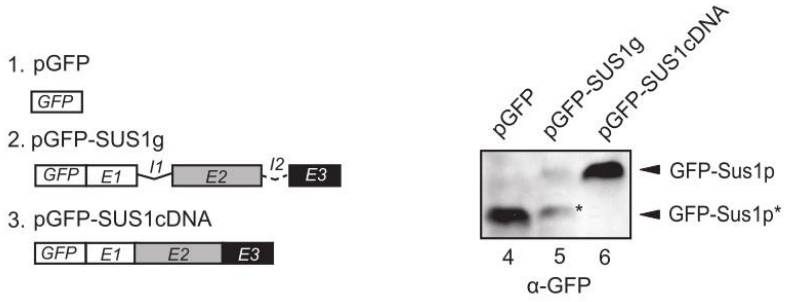
Suppl. Table 2. Conservation of exonic / intronic sizes in *SUS1* orthologues. ORF. Open Reading Frame. Number of nucleotides from ATG to STOP codon (including introns). CDS. Coding Sequence. Number of nucleotides from ATG to STOP codon (excluding introns). Exon 1: nucleotides from ATG to the first 5'ss. If the species does not contain introns, exon 1 boxes represent number of nucleotides from ATG to STOP codon. Exon 3: nucleotides from the last 3'ss to the STOP codon (included).

Supplementary Figure 1

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	Scerevisiae	300	2	Sparadoxus	300	90
1	Scerevisiae	300	3	Smikatae	300	78
1	Scerevisiae	300	4	Sbayanus	300	75
1	Scerevisiae	300	5	Skudriavzevii	300	59
1	Scerevisiae	300	6	Kthermotolerans	300	4
1	Scerevisiae	300	7	Cglabrata	300	4
1	Scerevisiae	300	8	Scastellii	300	6
1	Scerevisiae	300	9	Klactis	300	7
2	Sparadoxus	300	3	Smikatae	300	84
2	Sparadoxus	300	4	Sbayanus	300	74
2	Sparadoxus	300	5	Skudriavzevii	300	60
2	Sparadoxus	300	6	Kthermotolerans	300	5
2	Sparadoxus	300	7	Cglabrata	300	3
2	Sparadoxus	300	8	Scastellii	300	8
2	Sparadoxus	300	9	Klactis	300	7
3	Smikatae	300	4	Sbayanus	300	64
3	Smikatae	300	5	Skudriavzevii	300	62
3	Smikatae	300	6	Kthermotolerans	300	9
3	Smikatae	300	7	Cglabrata	300	4
3	Smikatae	300	8	Scastellii	300	8
3	Smikatae	300	9	Klactis	300	7
4	Sbayanus	300	5	Skudriavzevii	300	53
4	Sbayanus	300	6	Kthermotolerans	300	5
4	Sbayanus	300	7	Cglabrata	300	4
4	Sbayanus	300	8	Scastellii	300	7
4	Sbayanus	300	9	Klactis	300	10
5	Skudriavzevii	300	6	Kthermotolerans	300	5
5	Skudriavzevii	300	7	Cglabrata	300	12
5	Skudriavzevii	300	8	Scastellii	300	7
5	Skudriavzevii	300	9	Klactis	300	25
6	Kthermotolerans	300	7	Cglabrata	300	4
6	Kthermotolerans	300	8	Scastellii	300	4
6	Kthermotolerans	300	9	Klactis	300	11
7	Cglabrata	300	8	Scastellii	300	6
7	Cglabrata	300	9	Klactis	300	4
8	Scastellii	300	9	Klactis	300	5

Suppl. Figure 1. 3'UTR conservation in fungal *SUS1* orthologues. ClustalW2 tool was used for sequence alignments of 3'UTR sequences from *S. cerevisiae, S. mikatae, S. paradoxus and S. bayanus*. (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Supplementary Figure 2



Suppl. Figure 2. Elimination of *SUS1* introns (SUS1cDNA) causes accumulation of Sus1 protein when compared to SUS1g. Whole cell extracts from *sus1*Δ cells transformed with the indicated plasmids (left) were used to follow Sus1 protein levels by western blot using anti-GFP (right). Asterisk indicates the putative protein translated from *SUS1* transcripts retaining I1.

Supplementary Material and Methods

Strains

WT cells (BY4741 background) were used for RT-PCRs, qRT-PCRs, Northern blots and GFP-Sus1 detection and inmunoprecipitation. Figures 2A, 2B, 3B, 4 and 5C. *sus1Δ*, *mud2Δ* and *upf1Δ* strains were obtained from EUROFAN II BY4741 background (Brachmann et al., 1998). *mud2Δupf1Δ* strain was constructed by disruption of *UPF1* with a *URA3* cassette in a *mud2Δ* strain.
cup1Δmud2Δ strain, was obtained by replacing *MUD2* by a KanMX4 cassette in a *cup1Δ* strain (Lesser & Guthrie, 1993). Figures 3A and 6B.
Deletion of *SUS1* in CTY10-5d and W303 backgrounds, were obtained by replacement of *SUS1* locus with a KanMX4 cassette. *sus1Δ* cells (CTY10-5d background) were used for LexA-Sus1 protein expression (Figure 5A). *sus1Δ* cells (W303 background) were used for growth and FISH assays (Figure 7) and LexA-Taf14 protein expression (Figure 6A). The SL-9 strain is detailed at Rodriguez-Navarro et al. (Rodriguez-Navarro et al., 2004).

Plasmids

For protein expression analysis of N-terminally tagged LexA-Sus1, we have cloned different *SUS1* intron-versions in pSR1 plasmid. pSR2-4, were obtained by cloning of *SUS1* cDNA products, using RNA from BY4741 cultures as starting material. pSR5-8 were generated by cloning of fusion-PCR products. The pSR9, which contain *SUS1* with a single mutation in the intron 1 (making consensus the BS; TACTGAC>TACTAAC) was obtained using SR5 primer with the mentioned mutation, the pLexA-SUS1g as template and following the QuickChange® Multi Site-Directed Mutagenesis Kit instructions (from Stratagene). pSR1 vector was also used to clone *TAF14* and the *TAF14* fusion-PCR products bearing different *SUS1* fragments (pSR10-13). pSR14 vector was used to

clone *SUS1* gene, SL-9 or its cDNA version (from ATG to 163 nt after the STOP codon) fused N-terminally to GFP (pSR15 and pSR16 respectively). Growth and FISH assays were done with plasmids based in pSR17. SUS1 and SUS1cDNA plasmids (pSR18 and pSR19) were obtained by cloning genomic or fusion-PCR products, respectively. These plasmids contain *SUS1* with its own promoter (144 nt) and terminator (171nt) regions.
Plasmids for copper assays and primer extensions are based on the reporter construction ACT1-CUP1 (described at (Lesser & Guthrie, 1993)). The pSR23 was constructed in pSR22, by four consecutive cloning steps: (1) the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, (2) *SUS1* including 10 nt of 5'-UTR and the first 20 nt of E3, (3) *CUP1* and finally (4) the 200 nt following the STOP codon of *SUS1* as terminator. This plasmid was used to subclone other *SUS1* intron-versions (pSR24-27), amplified from pBTM116-*SUS1* plasmids (see later) or by fusion PCRs.

Code	Plasmid name	Description	Source
pSR1	pBTM116	2μ / TRP1 / LexA	Dr. Sternglanz
pSR2	pLexA-Sus1g	Based on pBTM116	Our laboratory
pSR3	pLexA-cDNASUS1	Based on pBTM116	Our laboratory

Code	Plasmid name	Description	Source
pSR4	pLexA-*I2Δ*	Based on pBTM116	Our laboratory
pSR5	pLexA-*I1Δ*	Based on pBTM116	This study
pSR6	pLexA-2XI1	Based on pBTM116	This study
pSR7	pLexA-I2&I1	Based on pBTM116	This study
pSR8	pLexA-2XI2	Based on pBTM116	This study
pSR9	pLexA-BP	Based on pBTM116	This study
pSR10	pLexA-TAF14	Based on pBTM116	This study
pSR11	pLexA-TAF14-I1SUS1	Based on pBTM116	This study

Code	Plasmid name	Description	Source
pSR12	pLexA-E1TAF14-I1SUS1-E2*TAF14-I2SUS1-E3*TAF14	Based on pBTM116	This study
pSR13	pLexA-E1TAF14-I1SUS1-E2SUS1-I2SUS1-E3*TAF14	Based on pBTM116	This study
pSR14	pNOPGFP1L	CEN / LEU2 / GFP	(Hellmuth et al., 1998)
pSR15	pGFP-SUS1g	Based on pNOPGFP1L	Our laboratory
pSR16	pGFP-SUS1cDNA	Based on pNOPGFP1L	This study
pSR17	pRS314	CEN / TRP	(Sikorski & Hieter, 1989)
pSR18	pSUS1g	Based on pRS314	This study

Code	Plasmid name	Description	Source
pSR19	pSUS1cDNA	Based on pRS314	This study
pSR20	pMM4c	Contains CUP1	Dr. Vilardell
pSR21	pACT1-CUP1	pCC71	Dr Vilardell
pSR22	pRS425	2μ / LEU2	(Sikorski & Hieter, 1989)
pSR23	pSUS1g-CUP1	Based on pRS425	This study
pSR24	pBS-CUP1	Based on pRS425	This study
pSR25	pSUS1-*I1Δ*-CUP1	Based on pRS425	This study
pSR26	pSUS1-*I2Δ*-CUP1	Based on pRS425	This study
pSR27	pSUS1(E2TAF14)-CUP1	Based on pRS425	This study

Code	Plasmid name	Description	Source
pSR28	pFA6a-MYC-HIS3	Myc / HIS3	(Longtine et al., 1998)
pSR29	pRS400-KanMX4		(Brachmann et al., 1998)
pSR30	pSL-9	Based on pRS314	This study

Primers

Code	Sequence 5'→3'	Description
SR1	aaaaaagcggccgcGGAAGTGTATTCGCTTGCC	pSR18 & pRS19
SR2	aaaaaactcgagAGGTGGGTAACGTGAATT	pSR18 & pRS19
SR3	TTTACCCAAATTTTATCCACCGTAGAACCCAAAGCATTAGAAATGGTATCGGATTCAACA	pRS19 & pRS16
SR4	TGCTTTAAAACGGTTTCCCTTGTTGAATCCGATACCATTTCTAATGCTTTGGGTTCTACG	pRS19 & pRS16
SR5	AAATACAACAATATCTTGTAGAATCAGGAAACTATGAACTAATTTCAAACGAACTAAAAG	pRS19 & pRS16
SR6	ACCTTCTTGAAGTAGTCTGGCTTTTAGTTCGTTTGAAATTAGTTCATAGTTTCCTGATTC	pRS19 & pRS16

Code	Sequence 5'→3'	Description
SR7	aaaaaagaattcATGACTATGGATACTG	pSR2-9 and for RT-PCR
SR8	aaaaaactgcagTCATTGTGTATCTACA	pSR2-9 and for RT-PCR
SR9	AACCGTCTTTCCTCCGTCGTAA	F-SCR1 qRT-PCR
SR10	AGAACTACCTTGCCGCACCA	R-SCR1 qRT-PCR
SR11	AAACTATGAACTAATTTCAAACG	F-mRNA & F-I2 qRT-PCR
SR12	CGATACCATTTCTAATGCTT	R-mRNA qRT-PCR
SR13	TTCAGATCACCGTCACATTT	F-I1 qRT-PCR
SR14	ATCCGATACCATTTCTAATGCTT	R-I1 qRT-PCR
SR15	AAAAATTCCACTGTTAGTAACG	R-I1&I2 & R-I2 qRT-PCR
SR16	TACTGACTATTCAGATCACCG	F-I1&I2 qRT-PCR

Code	Sequence 5'→3'	Description
SR17	aaaaaactcgagTAATTAATAGTAGTGATTTTCC	(F) To clone GPD in pSR23
SR18	aaaaaagtcgacTCGAAACTAAGTTCTTGGTG	(R) To clone GPD in pSR23
SR19	aaaaaaaagcttCACTCCAATGACTATGGATACTGCGC	(F) To clone *SUS1* in pSR23 & *SUS1* versions in pSR24-27
SR20	aaaaaactgcagTGTTGAATCCGATACCATTTC	(R) To clone *SUS1* in pSR23 & *SUS1* versions in pSR24-27 & 29
SR21	aaaaaactgcagTTCTCCGAATTAATTAACTTCC	(F) To clone *CUP1* in pSR23
SR22	aaaaaaggatccTTATTTCCCAGAGCAGCATG	(R) To clone CUP1 in pSR23
SR23	aaaaaaggatccAGGATACGAAAATGCACC	(F) To clone *SUS1*-3'UTR in pSR23
SR24	aaaaaagcggccgcTATATAAATAAAGAAAAGGC	(R) To clone *SUS1*-3'UTR in pSR23

Code	Sequence 5'→3'	Description
SR25		(F) For *SUS1* riboprobe
SR26	taatacgactcactatagGTGTATCTACAATCTCTTC	(R) For *SUS1* riboprobe
SR27	GGCACTCATGACCTTC	(R) For primer extensions of *CUP1*
SR28	GAACTGCTGATCATCTCTG	(R) For primer extensions of U6 snRNA
SR29	aaaaaactgcagATGACTATGGATACTGCGCAATTA	(F) pRS15-16
SR30	aaaaaactcgagTGGGTACGTGAATTAGGTT	(R) pRS15-16
SR31	aaaaaagaattcATGGTAGCTGTATGTTCGTTAATC	(F) pSR10
SR32	aaaaaaggatccTTACTCGGTATTTTTCTTAACG	(R) pSR10
SR33	aaaaaagaattcATGGTAGCTGTATGATAAAATATTATAGG	(F) pRS11
SR34	CCGTCACATTTATTAGACAGTAAAAAGAACCATCCG	(F) pRS11

Code	Sequence 5'→3'	Description
SR35	CGGATGGTTCTTTTTACTGTCTAATAAATGTGACGG	(R) pRS11
SR36	GTGATAAAATATTCACATACATTGTAGCGGGAATTTCTTTTCC	(R) pRS12
SR37	GAAATTCCCGCTACAATGTATGTGAATATTTTATCAC	(F) pRS12 & pRS29
SR38	GGTAAATAACTTTGTCAAAACTAGAAAAAAAAATTCC	(R) pRS12
SR39	GTGGAATTTTTTTTTCTAGTTTTGACAAAGTTATTTACC	(F) pRS12
SR40	aaaaaagaattcATGGTAGCTACGTATGATAAAATATTATAGGG	pRS13
SR41	GGTAAATAACTTTGTCAAAAACTAGAAAAAAAAATTCCACTG	pRS13
SR42	CAGTGGAATTTTTTTTTCTAGTTTTTGACAAAGTTATTTACC	pRS13
SR43	GGGTTTTTATACGGATGGTTCTTTTTCTAATAAATGTGACGGTGATCTG	pRS13
SR44	GGAAAAGAAATTCCCGCTACAATTTTTGGTATGTGAATATTTTATCAC	pRS13

Code	Sequence 5'→3'	Description
SR45	CAGATCACCGTCACATTTATTAGAAAAAGAACCATCCGTATAAAAACCC	pRS13
SR46	GTGATAAAATATTCACATACCAAAAATTGTAGCGGGAATTTCTTTTCC	pRS13
SR47	TAAATGTGACGGTGATCTGAATAG	pRS29
SR48	aaaaaaaagcttCACTCCAATGACTATGGATACTGCGCAATTAAAGAGTCAA ATACAACAATATCTTGTAGAATCAGGAAACTATGAACTGTATGATAAAA TATTATAGGG	pRS29

Antibodies used in this study

- Primary antibodies

Name	**Dilution**	**Source**
Anti-Myc (9E10)	1:2.000	Santa Cruz
Anti-GFP	1:15.000	Roche
Anti-LexA	1:5.000	Santa Cruz
Anti-Sus1(E1)	1:500	Our laboratory

- Secondary antibodies

Name	**Dilution**	**Source**
Anti-Mouse-IgG-ECL™HRP	1:4.000 (LexA) 1:30.000 (GFP) 1:5.000 (Myc)	GE Healthcare
Anti-Rabbit-IgG-ECL™HRP	1:10.000 (Sus1(E3)	GE Healthcare

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III.II. CAPÍTULO II

Los resultados de este capítulo fueron publicados en la revista científica BMC Cell Biology, en Marzo de 2010. Se adjunta el texto íntegro de esta publicación, que representa el contenido del capítulo II en el apartado de resultados.

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http://www.biomedcentral.com/1471-2121/11/19

BMC Cell Biology

RESEARCH ARTICLE Open Access

A novel link between Sus1 and the cytoplasmic mRNA decay machinery suggests a broad role in mRNA metabolism

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Abstract

Background: Gene expression is achieved by the coordinated action of multiple factors to ensure a perfect synchrony from chromatin epigenetic regulation through to mRNA export. Sus1 is a conserved mRNA export/ transcription factor and is a key player in coupling transcription initiation, elongation and mRNA export. In the nucleus, Sus1 is associated to the transcriptional co-activator SAGA and to the NPC associated complex termed TREX2/THSC. Through these associations, Sus1 mediates the nuclear dynamics of different gene loci and facilitate the export of the new transcripts.

Results: In this study, we have investigated whether the yeast Sus1 protein is linked to factors involved in mRNA degradation pathways. We provide evidence for genetic interactions between *SUS1* and genes coding for components of P-bodies such as *PAT1*, *LSM1*, *LSM6* and *DHH1*. We demonstrate that *SUS1* deletion is synthetic lethal with 5'→3' decay machinery components *LSM1* and *PAT1* and has a strong genetic interaction with *LSM6* and *DHH1*. Interestingly, Sus1 overexpression led to an accumulation of Sus1 in cytoplasmic granules, which can co-localise with components of P-bodies and stress granules. In addition, we have identified novel physical interactions between Sus1 and factors associated to P-bodies/stress granules. Finally, absence of *LSM1* and *PAT1* slightly promotes the Sus1-TREX2 association.

Conclusions: In this study, we found genetic and biochemical association between Sus1 and components responsible for cytoplasmic mRNA metabolism. Moreover, Sus1 accumulates in discrete cytoplasmic granules, which partially co-localise with P-bodies and stress granules under specific conditions. These interactions suggest a role for Sus1 in gene expression during cytoplasmic mRNA metabolism in addition to its nuclear function.

Background

During gene expression, the coordinated action of several multiprotein complexes couple transcription, mRNA biogenesis and export, to guarantee the proper maturation of transcripts before their translation in the cytoplasm [1]. mRNA levels are highly regulated by transcription rate adjustments and mRNA decay, to produce the appropriate number of transcripts competent for translation [2]. In yeast, two major cytoplasmic mRNA degradation pathways control transcript turnover: the cytoplasmic exosome and the 5'→3' mRNA decay. Moreover, 5'→3' mRNA decay and translation are interconnected processes providing an exquisite equilibrium between degradation, storage and translation that correlates with the type and localisation of the mRNP in the cell (reviewed in [3]). Work over the last few years has shown that different classes of mRNPs are found as discrete granules in the cytoplasm. In yeast, different sorts of cytoplasmic mRNP granules have been described. Among them, P-bodies (PBs) and stress gran- ules (SGs) are the best characterised (reviewed in [4,5]). P-bodies are implicated in translational repression, mRNA storage and 5'→3' mRNA decay [6]. The compo- sition of PBs has been thoroughly studied. They are made up of a set of proteins that form the core of the particules, such as the decapping enzyme Dcp1/Dcp2, activators of decapping Dhh1, Pat1, Lsm1-7, Edc3 and the 5'→3' exonuclease Xrn1 [7]. Other proteins involved in different processes, such as nonsense-mediated decay (Upf1-3) [8] and translation (eIF4E, eIF4G and Pab1) [9,10] have also been reported to accumulate in these granules, but only under specific conditions.

A second class of well studied cytoplasmic mRNP structures are the stress granules (reviewed in [4]). SGs are cytoplasmic mRNP accumulations that appear when translation initiation is impaired. Study of stress granule

formation has suggested that they contain mRNAs stalled in the process of translation initiation. In yeast they characteristically contain poly(A) mRNA, the poly (A)-binding protein Pab1, 40S ribosomal subunits and the translation factors eIF4E, eIF4G, eIF3 (reviewed in [4,5]).

In yeast but also in other organisms, both types of granules are interconnected (reviewed in [5]). Strikingly, assembly of stress granules depends on P-body formation and several factors are present in both granules, suggesting a crosstalk between them [6,10,11].

One key factor involved at different stages of nuclear mRNA metabolism is the conserved Sus1 protein, which is part of two stable nuclear complexes: the transcriptional coactivator SAGA and the nuclear pore associated TREX2 [12]. Biochemical and functional data have suggested a crucial nuclear role for Sus1 in coupling transcription activation and mRNA export. Previously, we have shown that Sus1 participates in histone H2B deubiquitination and histone H3 methylation together with the SAGA-DUB subunits Ubp8 and Sgf11 [13]. Sus1 mediates transcription activation through its associated with chromatin promoters as part of SAGA and is recruited to coding regions where it is necessary for transcription elongation [14]. Interestingly, Sus1 is also required for nuclear post-transcriptional events. After transcriptional shut off, Sus1 affects both the morphology as well as the persistent tethering of the mRNPs to their cognate gene, reinforcing the broad role of Sus1 in nuclear mRNA biogenesis [15]. Furthermore, Sus1 is crucial for TREX2-NPC interaction and its absence provokes a dramatic defect in mRNA export [12-14]. Altogether, Sus1 participates in many nuclear events from early epigenetic modifications to mRNA export through the nuclear pore (reviewed in [16]).

Strikingly, although Sus1's described functions take place in the nucleus it was also observed in the cytoplasm of yeast and Drosophila [14,17], thus suggesting additional roles outside of the nucleus.

In this study, we describe genetic and functional links between *SUS1* and several components of P-bodies and stress granules. We demonstrate that *SUS1* deletion is synthetic lethal with 5'→3' decay machinery components *LSM1* and *PAT1* and has a strong genetic interaction with *LSM6* and *DHH1*. Interestingly, Sus1 overexpression leads to an accumulation of Sus1 at cytoplasmic granules, which can co-localise with P-bodies and stress granules. In addition, through affinity purification of TAP tagged Sus1, we have identified novel physical interactions between Sus1 and factors associated with P-body/stress granule. Finally, absence of *LSM1* and *PAT1* slightly promotes association between Sus1-TREX2. Taken together, our results reveal a novel link between the transcription/export factor Sus1 and cytoplasmic mRNA decay factors. Thus, Sus1 plays a broad role in mRNA metabolism.

Results

Sus1 interacts genetically with components of the mRNA degradation machinery

Unveiling the network of genetic interactions for a given factor provides clues to understand its role in a cellular context. To further uncover Sus1's molecular function, we utilized The BIOGRID database [18], searching for the complete genetic network of *sus1Δ*. Among others, we found a number of factors involved in mRNA biogenesis, whose deletion enhanced the *sus1Δ* growth defect (Figure 1A). Moreover, recent analysis of wide yeast genetic interactions confirms these data and extends the list of *sus1Δ* interactors involved in mRNA processing (Figure 1B) [19,20].

To corroborate and extend this observation we generated double mutants of *SUS1* combined with deletions in *PAT1*, *LSM1*, *LSM6* or *DHH1* and the resulting phenotypes were analysed. Deletion of either

PAT1 or *LSM1* in *sus1Δ* cells provokes a synthetic lethal phenotype (Figure 2A). In addition, absence of *LSM6* or *DHH1* elicits an enhancement of the slow growth associated with *sus1Δ* (Figure 2B). In conclusion, Sus1 genetically interacts with key components of the cytoplasmic 5'→3' mRNA decay machinery.

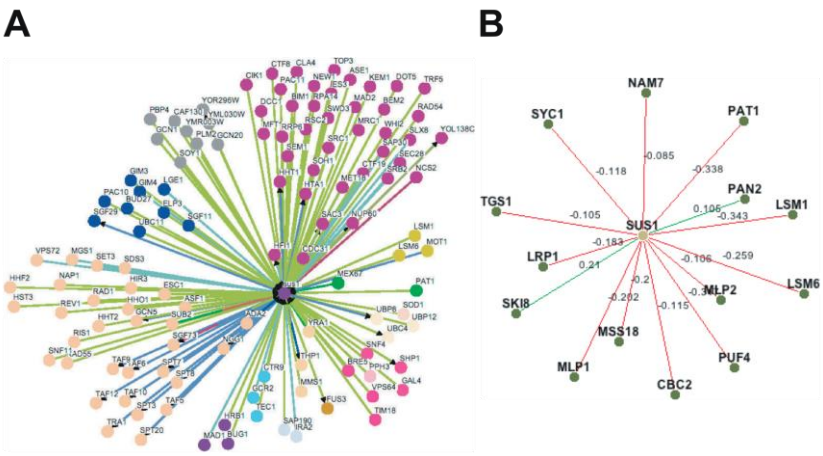


Figure 1. Network showing Sus1 interactions. (A) All known physical or genetic *SUS1* interactions were downloaded from the BioGRID database [18]. Osprey software was used to obtain the graphical representation of the Sus1 network by gene ontology and a complete legend of color settings can be found in [26]. (B) Network visualization of SGA genetic interactions involving genes that participate in mRNA processing. Positive SGA interactions are coloured in green, while negative SGA interactions are in red. A complete legend of colour setting and all information can be found in DRYGIN [20].

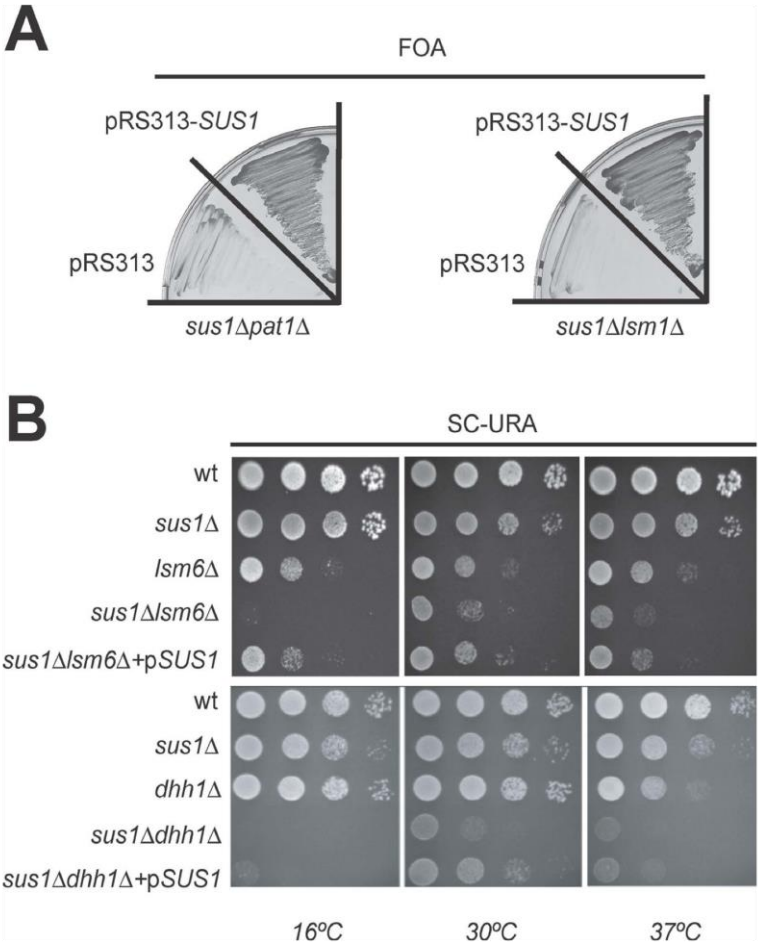


Figure 2. *SUS1* Interacts Genetically with Genes Encoding Components of the mRNA decay machinery. (A) Synthetic lethality of *sus1Δ* with *lsm1Δ* and *pat1Δ*. Double mutants containing a pRS316-SUS1 plasmid, were transformed with an empty vector (pRS313) or the same plasmid bearing a wild-type version of *SUS1* (pRS313-SUS1). Transformants were streaked onto 5-fluoroorotic acid (FOA) containing plates, which were incubated at 30°C for 3 days. No growth indicates synthetic lethality. (B) Synthetic sick phenotype of *sus1Δ* with *lsm6Δ* or *dhh1Δ*. Wild-type (wt), single and double mutants were transformed with an empty vector (pRS316). The double mutants were also transformed with a pRS316-SUS1 (pSUS1) in order to complement the phenotype. Cells were diluted in 10-1 steps, and equivalent amounts of cells were spotted on SC-URA plates.

When overexpressed, Sus1 accumulates at cytoplasmic granules

Work from many laboratories has shown that some proteins involved in mRNA metabolism are constituents of mRNP cytoplasmic granules (reviewed in [4,5]). In light of the fact that there are genetic interactions between Sus1 and some of these factors, we asked whether Sus1 could accumulate at discrete cytoplasmic granules under specific conditions. Interestingly, after glucose starvation or in stationary phase, we observed an accumulation of Sus1 in discrete granules at the cytoplasm (data not shown). Moreover, overexpression of Sus1 from its cDNA (Cuenca-Bono et al., manuscript in preparation) enhanced this accumulation. Several types of cytoplasmic granules have been characterized in yeast including P-bodies and stress granules [5]. To address the nature of Sus1-containing granules, we co-localised the P-body component Dcp2 and Sus1 in cells expressing *SUS1* cDNA. As shown in Figure 3A, Sus1 partially co-localises with P-bodies under these conditions. From this, we conclude that Sus1 can enter the P-bodies when overexpressed.

Different factors contribute to the assembly of P-bodies under glucose deprivation. The general decapping activators Dhh1, Pat1 and Lsm1 have different roles in P-body assembly and composition [21]. Since absence of Lsm1 or Pat1 is synthetic lethal with *sus1Δ*, we checked for the contribution of these factors to Sus1 presence at cytoplasmic granules. Cells lacking either *LSM1* or *PAT1* were transformed with pGFP-*SUS1-cDNA* and pDcp2-RFP; and localisation analysis of both proteins by fluorescence microscopy was performed. As shown in Figure 3B, Sus1 and Dcp2 still co-localise in these mutant backgrounds. Therefore, we conclude that Sus1 co-localises with P-bodies independently of Lsm1 and Pat1. To analyze whether Sus1 might have a role in the assembly of cytoplasmic structures as P-bodies or stress granules, we localized Dcp2 or Pab1 in *sus1Δ* cells. As

illustrated in Figure 3C Sus1 is dispensable for the formation of P-body and stress granule. Thus, we conclude that Sus1 appears not to be a structural component of P-bodies or stress granules.

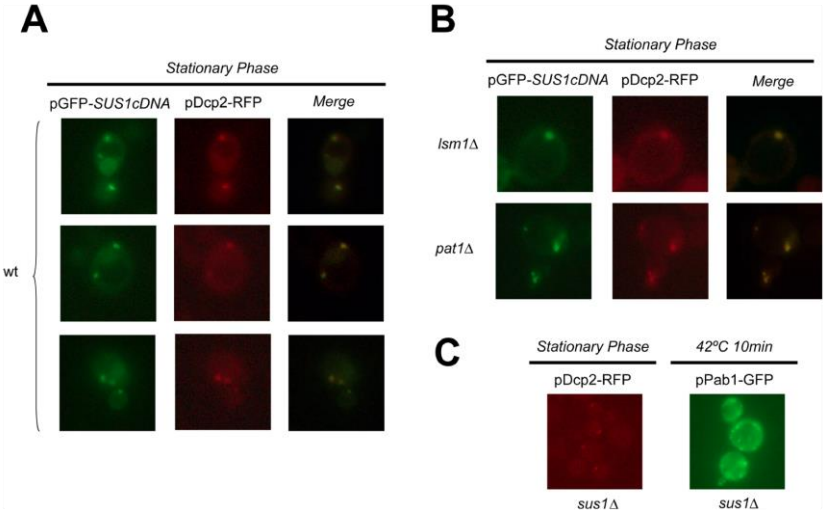


Figure 3. Sus1 co-localises with P-bodies. (A) Sus1 partially localises at P-bodies in stationary phase. Wild-type (wt) cells co-transformed with a plasmid containing the cDNA of *SUS1* (pGFP-SUS1cDNA) and a plasmid containing Dcp2 (pDcp2-RFP) were observed in stationary phase. Partial co-localisation indicates localisation of Sus1 in P-bodies. Pictures were obtained by fluorescence microscope. (B) Sus1 is present at P-bodies independently of Lsm1 or Pat1. Cells expressing SUS1cDNA and Dcp2 in the different mutants were observed in stationary phase. Pictures were taken with a camera mounted onto a fluorescence microscope. (C) *SUS1* is not required for P-bodies or stress granules formation. Cells expressing Dcp2-RFP or Pab1-GFP were transformed in *sus1Δ* and observed in stationary phase or after heat shock respectively. Images were generated using a fluorescence microscope.

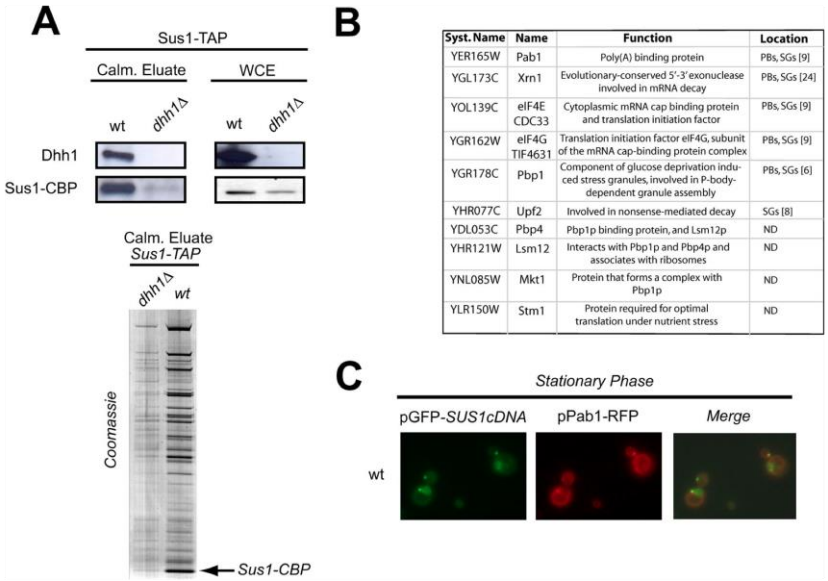
Sus1 interacts physically with components of P-bodies and stress granules

Taken together our genetic and localisation results suggest that Sus1 could transiently enter P-bodies. A tentative idea is that Sus1 interacts with this mRNP early

during transcription and a minor pool of the proteins could travel to the cytoplasm. To substantiate this idea, we studied whether Sus1 physically interacts with proteins found in P-bodies. Based on our genetic data we first tested the association between Sus1 and Dhh1. Cells expressing Sus1-TAP were grown in standard conditions and TAP purification was performed as described previously [12] and in Methods. As shown in Figure 4A, specific interaction between Sus1 and Dhh1 was demonstrated by western blot using anti-Dhh1 anti-bodies. Notably, we observed a reduction in Sus1 expression in whole cell extracts (WCE) of cells lacking *DHH1*. This decrease could impact on Sus1 stability since the profile of the enriched Sus1-TAP calmoduline eluate in *dhh1Δ* is drastically affected (Figure 4A lower panel). Hence, we conclude that Sus1 physically interacts with Dhh1 and loss of *DHH1* affects Sus1 stability/ expression.

To extend this observation, we performed Sus1-TAP purification and the enriched calmoduline eluate was this time analysed by multidimensional protein identification technology (MudPIT) in order to identify the polypeptide mixture present in our affinity purification. This kind of analysis has been extensively used to discover new interactors of a known protein and to study in more detail the proteomic characterization of different pathways [22]. Our MudPIT analysis (Figure 4B) reveals that besides SAGA and TREX2 subunits (data not shown), Sus1 co-purifies with the ribosome-associated factor Stm1 and the translation initiation factors eIF4E and eIF4G. Moreover, we also identified the poly(A) binding proteins Pab1, Pbp1, Pbp4, Lsm12 and Mkt1. Furthermore, peptides corresponding to the 5'→3' exonuclease Xrn1, present at PBs and SGs and Upf2 protein (involved in NMD and present at SGs) were identified in our purification. Altogether, our Mud-PIT analysis reinforces our genetic and localisation data thereby revealing new physical connections between Sus1 and factors involved in translation and mRNA metabolism, some of which are

localised to P-bodies and/or stress granules. To strengthen our biochemical result showing physical interaction between Sus1 and the SGs/PBs component Pab1, we checked for co-localisation between them in wild-type cells during stationary phase. As illustrated in Figure 4C, Sus1 and Pab1 co-localise at discrete cytoplasmic granules. Thus, we conclude that Sus1 is present in Pab1-containing granules, which support its presence at P-bodies and/or stress granules.



Syst. Name	Name	Function	Location
YER165W	Pab1	Poly(A) binding protein	PBs, SGs [9]
YGL173C	Xrn1	Evolutionary-conserved 5'-3'exonuclease involved in mRNA decay	PBs, SGs [24]
YOL139C	eIF4E CDC33	Cytoplasmic mRNA cap binding protein and translation initiation factor	PBs, SGs [9]
YGR162W	eIF4G TIF4631	Translation initiation factor eIF4G, subunit of the mRNA cap-binding protein complex	PBs, SGs [9]
YGR178C	Pbp1	Component of glucose deprivation induced stress granules, involved in P-body-dependent granule assembly	PBs, SGs [6]
YHR077C	Upf2	Involved in nonsense-mediated decay	SGs [8]
YDL053C	Pbp4	Pbp1p binding protein, and Lsm12p	ND
YHR121W	Lsm12	Interacts with Pbp1p and Pbp4p and associates with ribosomes	ND
YNL085W	Mkt1	Protein that forms a complex with Pbp1p	ND
YLR150W	Stm1	Protein required for optimal translation under nutrient stress	ND

Figure 4. Sus1 interacts with Dhh1 and co-localise with Pab1. (A) Sus1 physically interacts with Dhh1. Sus1 co-purification with Dhh1 was revealed by western-blot of Sus1-TAP calmoduline eluates purificied from wild-type (wt), and *dhh1Δ* cells using anti-Dhh1 antibodies (left panel). Prior to purification, Sus1 and Dhh1 presences were confirmed by western blot analysis of whole cell extracts (WCE) (right panel). Lower panel shows the enriched calmoduline eluates from Sus1-TAP (wt), and Sus1-TAP*dhh1Δ* purifications analysed by SDS 4-12% gradient polyacrylamide gel electrophoresis stained with Coomassie (B) MudPIT analysis of Sus1-TAP. List of proteins co-purified with Sus1 identified by MudPIT. PBs (P-bodies); SGs (stress granules); ND (Not Determined) (C) Sus1 is present at cytoplasmic Pab1-containing granules. Sus1 co-localises with Pab1 in stationary phase.

mRNA decay components affect Sus1 protein interactions

Sus1 is part of two multiprotein assemblies, the SAGA and TREX2 complexes (reviewed in [16]). The levels of Sus1 binding to each complex is important for their correct functionality. The fact that *LSM1*, *PAT1* and *DHH1* are genetically linked to *SUS1*, prompted us to test whether Sus1 association with SAGA or TREX2 components was disrupted in the absence of these proteins. Hence, we genomically TAP-tagged Sus1 in *lsm1Δ* and *pat1Δ* strains and conducted Sus1-TAP purification using standard conditions. As illustrated in Figure 5A, Sus1 is associated with both SAGA and TREX2 in lsm1Δ and *pat1Δ* strains. However, comparison between the calmoduline eluates from the three purifications by coomassie staining revealed a reproducible enrichment of Sac3 (TREX2 subunit) in *lsm1Δ* and *pat1Δ* compared to the wild-type. We conclude that the absence of *LSM1* and *PAT1* improves Sus1-Sac3 association. Both Sac3 and Sus1 bind to the essential mRNA exporter Mex67. If loss of *LSM1* and *PAT1* promotes the interaction between Sus1 and TREX2 it may increase the association between Sus1 and Mex67. To test this possibility, we used western blot analysis to look for the presence of Mex67 in the calmoduline eluates from our purifications. As shown in Figure 5B a slight increase of the associated mRNA export factor Mex67 is observed, whereas no enrichment of the SAGA subunit Taf6 was found. As loading controls, western blots of whole cell extracts (WCE) revealed similar expression for these proteins in wild-type and in both mutants strains (Figure 5C). Taken together, these results suggest that loss of Lsm1 or Pat1 alters the kinetics of Sus1 associations.

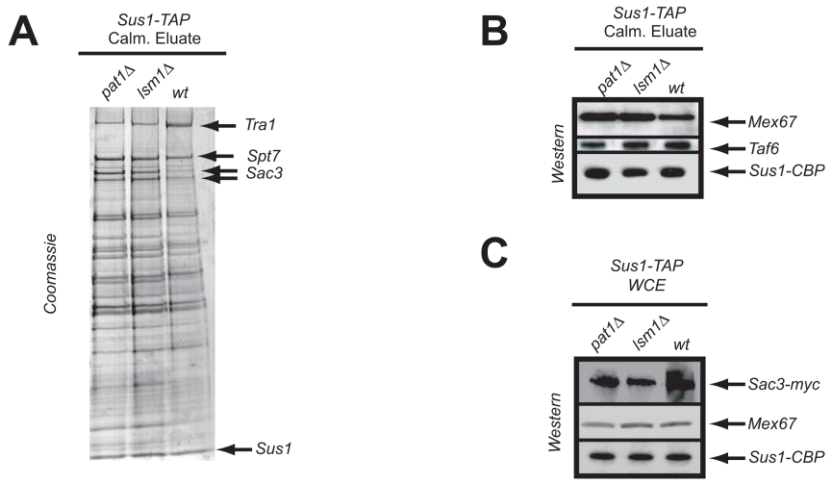


Figure 5. Absence of the decay mutants partially enhances Sus1 binding to TREX2 and Mex67. Sus1 association to TREX2 is enhanced in *pat1Δ* and *lsm1Δ*. Sus1-TAP was affinity-purified from wild-type (wt), *pat1Δ* and *lsm1Δ* strains. The enriched calmoduline eluates from all purifications were analysed by SDS 4-12% gradient polyacrylamide gel electrophoresis and proteins stained with Coomassie. Sus1, Tra1, Spt7 and Sac3 bands were verified by mass spectrometry. (B) Sus1 association to Mex67 is increased in the absence of *PAT1* or *LSM1*. Enrichment of Mex67 in the calmoduline eluates from the respectives deletion strains is demonstrated by western blotting using anti-Mex67 antibodies, whereas no differences were observed for Taf6. (C) Sac3, Mex67 and Sus1 expression levels were confirmed by detecting similar loading from inputs by immunoblot.

Discussion

Sus1 is genetically linked to proteins involved in different stages of gene expression. Previously, we reported that Sus1 deletion is synthetic lethal with deletions in mRNA export/biogenesis factors, namely Mex67, Yra1, Dbp5, Nab2 and Sub2 [12]. In this study we show strong genetic interactions between *SUS1* and *PAT1* or *LSM1* (synthetic lethality), and *DHH1* or *LSM6* (synthetic enhancement). Lsm1, Lsm6, Pat1 and Dhh1 are proteins involved in mRNA degradation that are loca- lised at cytoplasmic P-bodies [21]. Thus these new genetic

interactions between Sus1 and these factors suggest a connection between Sus1 and cytoplasmic mRNA metabolism.

To gain knowledge about the functional meaning of these genetic interactions, we have investigated the presence of Sus1 at P-bodies. In addition to the genetic link between Sus1 and P-body components, we have been able to localise Sus1 at cytoplasmic granules, by showing that it partially co-localises with Dcp2 and Pab1. Moreover, Sus1-Dcp2 co-localisation in the cytoplasmic granules is independent of Lsm1 or Pat1 and we have demonstrated that Sus1 is dispensable for PB or SG assembly.

We showed previously that under standard growth conditions Sus1-GFP is localized at the NPC and the nuclear lumen, but that a weak signal could be detected in the cytoplasm [12]. However, Sus1 was not visible at these granules suggesting that Sus1 co-localisation with P-bodies might be very dynamic and transient. Accordingly, we have shown that Sus1 co-purifies with P-body components Dhh1 and Xrn1, after standard Sus1-TAP purification. Thus it is likely that a minor pool of the protein, undetectable by our fluorescence analysis, is in fact associated with cytoplasmic structures in physiological conditions. Strikingly, during the revision process of this work, Drosophila Sus1 (ENY2) has been observed in the cytoplasm [17]. Kopytova and coworkers detected a significant amount of ENY2 in the cytoplasm of Drosophila S2 cells and they conclude that ENY2 may also be of significance for the fate of mRNPs in the cytoplasm.

Sus1 also co-purifies with factors involved in translation initiation and poly(A) binding proteins, which have also been reported to enter P-bodies and to be constituents of stress granules [8-10,23]. By MudPIT analysis of our standard Sus1-TAP purification, peptides for eIF4E, eIF4G, Pab1, Pbp1, Pbp4, Lsm12, Mkt1 and Stm1 were found. In glucose starvation conditions, eIF4E, eIF4G and Pab1 were shown to keep away from ribosomes and to localise at cytoplasmic granules

originally named EGP-bodies (eIF4E, eIF4G, Pab1) [9]. Observations by Hoyle and co-workers demonstrate that these mediators accumulate both in P-bodies and in EGP-bodies. In these lines, co-localisation of Sus1 and Pab1 demonstrates that Sus1 is present in Pab1-containing granules during stationary phase, which supports our previous data and opens the possibility of Sus1 presence also at SG and/or EGP-bodies. In addition, Sus1 also interacts with Stm1, which promotes Dhh1 function in translation repression and mRNA decay [24].

What is Sus1 function in the cytoplasmic mRNA cycle? We speculate that Sus1 can travel with the mRNPs, especially since it is loaded early during transcription. It may then facilitate the interaction with other factors thereby providing a way to connect transcription and translation through mRNP metabolism. A possible scenario is that Sus1 mediates different physical interactions between the P-body machinery and translation, to contribute to the fate of the mRNA in the cytoplasm. In support of this model, the resolution of the Sus1 structure in association with TREX2 or SAGA has revealed that its structure is compatible with multiple associations [25]. The extended fold of Sus1 creates a surprisingly large surface area for a protein of this size and this could facilitate the association of Sus1 with other factors. Previous work suggested that eIF4E, eIF4G and Pab1 could be present on a subpopulation of mRNAs in the P-bodies [10]. It was suggested that such mRNA subpopulations could be a specific set of transcripts. Based on these arguments it is possible that Sus1 contributes to the mRNA metabolism of SAGA dependent genes in the cytoplasm as well as in the nucleus. Further experiments to characterize the composition of Sus1 cytoplasmic structures will help us to verify this hypothesis. Remarkably, a possible cytoplasmic role for Sus1 is likely to be conserved through evolution since ENY2 is present in the cytoplasm of *Drosophila* S2 cells [17], a finding which strongly supports our data. Future work will help us to reveal new insights in to how Sus1

participates in gene expression from chromatin modifications to mRNA metabolism in the cytoplasm.

Conclusions

Sus1 is genetically linked to factors involved in mRNA decay. Sus1 is observed at P-bodies and stress granules when overexpressed and it interacts physically with components of these cytoplasmic structures in normal conditions.

Methods

Yeast Strains, Plasmids, Microbiological Work

Yeast strains and plasmids used in this study are listed in Additional file 1 (Supplemental Table 1 and Supplemental Table 2, respectively). Microbiological techniques, yeast plasmid transformation, mating, sporulation of diploids and tetrad analysis were done essentially as described previously [14]. For spotting analyses, cells were grown on synthetic selective medium (Synthetic complete medium: glucose 2%, ammonium sulphate 0,4%, yeast nitrogen base 0,34%, and supplements (Dropout)) to 0.5 OD_{600} and subjected to 10-fold serial dilutions. Chromosomal integration of TAP and MYC as C-terminal tags were performed as described in [14]. Genetic interaction studies were performed by growing the double mutants under appropriated conditions. An empty plasmid (pRS313) and the same plasmid containing *SUS1* were used for synthetic lethal assays. The dot-spot assays were performed by transforming the cells with an empty plasmid (pRS316) and the same plasmid backbone bearing *SUS1* for complementation.

TAP Purifications, MudPIT and Western Blot Analysis

TAP purifications of wild-type (BY4741) and mutant strains were performed as described previously [12]. Sus1-TAP fusion protein and associated proteins were recovered from cell extracts by affinity selection on an IgG matrix. After washing, the TEV protease is added to release the bound material. The eluate is incubated with calmodulin-coated beads in the presence of calcium. This second affinity step is required to remove the TEV protease as well as traces of contaminants remaining after the first affinity selection. After washing, the bound material is released with EGTA. This enriched fraction is called Calmoduline eluate. Calmoduline eluates from the TAP-purified complexes were analyzed by SDS-PAGE by using Novex 4-12% gradient gels (Invitrogen) and visualized by staining with Novex Colloidal Blue staining kit (Invitrogen). MudPIT analyses were performed by mass spectrometry as described in [22]. Western blot analysis was performed using anti-Mex67, anti-Taf6, anti-Dhh1, anti-MYC and anti-TAP according to standard procedures.

Preparation of Cells for Fluorescence Microscopy

For observation at stationary phase, cells were grown for two days in synthetic selective medium supplemented with amino acids. Cells were then washed and resuspended in fresh media prior to observation. For observation at 42°C, cells were treated for 10 minutes at 42°C and immediately observed. Pictures were made using a Leica DM6000B fluorescence microscope.

Sus1 Network Representation

Data available from the BIOGRID [18] were downloaded and used to represent Sus1 interactions with the Osprey 1.2.0 software available at [18]. A complete legend of color settings can be found in [18]. Network visualization of SGA genetic interactions involving genes

that participate in mRNA processing were downloaded from DRYGIN database. A complete legend of colour settings and all information can be found in [18].

List of abbreviations

mRNA: messenger RiboNucleic Acid
SAGA: Spt-Ada-Gcn5-acetyltransferase
NPC: Nuclear Pore Complex
TREX2/THSC: Sac3-Thp1-Cdc31-Sus1 complex
P-bodies/PBs: Processing bodies
SGs: Stress Granules
mRNP: messenger RiboNucleoProtein
Poly(A): Polyadenilated mRNA tail
SAGA-DUB: SAGA DeUbiquitination Module
MudPIT: Multidimensional Protein Identification Technology
NMD: Nonsense Mediated Decay
EGP-bodies: eIF4E, eIF4G and Pab1p bodies

Additional files

Additional file 1: Supplementary tables. Yeast strains and plasmids used in this study.
[http://www.biomedcentral.com/content/supplementary/1471-2121-11- 19-S1.PDF]

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Authors' contributions

BCB participates in the design of the study, carried out the molecular genetic studies, microscopy work and helped to draft the manuscript. VGM contributed to the microscope work, genetic studies and strain construction. PPG carried out the biochemical analysis and strains construction. EGO participates in the TAP purification and western blot analysis. ALL assisted technically in all parts of the work. SRN conceived the study, participated in microscope and biochemical analysis, coordinated the work and wrote the paper. All authors read and approved the final manuscript.

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Supplementary Table 1 Cuenca-Bono et al., 2010

Yeast strain	Genotype	Reference
BY4741	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0	EUROSCARF
lsm1Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, lsm1::KanMX4	EUROSCARF
lsm6Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, lsm6::KanMX4	EUROSCARF
pat1Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, pat1::KanMX4	EUROSCARF
dhh1Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, dhh1::KanMX4	EUROSCARF
sus1Δ	Mat α, his3-Δ1, leu2-Δ0, lys2-Δ0, ura3-Δ0, sus1::KanMX4	EUROSCARF
lsm1Δ sus1Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, lsm1::KanMX4, sus1::KanMX4	This study
lsm6Δ sus1Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, lsm6::KanMX4, sus1::KanMX4	This study
pat1Δ sus1Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, pat1::KanMX4, sus1::KanMX4	This study
dhh1Δ sus1Δ	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, dhh1::KanMX4, sus1::KanMX4	This study
SUS1-TAP	Mat α, ade2, his3, leu2, trp1, ura3, SUS1-TAP::TRP1	[1]
lsm1Δ SUS1-TAP	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, lsm1::KanMX4, SUS1-TAP::URA	This study
pat1Δ SUS1-TAP	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, pat1::KanMX4, SUS1-TAP::URA	This study
dhh1Δ SUS1-TAP	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, dhh1::KanMX4, SUS1-TAP::URA	This study
SUS1-TAP SAC3-MYC	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, SUS1-TAP::URA, SAC3-MYC::HIS	This study
lsm1Δ SUS1-TAP SAC3-MYC	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, lsm1::KanMX4, SUS1-TAP::URA, SAC3-MYC::HIS	This study
pat1Δ SUS1-TAP SAC3-MYC	Mat a, leu2-Δ0, his3-Δ1, met15-Δ0, ura3-Δ0, pat1::KanMX4, SUS1-TAP::URA, SAC3-MYC::HIS	This study

Supplementary Table 2 Cuenca-Bono et al., 2010

Name	Description	Source
pNOPGFP1L	*GFP, LEU2, CEN*	[2]
pGFP-*SUS1*	*GFP-SUS1, LEU2, CEN* (based in pNOPGFP1L)	This study
pGFP-*SUS1cDNA*	*GFP-SUS1cDNA, LEU2, CEN* (based in pNOPGFP1L)	This study
pRS313	*HIS3, CEN*	[3]
pRS313-*SUS1*	*SUS1, HIS3, CEN*	This study
pRS316	*URA3, CEN*	[3]
pRS316-*SUS1* (p*SUS1*)	*SUS1, URA3, CEN*	This study
pDcp2-RFP	*DCP2-RFP, URA3, CEN*	[4]
pPab1-RFP	*PAB1-RFP, URA3, CEN*	[5]
pPab1-GFP	*PAB1-GFP, URA3, CEN*	[5]

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IV. DISCUSIÓN

IV. DISCUSIÓN

IV.I. Un complejo mecanismo encargado de regular la expresión del gen *SUS1*.

SUS1 ha sido objeto de estudio en nuestro laboratorio por sus implicaciones en la coordinación de distintos procesos durante la biogénesis del mRNA (Pascual-García & Rodriguez-Navarro, 2009). La existencia de dos intrones interrumpiendo su secuencia codificante y la presencia de secuencias no consenso en sus sitios de splicing, motivó el inicio de investigaciones para elucidar el papel de los intrones de *SUS1* en la regulación de su expresión.

A través del estudio evolutivo de la secuencia de *SUS1*, hemos constatado que este gen ha perdido e incorporado intrones en su secuencia con el fin de adaptarse al contexto de cada organismo. A pesar de la presencia o no de intrones, el mensaje que codifica a lo largo de las diferentes especies eucariotas parece ser muy similar (Rodriguez-Navarro, 2009). Estos hechos han reforzado la relevancia de Sus1, participando en la respuesta adaptativa frente a diferentes tipos de estrés y en la regulación y coordinación de procesos durante la transcripción y la biogénesis del RNA mensajero a lo largo de la evolución.

Durante la evolución de los organismos eucariotas, la densidad de intrones en los genomas ha ido adaptándose a los diferentes saltos evolutivos y a la complejidad de los organismos (Jeffares et *al*., 2006). En *S. cerevisiae*, pocos son los genes que contienen intrones si los comparamos con otros eucariotas. Aun así, un buen número de genes requieren del splicing para la correcta regulación de su expresión (Meyer & Vilardell, 2009).

Al igual que en otros genes con intrones implicados en el metabolismo del mRNA (*YRA1*), *SUS1* es capaz de ejercer una respuesta adaptativa regulando el procesamiento de sus intrones en función de los requerimientos celulares. De acuerdo a los resultados presentados en esta Tesis, ante un estrés térmico o durante el periodo de adaptación a un cambio en la fuente de carbono, un complejo mecanismo actúa para equilibrar el balance de transcritos de *SUS1* en respuesta a un cambio de las condiciones de crecimiento. Los cambios en el balance de transcritos, observados por reacción en cadena de la polimerasa cuantitativa con transcriptasa inversa (RT-qPCR), en las distintas condiciones de crecimiento (30ºC, 42ºC y Galactosa), podrían estar directamente relacionados con el rol fisiológico que cada transcrito de *SUS1* puede regular. La especie más abundante detectada es el transcrito maduro, pero

también es posible detectar pre-mRNAs con ambos o con solo uno de los intrones. La forma mayoritaria después del mRNA es el pre-mRNA de *SUS1* con el intrón 1 (I1) retenido. Este hecho sugiere una posible implicación de la retención del I1 en la regulación de los niveles de transcrito maduro en la célula.

Hemos mostrado como las secuencias no consenso del I1 son esenciales para controlar la cantidad de transcritos de *SUS1* en la célula y cuanto mRNA estará disponible para formar la proteína Sus1. En este trabajo centramos inicialmente nuestros esfuerzos en identificar la relevancia del BS del I1 de *SUS1* en la regulación de su splicing. De este modo detectamos que esta secuencia en *cis* es esencial para controlar los niveles de transcritos que se acumulan en la célula. Así mismo, la presencia del I1 de *SUS1*, limita la cantidad de mRNA detectable e influye en la eficiencia del splicing de *SUS1*.

Por otro lado, la detección de transcritos con solamente el intrón 2 (I2) retenido nos hace pensar que en un porcentaje de los pre-mRNAs de *SUS1*, el I1 puede ser procesado cotranscripcionalmente y antes de que se procese el segundo de los intrones. Al mismo tiempo, la detección de transcritos con el I1 retenido, implica que existe un mecanismo de regulación que permite que el I2

sea procesado con antelación. Una sugerente hipótesis contemplaba la posibilidad de que durante la transcripción y en respuesta a las necesidades de la célula, existe una coordinación de factores que regula los porcentajes de especies de transcritos de *SUS1* durante los primeros estadios de la formación de la ribonucleopartícula.

Datos mostrados por otros autores (Miura et al. 2006) indican que los transcritos que retienen el I1 de *SUS1* poseen una región no traducible en 3' (3'UTR) de mayor tamaño. Análisis preliminares de la estructura secundaria del exón 2 (E2) de *SUS1* y de su región 3'UTR indican un posible papel funcional de estas regiones y serán objeto de estudio en años posteriores.

En este trabajo no hemos profundizado en el papel del 5'ss no consenso del I1, pero otros autores han demostrado la importancia de esta secuencia para regular el splicing de *SUS1* (Hossain *et al*., 2011).

El I2 de *SUS1* también se muestra implicado en el correcto procesamiento de los transcritos a pesar de poseer secuencias consenso de reconocimiento de splicing. La eliminación del I2 de la secuencia de *SUS1* influye en la eficiencia de su splicing, como se evidencia en la disminución de la detección de Sus1 por western blot respecto a células que poseen una copia completa de

SUS1. Además, la presencia del I2 de *SUS1* en una construcción con TAF14, bloquea el splicing de los intrones de *SUS1*. Cuando se sustituye un E2 artificial de TAF14 por el de E2 de *SUS1*, flanqueado por los intrones de *SUS1*, se evidencia que la secuencia de este exón también es responsable del eficiente splicing de *SUS1*. Estos datos sostienen que existe una relación interdependiente entre ambos intrones de *SUS1* y que además el E2 influye en el proceso de splicing.

Como representamos en la Figura IV.1, distintas regiones del DNA de *SUS1* están implicadas en en la regulación de su procesamiento.

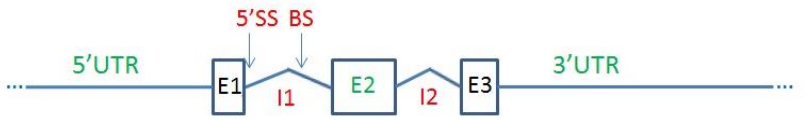

Figura IV.1 Diferentes regiones en el DNA de *SUS1* implicadas en la regulación del splicing de los intrones de *SUS1*. Los exones E1, E2 y E3 están separados por los intrones I1 e I2 respectivamente. Se representa la posición de 5'ss y del BS del I1 y se flanquea *SUS1* por las regiones 5'UTR (izquierda) y 3'UTR (derecha). En rojo, regiones del DNA implicadas en la regulación del splicing de *SUS1*. En verde regiones que muestran evidencias de su funcionalidad en el splicing de *SUS1*.

La identificación de una funcionalidad ligada al BS del I1, nos impulsó a identificar que factores de splicing pudieran estar implicados específicamente en el reconocimiento de esta secuencia. De este modo

descubrimos a Mud2 como un factor (no esencial para el splicing de la mayoría de intrones en levadura) fundamental para el correcto procesamiento de los intrones de nuestro gen de interés. Aunque existe un efecto directo en el reconocimiento del BS del I1 de *SUS1* por parte de Mud2, los resultados mostrados en esta Tesis sugieren que otras secuencias en este intrón pueden estar sujetas a la regulación por parte de Mud2. La eliminación del I1 de *SUS1* mejora el crecimiento de células en medio con cobre en comparación con células que poseen una mutación en el BS del I1 de *SUS1*. Otro dato relevante es el aumento de la relación de transcritos con ambos intrones retenidos en células *mud2Δ* respecto a una cepa WT. Este dato sugiere una dependencia entre ambos intrones para su correcto procesamiento. Sin embargo, Mud2 no es esencial para el procesamiento completo de los intrones de *SUS1*, aunque si determina enormemente la eficiencia de su splicing.

Puesto que en células eucariotas, los transcritos que retienen intrones son reconocidos y degradados para evitar la traducción de proteínas truncadas, pensamos inmediatamente en los posibles mecanismos encargados de su degradación. Así pues, evaluamos si la maquinaria NMD pudiese estar implicada en la identificación y degradación de este tipo de transcritos. Nuestros

resultados mostraron que esta maquinaria regula los niveles de transcritos de *SUS1* al ser capaces de identificar una acumulación de pre-mRNAS de *SUS1* en una cepa *upf1Δ*. Además, la evaluación de los niveles de transcritos de *SUS1* en una cepa *upf1Δmud2Δ*, reforzó la importancia de NMD en la regulación de los niveles de transcritos de *SUS1* en la célula. De nuevo, observamos en esta cepa doble mutante, que la relación de los niveles de transcritos no procesados aumenta considerablemente, indicando como se menciona en esta discusión para células *mud2Δ* que ambos intrones dependen del otro para regular eficientemente el splicing de *SUS1*.

De este modo descubrimos que en la regulación de los niveles de transcritos de *SUS1,* diferentes etapas durante la biogénesis de este transcrito (transcripción, splicing y degradación) se coordinan para regular su expresión. Además, mostramos que este mecanismo de regulación no es compartido completamente con el de *YRA1*, a pesar de estar íntimamente relacionado e implicado en procesos de transcripción, exporte y degradación de mRNAs (Rodríguez-Navarro *et al*., 2002; Dong *et al.*, 2007; Dong *et al*., 2010). De este modo, *SUS1* representa un caso particular en levadura y se

presenta además como modelo para el estudio de la regulación del splicing en otros organismos.

La cantidad de transcritos que se acumulan en la célula afectan a la cantidad de proteína Sus1 detectable por Western blot (WB). Nuestros ensayos etiquetando a Sus1 en posición N-terminal con LexA y expresando esta proteína desde un plásmido, muestran que la ausencia de intrones en *SUS1* provoca que la proteína Sus1 sea identificada en mayor cantidad que cuando los intrones están presentes. Además, a partir de cualquier versión de *SUS1* con el I1 en su posición, se traducen dos proteínas a partir de los distintos transcritos de *SUS1*. Una de ellas corresponde a la traducción del mRNA, mientras que la segunda es producto de la traducción de un transcrito que retiene al menos el I1. Este hecho podría explicarse si los transcritos que retienen el I1 de *SUS1* fueran conducidos al citoplasma y allí traducidos.

Una proteína con el E1 más 6 aminoácidos, corresponde por tamaño con la banda identificada. Un codón de parada en el I1 de *SUS1* detendría la traducción del transcrito en ese punto. Es bien conocido que la maquinaria NMD conduce a los transcritos a degradación tras una ronda inicial de traducción. En este momento nos planteamos si la detección de esta proteína Sus1 de

menor tamaño, estaba siendo detectada debido al tipo de marcaje y expresión desde un plásmido. Para ello reprodujimos los experimentos con otras proteínas de fusión (GFP, TAP). Intentamos conseguir cepas de levadura con etiquetas antes del codón de iniciación de *SUS1* y una cepa de levadura sin los intrones, pero ambas aproximaciones resultaron infructuosas. Al parecer, es inviable el marcaje de *SUS1* en su extremo 5' y sugerimos que la región 5'UTR pueda poseer un posible papel en la regulación de la expresión de *SUS1* aún por descubrir. Además, bajo el control de los propios elementos de regulación de *SUS1*, no es posible la obtención de una cepa sin los intrones. De estos experimentos se observa que la presencia de ambos intrones en su posición es imprescindible para que se produzca una determinada detección de proteína LexA-Sus1 en la célula.

Aunque iniciamos experimentos FISH para detectar específicamente la localización en la célula de transcritos de *SUS1* con o sin intrones, estos experimentos no se concluyeron durante la realización de esta Tesis. Por tanto, suponemos por las evidencias experimentales que los transcritos que retienen el I1 son conducidos al citoplasma y traducidos.

Resultaba evidente que la retención específica del I1, como consecuencia de un splicing alternativo de *SUS1*, poseía al menos una función como reguladora de la cantidad de transcrito maduro disponible. A partir de la detección de que una proteína alternativa podia traducirse desde transcritos que retienen al menos el I1, descubrimos que esta proteína es funcional, ya que es capaz de complementar parcialmente las funciones de Sus1 en el crecimiento celular. Mientras que la transformación de células ***sus1*Δ,** con un plásmido que contiene la versión SL9 de *SUS1* (no puede procesar el I1) solo produce la proteína truncada, el cDNA de *SUS1* (solo produce mRNA) no es capaz de complementar las funciones de Sus1 en exporte de mRNAs y crecimiento celular. Como se evidencia en los resultados presentados, este último hecho no es debido a una menor cantidad de proteína Sus1. Estos datos indican que tanto la proteína Sus1 como la proteína corta (Sus1 “E1+6 aminoácidos”), complementan parte de las funciones de *SUS1,* sugiriendo una implicación biológica para ambas proteínas.

La presencia de los intrones de *SUS1* fue necesaria para la funcionalidad de los productos de la expresión de *SUS1*. Cuando ambos intrones estaban ausentes de la secuencia de *SUS1*, no fuimos capaces de

complementar los defectos de crecimiento, ni el defecto de exporte de mRNAs poliadenilados a 37ºC en una cepa *sus1*Δ. Remarcamos por tanto, que los intrones de *SUS1* son requeridos para la función que han de ejercer sus transcritos.

Sorprendentemente cuando localizamos la proteína producida a partir de una versión de Sus1 sin sus intrones, en células en fase estacionaria, observamos nuestra proteína en una localización inesperada. La expresión de esta proteína (etiquetada con GFP) desde un plásmido que sobreexpresaba Sus1 permitió detectarla en agregados citoplasmáticos. Aunque otros trabajos habían detectado a Sus1 distribuido uniformemente por el citoplasma (Pascual *et al*., 2008), nunca antes se había observado esta localización en gránulos. Este resultado impulsó un proyecto paralelo que da lugar al segundo capítulo de esta Tesis.

La investigación llevada a cabo durante esta Tesis indica que la expresión de *SUS1* se ve condicionada por una intrincada coordinación de factores que actúan durante la biogénesis de sus transcritos (Figura IV.2). Los intrones de *SUS1* se muestran determinantes, tanto en su secuencia como en su posición, para poder mantener los balances adecuados de transcritos y la funcionalidad de

los transcritos con el I1 retenido. La evidencia de una proteína de menor tamaño a partir de la traducción de *SUS1* abre la posibilidad de nuevos estudios para investigar su rol funcional.

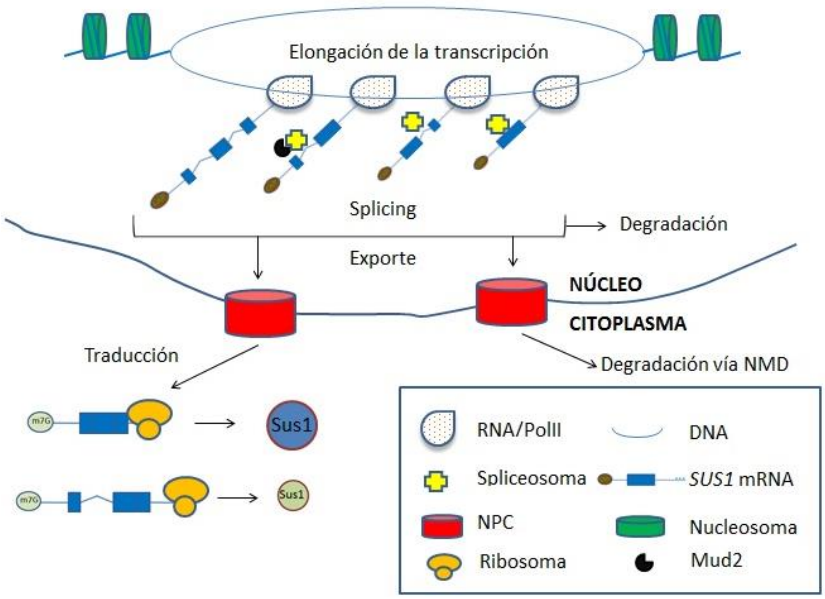

Figura IV.2 Representación de procesos identificados durante la biogénesis de los transcritos de *SUS1* en la célula de levadura.

IV.II. Sus1 actúa como un factor capaz de coordinar eventos durante la biogénesis del mRNA tanto en el núcleo como en el citoplasma

Existen diferentes ejemplos de factores que se reclutan cotranscripcionalmente a los transcritos y acompañan a la mRNP a lo largo de la vida de los transcritos. Sus1 está física y genéticamente relacionada con multitud de proteínas implicadas en diferentes etapas de la expresión génica. La localización de Sus1 en agregados citoplasmáticos y la interacción genética de *SUS1* con genes implicados en la degradación de mRNAs en el citoplasma, sugirió un papel de Sus1 más allá del núcleo.

SUS1 es sintético letal con los genes *LSM1* y *PAT1*, mientras que presenta un fenotipo de sintético enfermo con *DHH1*, *LSM6* y *RRP6*. Rrp6 forma parte del exosoma nuclear, mientras que Lsm1, Lsm6, Pat1 y Dhh1 son proteínas que participan en la degradación citoplasmática en dirección 5'→3' y se localizan en gránulos citoplasmáticos denominados P-bodies. Estas relaciones genéticas sugerían una conexión entre Sus1 y mecanismos de degradación del mRNA, tanto en el núcleo como en el citoplasma.

Con tal de averiguar qué tipo de agregados citoplasmáticos son los que distinguimos para Sus1,

iniciamos nuestros experimentos intentando verificar si se trataba de P-bodies. Se demostró la colocalización de Sus1 con Dcp2 y Pab1 y además, que la localización de Sus1 en estos gránulos es independiente de Lsm1 y Pat1. También hemos demostrado que Sus1 no es necesario para la formación de los P-bodies y SGs, puesto que no somos capaces de observar la acumulación de Sus1 en gránulos citoplasmáticos en condiciones normales de crecimiento. Es posible que las interacciones con los P-bodies sean muy dinámicas y transitorias, debido a que solamente en condiciones de sobreexpresión de Sus1 hemos podido llegar a observar su aparición en estas estructuras.

Por otro lado, a través de purificaciones TAP, hemos demostrado que Sus1 copurifica con componentes de los P-bodies, como Dhh1 y Xrn1, en condiciones de crecimiento estándar. Sus1 también copurifica con factores proteicos implicados en inicio de traducción y con proteínas de unión a la cola poli(A); previamente identificadas como constituyentes de PBs y SGs. Ensayos MudPIT de nuestras purificaciones TAP, identificaron las proteínas eIF4E, eIF4G, Pab1, Pbp1, Pbp4, Lsm12, Mkt1 y Stm1. En condiciones de inanición de glucosa, eIF4E, eIF4G y Pab1 se han descrito como componentes de los EGP-bodies y P-bodies (Hoyle *et al*., 2007; Brengues &

Parker, 2007). La colocalización de Sus1 y Pab1 demuestra que Sus1 está formando parte de los mismos gránulos en los que se encuentra Pab1, abriendo la posibilidad de que Sus1 no solo se encuentre en PBs. Además, como hemos mencionado, Sus1 interacciona con Stm1, el cual promueve las funciones de Dhh1 en la represión de la traducción y degradación de mRNAs (Balagopal & Parker, 2009b).

Nuestra hipótesis es que Sus1 viaja con la mRNP desde el núcleo hasta el citoplasma, donde actúa como un factor capaz de coordinar multitud de procesos durante la biogénesis de los transcritos (Figura IV.3).

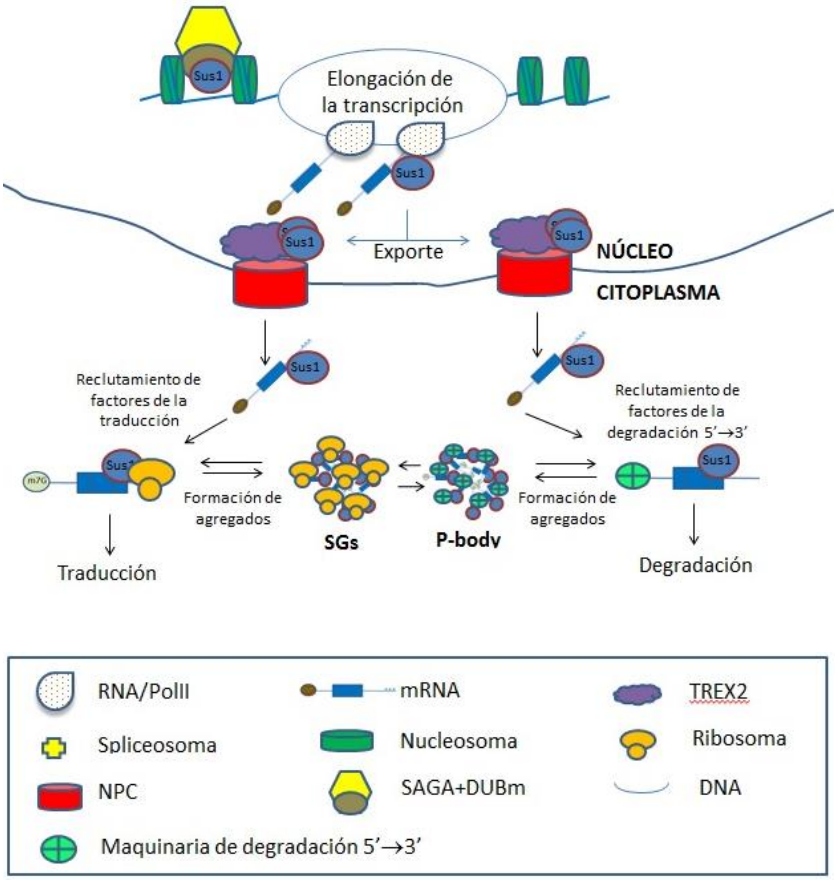

Figura IV.3. Modelo de nuevas funciones atribuidas a Sus1 en el citoplasma.

La cristalización de Sus1 muestra una estructura capaz unirse a multitud de factores proteicos (Wilmes & Guthrie, 2009). Como se comentó en la introducción de esta Tesis, al menos todos los pasos nucleares durante la biogénesis del mRNA están acoplados, pero más allá del núcleo, esta biogénesis se encuentra coordinada por diferentes factores proteicos.

¿Es posible que Sus1 se una a transcritos regulados por SAGA para regular su biogénesis más allá del núcleo? ¿Podría Sus1 unirse a la mRNP de sus propios transcritos? ¿Cómo participa la regulación de la expresión de *SUS1* en la funcionalidad de sus transcritos y/o de la proteína resultante durante la biogénesis de otros transcritos?

Las implicaciones de Sus1 durante la biogénesis de mRNAs, junto a los resultados y las evidencias mostradas en esta Tesis, sugieren múltiples hipótesis que podrán servir de base para el abordaje de nuevos estudios.

V. CONCLUSIONES

V. CONCLUSIONES

Las principales conclusiones de esta Tesis se enumeran a continuación:

1. Secuencias no consenso en los intrones de *SUS1* son relevantes para su procesamiento.
2. Mud2, componente de NMD, participa en la regulación de la expresión de *SUS1*.
3. La degradación de pre-mRNAs de *SUS1* está regulada por NMD.
4. La presencia y posición de los dos intrones de *SUS1* son esenciales para el correcto procesamiento del transcrito y la regulación de la expresión de Sus1.
5. La retención del I1 de *SUS1* conduce a la traducción de una proteína Sus1 de menor peso molecular.
6. La presencia del E2 de *SUS1* es esencial para la expresión de *SUS1*.
7. Los intrones de *SUS1* son esenciales para su correcta expresión y funcionalidad de Sus1 en la célula.
8. *SUS1* interacciona genéticamente con genes que intervienen en el metabolismo del mRNA en el citoplasma.
9. La delección de *SUS1* es letal cuando se combina con la de *LSM1* o con *PAT1* y tiene una fuerte relación genética con la de *LSM6* o *DHH1*.
10. Sus1 interacciona físicamente con componentes citoplasmáticos que intervienen en el metabolismo del mRNA.
11. La sobreexpresión de Sus1 permite su identificación en gránulos citoplasmáticos que colocalizan con componentes de los P-Bodies y de los gránulos de estrés.

12. Sus1 interacciona bioquímicamente con componentes de los P-Bodies y de los gránulos de estrés.
13. La ausencia de *LSM1* o de *PAT1* intensifica la asociación de Sus1 a TREX2.

VI. ARTÍCULOS COMPLEMENTARIOS

VI. ARTÍCULOS COMPLEMENTARIOS

La participación en otros proyectos, dentro del laboratorio de la Dra. Susana Rodríguez-Navarro, ha dado lugar a otra publicación. Dada la relación de estos trabajos con el tema general de esta Tesis en transcripción y metabolismo del RNA, y puesto que este trabajo no se desarrolla en esta Tesis, hago mención a través de su cita.

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.

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