REPARACIÓN POSTREPLICATIVA DE CORTES DE DOBLE CADENA EN *Saccharomyces cerevisiae*

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Tesis Doctoral

Universidad de Sevilla

2006

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REPARACIÓN POSTREPLICATIVA DE CORTES DE DOBLE CADENA EN *Saccharomyces cerevisiae*

Trabajo realizado en el Departamento de Genética, Facultad de Biología, Universidad de Sevilla para optar al grado de Doctor en Biología por el Licenciado Felipe Cortés Ledesma

Sevilla, Mayo del 2006

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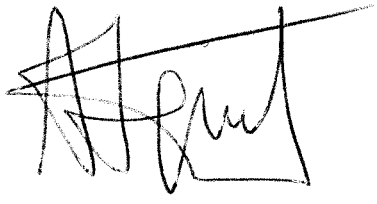

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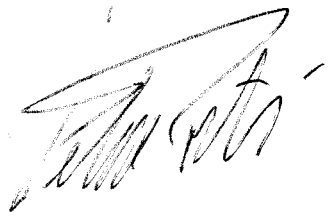


Felipe Cortés Ledesma

ÍNDICE

1- INTRODUCCIÓN

1. 1- Reparación de cortes de doble cadena

Entendemos como corte de doble cadena (*d*ouble-*s*trand *b*reak, DSB) toda interrupción en la continuidad de una molécula de ADN que afecte simultáneamente a las dos cadenas que la componen. A pesar de que DSBs programados son necesarios para ciertos procesos celulares, tales como la meiosis (Keeney, 2001), la generación de variabilidad en inmunoglobulinas (Lieber et al., 2004) y el cambio de sexo en levaduras (Haber, 1998b), en general los DSBs constituyen una grave lesión en el ADN. Por lo tanto deben ser reparados correctamente para asegurar la supervivencia de la célula (Resnick and Martin, 1976) y evitar la inestabilidad genética (Chen and Kolodner, 1999; Richardson and Jasin, 2000), un fenómeno que está asociado con la mayoría de los procesos tumorales y algunas enfermedades genéticas (Lengauer et al., 1998). Por consiguiente, el estudio de la reparación de DSBs es fundamental para entender los elementos que aseguran el mantenimiento de la estabilidad del genoma. En células eucariotas existen dos mecanismos fundamentales de reparación de DSBs, la unión de extremos no homólogos (*n*on-*h*omologous *e*nd-*j*oining, NHEJ) y la recombinación homóloga (RH). NHEJ consiste simplemente en la ligación de los extremos de un DSB. En cambio, RH repara un DSB usando como molde una secuencia de ADN homóloga, dando como resultado una transferencia de información genética hacia la molécula que ha sufrido el DSB (*c*onversión *g*énica, CG), que puede estar o no asociado a un intercambio recíproco (*c*ross-*o*ver, CO) entre las dos moléculas de ADN (Figura 1). Por lo tanto, el producto de RH depende en gran parte de la naturaleza de la molécula donadora, que puede ser la cromátida

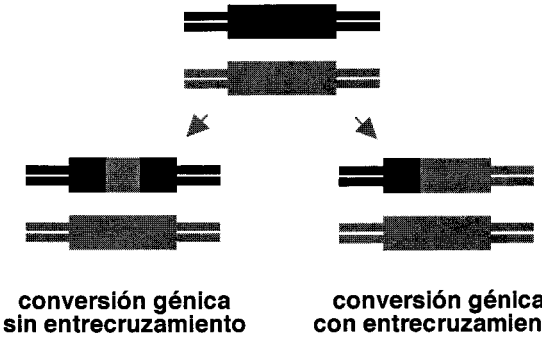


Figura1. Productos de RH. Un suceso de RH puede conducir a la transferencia unidireccional de información genética (conversión génica, CG), asociado o no a un entrecruzamiento de las regiones adyacentes (cross-over, CO).

hermana (*s*ister-*c*hromatid *r*ecombination, SCR), el cromosoma homólogo (recombinación alélica) o secuencias homólogas localizadas en otra parte del genoma (recombinación ectópica).

1. 2- Mecanismos y proteínas implicadas en NHEJ

NHEJ es un mecanismo bastante conservado en eucariotas (revisado en (Hefferin and Tomkinson, 2005) Figura 2). En *Saccaromyces cerevisiae* comienza con la unión del heterodímero yKu (yKu70/yKu80) a los extremos del DSB, lo que los protegería de degradación. El complejo MRX (Mre11/Rad50/Xrs2), que también funciona en los primeros pasos de RH así como en la señalización de DSBs y mantenimiento de telómeros (revisado en (Haber, 1998a), intervendría en el procesamiento (si es necesario) y/o unión de los dos extremos del DSB. En último término la actividad ligasa de Dnl4 (Lig4) actúa junto con Lif1 para sellar el DSB. La ausencia de cualquiera de estas funciones disminuye fuertemente la eficiencia de NHEJ.

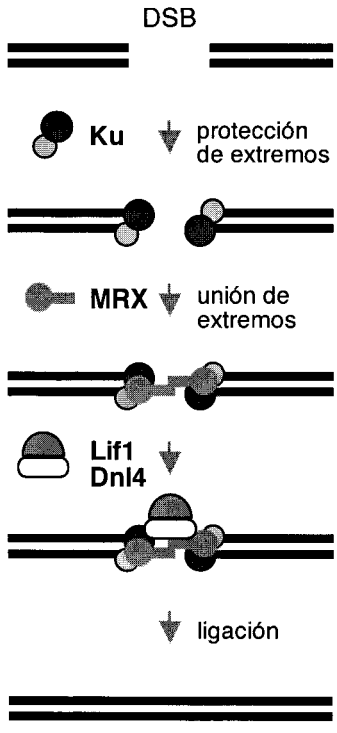


Figura 2. Mecanismo de NHEJ. El heterodímero Ku se une a los extremos del corte protegiéndolos de degradación. El complejo MRX actúa procesando y uniendo los extremos para su posterior ligación llevada a cabo por Dnl4 junto con Lif1.

1. 3- Mecanismos y proteínas implicados en RH

La mayor parte del conocimiento que tenemos sobre RH en eucariotas proviene del estudio de la recombinación alélica y ectópica en *S. cerevisiae* (revisado en (Paques and Haber, 1999; Prado et al., 2003; Sung et al., 2000;

Symington, 2002); Figura 3). Los extremos 5' de un DSB se reseccionan dejando extremos 3'-OH de cadena sencilla, un proceso en el que interviene el anteriormente mencionado complejo MRX. Dicho complejo posee actividad nucleasa que reside en la proteína Mre11 (Trujillo and Sung, 2001). Sin embargo otras funciones sin identificar deben actuar en este paso, ya que en mutantes nulos de MRX la RH mitótica sólo está levemente afectada (revisado en (Symington, 2002). Además, mutantes *mre11* sin actividad nucleasa no presentan un defecto evidente en resección, a pesar de ser sensibles a agentes que causan DSBs como el metil-metano sulfonato (MMS) y la radiación ionizante (Ajimura et al., 1993; Game and Mortimer, 1974; Ivanov et al., 1992). Sae2 parece controlar la actividad nucleasa del complejo, ya que mutantes *sae*Δ y MRX nucleasa deficientes presentan los mismos fenotipos (Lisby et al., 2004; McKee and Kleckner, 1997; Prinz et al., 1997; Rattray et al., 2001). Una proteína candidata para actuar en la resección es Exo1, una exonucleasa 5'-3' que presenta varias interacciones genéticas con MRX (Fiorentini et al., 1997; Moreau et al., 2001; Symington et al., 2000; Tsubouchi and Ogawa, 2000). Rad52, una proteína necesaria para todos los sucesos de RH en *S. cerevisiae* con capacidad de aparear cadenas de ADN (Mortensen et al., 1996); revisado en (Symington, 2002), promueve la invasión de los extremos 3'-OH de cadena sencilla generados por la resección sobre la secuencia de ADN homóloga. La proteína Rad59, que es homóloga a la parte amino-terminal de Rad52 y también posee capacidad de aparear ADN (Davis and Symington, 2001; Petukhova et al., 1999), podría actuar facilitando este paso. Esta invasión puede ser estabilizada por un intercambio de cadena catalizado por Rad51, un homólogo de RecA bacteriano, con la ayuda de sus homólogos Rad55 y Rad57, y la ATPasa de la familia SNF-SWI Rad54 (revisado en (Sung et al., 2000; Symington, 2002). Esto genera un heterodúplice en el que los extremos 3' invasores ceban la síntesis de ADN usando como molde la secuencia homóloga invadida. Estos pasos son comunes a los dos modelos aceptados mayoritariamente para explicar la RH, apareamiento de cadena dependiente de síntesis (*s*ynthesis-*d*ependent *s*trand *a*nnealing, SDSA) (Nassif and Engels, 1993) y reparación de cortes de doble cadena (*d*ouble-*s*trand *b*reak *r*epair, DSBR) (Szostak et al., 1983). En SDSA el intercambio de cadena se revierte y las dos cadenas de ADN recién

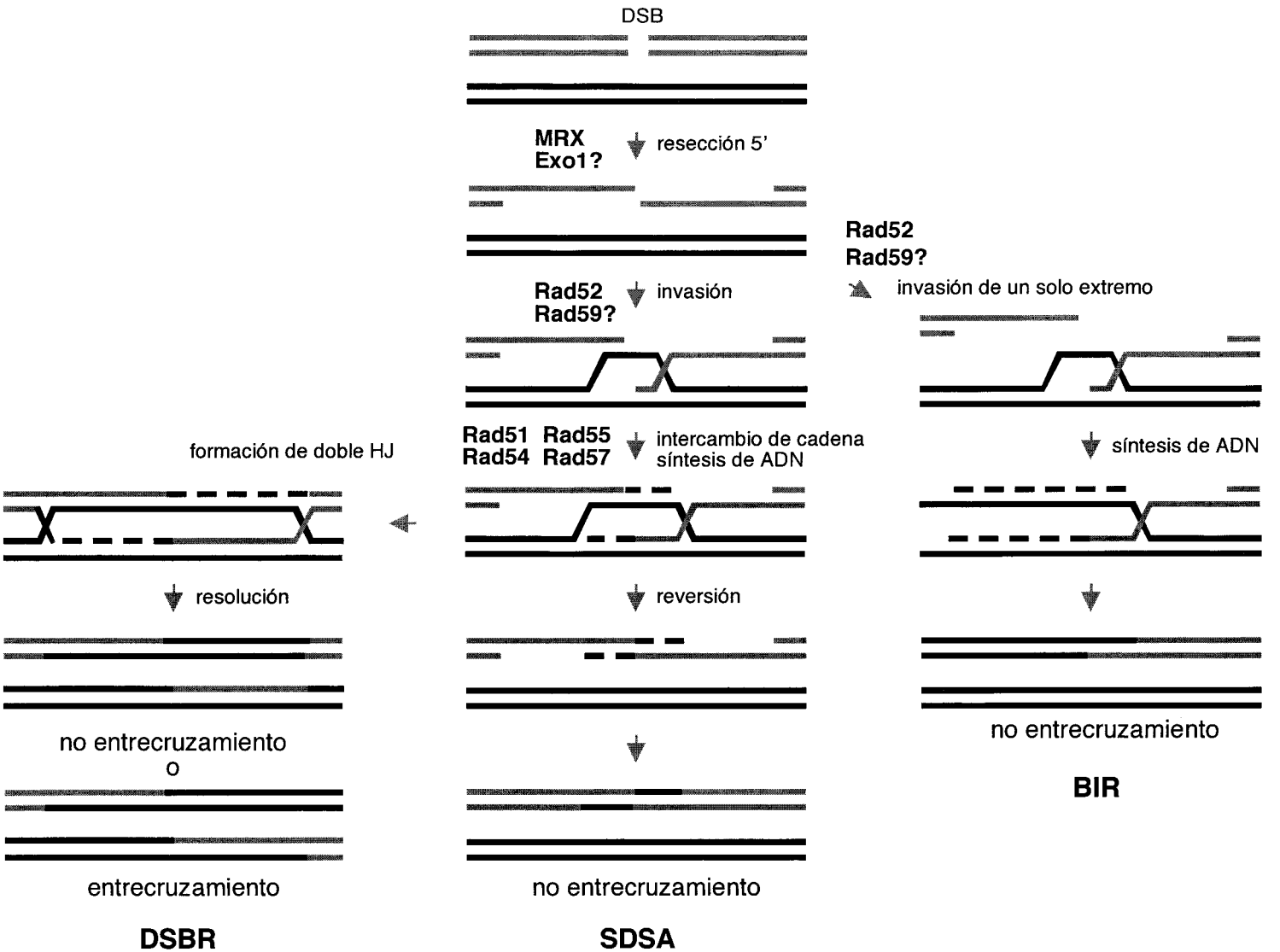


Figura 3. Mecanismos de RH. Los dos modelos principales de RH (SDSA,centro; y DSBR, izq.) comparten los primeros pasos de resección, invasión, intercambio de cadena y síntesis de ADN. En SDSA el intercambio de cadena se revierte generando un producto sin CO. En DSBR el intercambio de cadena y la síntesis de ADN continúan hasta formar una doble HJ, cuya resolución puede generar o no CO. En BIR (dcha.) la síntesis de ADN continúa a lo largo de la molécula invadida, generando largos tramos de CG no asociados a CO.

sintetizadas aparean generando CG (Figura 3, centro). Por el contrario, en DSBR el intercambio de cadena y la síntesis de ADN continúan hasta alcanzar los extremos reseccionados, generando dos estructuras de Holliday (Holliday junction, HJ) cuya resolución genera una CG que puede estar asociada o no a un CO (Figura 3, izquierda). Por otro lado, la síntesis de ADN a partir del extremo 3' invasor puede continuar a lo largo de la molécula invadida sin ser capturada por el otro extremo del DSB en un proceso conocido como replicación dependiente de rotura (*b*reak-*i*nduced *r*eplication, BIR) (Figura 3, derecha) (Malkova et al., 1996; Voelkel-Meiman and Roeder, 1990). Los datos actuales sugieren que el mecanismo mayoritario de RH mitótica es SDSA, ya que la mayor parte de la CG no está asociada a CO (revisado en (Paques and Haber, 1999; Prado et al., 2003; Symington, 2002)). Además, un mecanismo adicional de RH conocido como apareamiento de cadena sencilla (*s*ingle-*s*trand *a*nnealing, SSA) (Lin et al., 1990) parece ser responsable de gran parte de las deleciones en sistemas de recombinación basados en repeticiones directas (revisado en (Paques and Haber, 1999; Prado et al., 2003) (Figura 4). En este mecanismo, tras la resección, las cadenas homólogas expuestas aparean y las colas 3' sobrantes son eliminadas por el complejo Rad1-Rad10, generando la deleción.

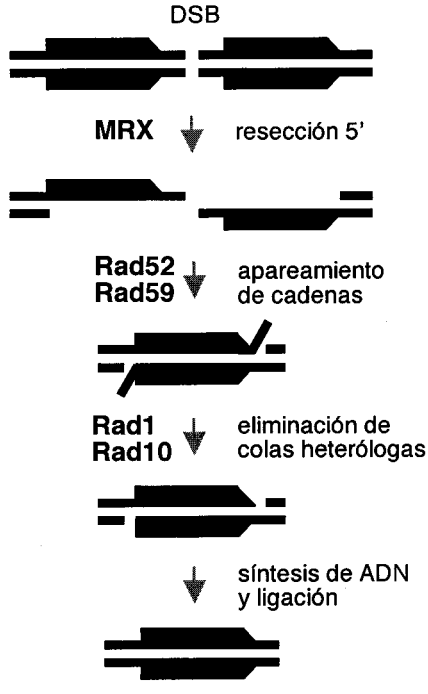


Figura 4. SSA como mecanismo responsable de deleciones. La resección de los extremos de un DSB entre repeticiones directas expone fragmentos homólogos de cadena sencilla que pueden aparear. Tras ser eliminadas las colas heterólogas, se ligan los extremos generando la deleción.

A pesar de compartir gran parte de la ruta y requerimientos genéticos, algunos sucesos de RH son menos dependientes de intercambio de cadena mediado por Rad51. Así, BIR puede ocurrir en ausencia de Rad51 (Malkova et al., 1996), aunque se ha demostrado que Rad51 participa cuando se deja homología en sólo un lado del DSB

para forzar a que la reparación ocurra por BIR (Davis and Symington, 2004). Además SSA, que no requiere intercambio de cadena (Figura 4), es totalmente independiente de Rad51 (Ivanov et al., 1996). Por otro lado, un CO entre repeticiones invertidas da como resultado una inversión de la zona intermedia. Sin embargo, al contrario de lo esperado, estas inversiones no se reducen de forma significativa en *rad51*Δ (revisado en (Prado et al., 2003; Symington, 2002), sugiriendo que no ocurren necesariamente por CO, sino por un doble suceso que implica BIR seguido de SSA (Bartsch et al., 2000) (Figura 5). Numerosos datos genéticos apoyan este modelo (Gonzalez-Barrera et al., 2002; Ira and Haber, 2002; Kang and Symington, 2000; Malagon and Aguilera, 2001; Rattray and Symington, 1995).

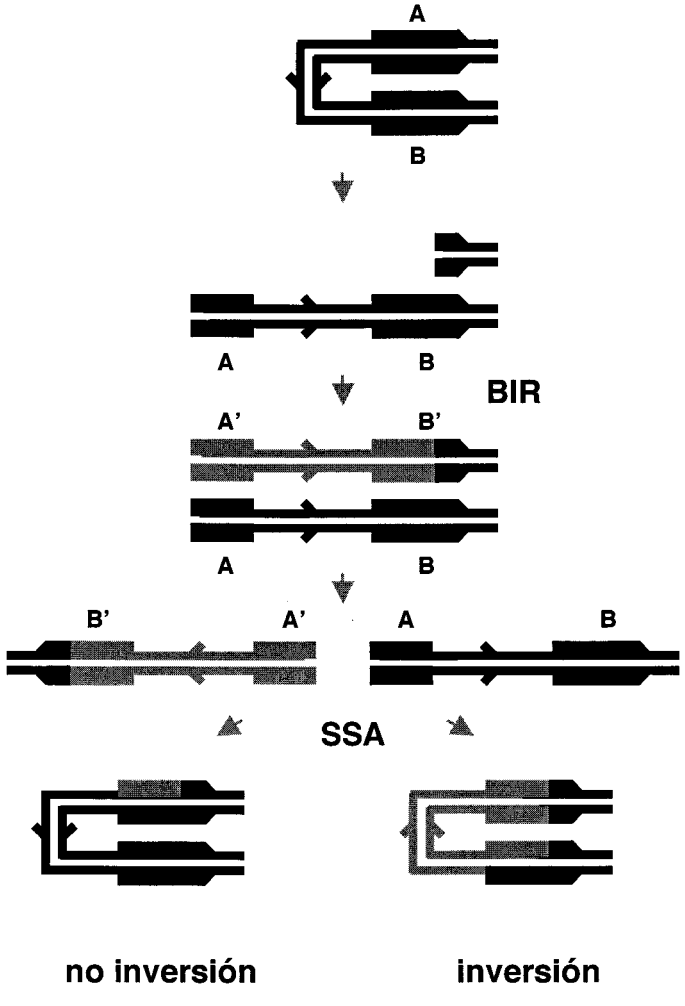


Figura 5. BIR seguido de SSA como mecanismo responsable de inversiones. Un DSB en una repetición invertida genera un sustrato para que un suceso de BIR duplique las repeticiones. Como consecuencia aparecen dos series de repeticiones directas, A-B' y A'-B, cuya resolución por SSA generaría, respectivamente, un producto sin y con una inversión asociada.

1. 4- La RH como mecanismo de reparación de daño replicativo

En todos los organismos estudiados RH y NHEJ contribuyen a la reparación de DSBs (Aylon and Kupiec, 2004; Couedel et al., 2004; Mills et al., 2004; Takata et al., 1998). Sin embargo, la incidencia relativa de ambos mecanismos varía a lo largo del ciclo celular. En células de vertebrados se ha observado una mayor actividad de RH en fase S que en G1 (Rothkamm et al., 2003; Saleh-Gohari and Helleday, 2004; Takata et al., 1998). De hecho, en levaduras se ha comprobado que durante G1 la RH está prácticamente ausente y los DSBs se reparan fundamentalmente por NHEJ, mientras que en S y G2 la RH está activa y disminuye la eficiencia de NHEJ (Aylon et al., 2004; Ira et al., 2004). Además el análisis citológico de Rad52 fusionada a la proteína fluorescente verde ha demostrado que se forman focos de recombinación, tanto espontáneos como inducidos, sólo durante las fases S y G2 (Lisby et al., 2001). Estas observaciones han venido a confirmar la idea de que la mayor parte de los DSBs espontáneos surgen por problemas durante la replicación, en cuyo caso la RH no sólo repararía el DSB sino que podría ser importante para el reinicio de horquillas de replicación colapsadas mediante un mecanismo de BIR (revisado en (Branzei and Foiani, 2005; Cox et al., 2000; Haber, 1999; Kogoma, 1997; Kuzminov, 2001a; Michel et al., 2001; Rothstein et al., 2000).

Una de las principales fuentes de colapso de horquillas puede ser la replicación a través de mellas o cortes de cadena sencilla (*s*ingle-*s*trand *b*reak, SSB), que son lesiones en el ADN frecuentemente generadas por daño oxidativo (Lindahl, 1993). En levaduras se ha visto que la inducción de un SSB, usando el gen *II* del fago f1, estimula RH (Strathern et al., 1991) de forma dependiente de fase S (Galli and Schiestl, 1998). La conversión de SSBs en DSBs se ha mostrado usando camptotecina, que produce un bloqueo en la acción de la topoisomerasa I generando SSBs (Strumberg et al., 2000); y se ha propuesto para mutantes afectados en la maduración de los fragmentos de Okazaki, que requieren RH para su viabilidad (revisado en (Aguilera et al., 2000; Kuzminov, 1995). Se han obtenido evidencias moleculares directas de este fenómeno usando un sistema bacteriano en el que se introduce la diana de SSB del fago M13 en el cromosoma del fago λ (Kuzminov, 2001b).

Cabe destacar que, al usar RH como mecanismo de reparación de daños ocurridos durante la replicación, la célula se asegura la presencia de la cromátida hermana que puede ser usada como molde para la reparación. La SCR, al ser ambas cromátidas hermanas iguales, garantiza el mantenimiento de la estabilidad del genoma, al contrario que la recombinación alélica o ectópica que pueden resultar en pérdida y/o reordenación de la información genética. De hecho la cromátida hermana es el sustrato preferido para la reparación de DSBs por RH en levaduras y células de mamífero (Gonzalez-Barrera et al., 2003; Johnson and Jasin, 2000; Kadyk and Hartwell, 1992). Sin embargo poco se conoce acerca de los mecanismos y requerimientos genéticos de SCR. Esto se debe en gran parte a que el hecho de las cromátidas hermanas son idénticas impide el análisis de SCR por los métodos por los que tradicionalmente se ha medido RH, fundamentalmente alélica y ectópica. Sin embargo se han desarrollado métodos que han permitido establecer la importancia de la SCR, así como algunos indicios sobre sus requerimientos génicos y posibles mecanismos.

1. 5- Métodos para la detección de SCR

La SCR se detectó originalmente en eucariotas superiores de forma citológica; sin embargo, las numerosas aproximaciones genéticas y moleculares desarrolladas con posterioridad ponen de manifiesto la dificultad del análisis de SCR así como de su mecanismo molecular.

A. Marcaje con 5-Bromodeoxiuridina.

Desarrollado en células de mamífero, este método clásico permite la visualización de intercambios entre cromátidas hermanas (*s*ister-chromatid *e*xchange, SCE) (Figura 6A) (revisado en (Wolff, 1977). Las células se incuban durante dos rondas de replicación en presencia del análogo de la timidina, 5-bromodeoxiuridina (BrdU), resultando en un marcaje diferencial de cada cromátida hermana que puede seguirse tras una tinción del ADN. Se observan SCEs como cromátidas hermanas que están teñidas de forma discontinua y de acuerdo con un patrón recíproco.

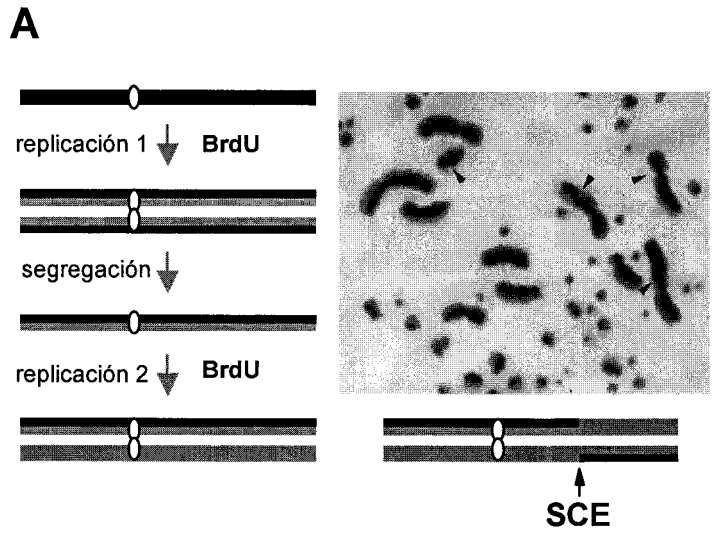
Este método ha sido útil para detectar niveles altos de SCE, como los que pueden observarse al tratar las células con agentes que dañan el ADN (revisado en (Wolff, 1977) o inhibidores de enzimas del metabolismo del ADN como la camptotecina y el m-AMSA (Cortes et al., 1993; Degrassi et al., 1989), así como en líneas celulares afectadas en la integridad del ADN (Bartram et al., 1976; Thompson et al., 1982; Wang et al., 1997). Sin embargo, el marcaje por BrdU no es válido para el análisis de SCE espontáneo, ya que la incorporación de BrdU induce SCE *per se* (Kato, 1974).

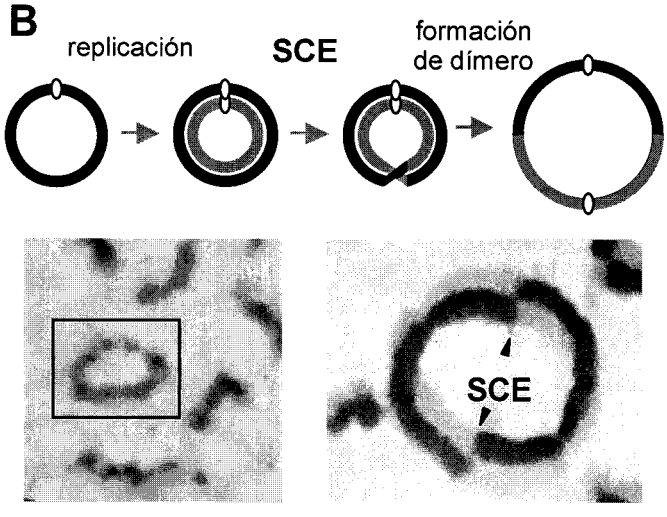
B. Detección de SCE en moléculas circulares.

Este método se basa en el hecho de que un único CO entre dos cromátidas hermanas circulares resulta en un dímero (Figura 6B). La visualización citológica de anillos dicéntricos en células de maíz fue la primera evidencia de SCR (McClintock, 1938). Dímeros similares se observaron en células humanas y de *Drosophila* con cromosomas circulares (Brewen, 1969; Gatti et al., 1979). Los cromosomas de levaduras no se pueden visualizar a nivel citológico; sin embargo, en *S. cerevisiae* se han detectado dímeros resultantes de SCE en un cromosoma III circular mediante Southern-blot de cromosomas separados por electroforesis de campo pulsado (Game et al., 1989).

C. Sistemas genéticos basados en repeticiones directas.

La RH se ha medido tradicionalmente de forma genética mediante el uso de dos heteroalelos mutantes, que por RH reconstituyen el gen silvestre, de forma que puede seleccionarse fenotípicamente, por prototrofía o resistencia a drogas. En principio recombinación entre dos moléculas idénticas, como son las cromátidas hermanas, no puede resultar en un fenotipo seleccionable, haciendo su detección genética imposible. Este problema se soluciona usando repeticiones directas, de forma que la RH puede tener lugar con la misma repetición (SCR igual), que es indetectable, pero también con la otra repetición (SCR desigual) de la cromátida hermana, generando una triplicación que puede resultar en un recombinante seleccionable (Figura 6C). El resultado en la otra cromátida es o bien la secuencia original inalterada, o una deleción dependiendo de si el suceso ha sido una CG (SC-CG) o un SCE, respectivamente (Figura 6C). El SCE desigual se observó por primera vez en





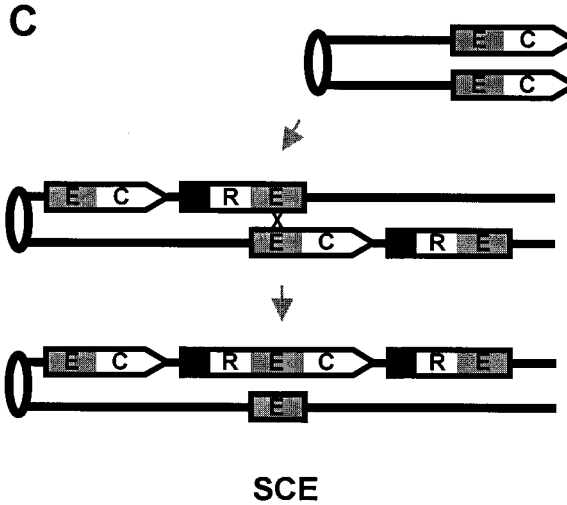


Figura 6. Detección citológica y genética de SCR.
A. Marcaje con BrdU. La incubación con BrdU durante dos rondas de replicación permite la tinción diferencial de las cromátidas hermanas (izq.), y la visualización de SCE (abajo dcha.). Las cadenas de ADN sustituidas con BrdU se indican en gris. Se muestran SCEs espontáneos en células de pollo (arriba dcha.). **B. SCE en moléculas circulares.** Un único CO entre dos cromátidas hermanas circulares genera un dímero (arriba). Se muestran dímeros dicéntricos en células de *Drosophila* (abajo izq.) y Hamster (abajo dcha.). **C. Sistemas de repeticiones directas.** Las repeticiones están orientadas de forma que sólo SCR genera un recombinante seleccionable (REC). Los sucesos que implican CO (SCE, izq.) o no (SC-CG, dcha.) pueden distinguirse por el resultado en la otra cromátida.

levaduras con un gen *LEU2* insertado en el locus del ADNr por la aparición de colonias en las que una mitad había perdido y la otra había duplicado *LEU2* (Petes, 1980; Szostak and Wu, 1980). En este sentido la expansión de repeticiones en el ADNr se ha tomado como indicador de SCR desigual recientemente (Kobayashi et al., 2004). Estos sistemas basados en repeticiones directas se han usado extensamente en levaduras para determinar niveles de SCR espontáneo (Dong and Fasullo, 2003; Fasullo et al., 2001; Fasullo and Davis, 1987; Jackson and Fink, 1981) e inducido por daño (Dong and Fasullo, 2003; Fasullo et al., 2001; Fasullo and Davis, 1987; Kadyk and Hartwell, 1992; Kadyk and Hartwell, 1993). En este tipo de sustratos también se ha estudiado SCR inducido por DSB usando endonucleasas específicas de sitio, tanto en células de mamífero (Johnson and Jasin, 2000; Puget et al., 2005; Saleh-Gohari et al., 2005; Xie et al., 2004) como en levaduras (Dong and Fasullo, 2003; Fasullo et al., 2001), pero esto crea una situación artificial en la que las dos cromátidas están rotas, lo que evita la SCR igual. Además, al tratarse de repeticiones directas SCR compite con SSA, que, aunque no puede dar lugar a un recombinante, es responsable de gran parte de la reparación. Estos ensayos genéticos han sido importantes para establecer la importancia de SCR en la reparación de DSBs así como algunos de sus requerimientos genéticos tanto en levaduras como en células de mamífero (Dong and Fasullo, 2003; Dronkert et al., 2000; Fasullo et al., 2001; Fasullo and Davis, 1987; Jackson and Fink, 1981; Johnson and Jasin, 2000; Kadyk and Hartwell, 1992; Kadyk and Hartwell, 1993).

D. Análisis molecular de SCE.

La mayor limitación para el análisis molecular de SCR es la dificultad para detectar sucesos espontáneos. Así, la detección de SCR a nivel molecular requiere inducción, para lo que se han usado frecuentemente endonucleasas específicas de sitio, como HO en *S. cerevisiae*, que crean un DSB en un lugar determinado, pudiendo analizarse molecularmente su posterior reparación por RH (Paques and Haber, 1999). Sin embargo esta aproximación no es válida para la detección de SCR, porque, como hemos mencionado anteriormente, tras la replicación, endonuleasas como HO cortan en ambas cromátidas. En un ensayo molecular desarrollado recientemente en *S. cerevisiae* se evita este

problema usando un plásmido (pRS316-TINV) con una diana de HO incompleta (21-pb en vez de 117-pb) (Gonzalez-Barrera et al., 2003), que se corta con muy baja eficiencia (10%), lo que favorece que, en la mayoría de los casos, los DSBs ocurran en una sola cromátida, mientras que la otra se mantiene intacta y puede usarse como molde para la reparación. Así, se puede detectar SCE mediante Southern-blot, por la aparición de dímeros en cinéticas de reparación. Además, la presencia de repeticiones invertidas permite la detección de SCE desigual por la aparición de fragmentos de restricción específicos.

1. 6- Posibles mecanismos de SCR

La SCR parece ocurrir principalmente por SDSA (Figura 7, b-d), por lo que sería mecanísticamente similar a otros sucesos de RH mitótica, ya que la CG no asociada a CO es el principal producto de SCR en levaduras y células de ratón (Johnson and Jasin, 2000; Kadyk and Hartwell, 1992). La importancia del SDSA viene también avalada por los requerimientos genéticos de SCR. Estudios genéticos en levaduras han mostrado que la SCR inducida por DSB o daño en el ADN se elimina completamente en *rad52* (Kadyk and Hartwell, 1992; Kadyk and Hartwell, 1993), se reduce entre 2 y 10 veces en *rad51*, *rad54*, *rad55* y *rad57* (Dong and Fasullo, 2003; Fasullo et al., 2001) y no se afecta en *rad59* (Dong and Fasullo, 2003). En células de ratón la SCR inducida por daño se reduce 2 veces en ausencia de Rad54 (Dronkert et al., 2000). Sin embargo, como hemos mencionado anteriormente, SCE, que requiere un CO y por lo tanto no puede ocurrir por SDSA, también se observa en levaduras y eucariotas superiores. El análisis a nivel molecular de reparación de DSB en levaduras ha demostrado recientemente la importancia de Rad52, Rad51 y Rad59 en SCE (Gonzalez-Barrera et al., 2003), y estudios citológicos han revelado que SCE depende de Rad51 y Rad54 en células de vertebrados (Dronkert et al., 2000; Sonoda et al., 1999).

Los múltiples factores que comparten la recombinación alélica y ectópica con SCR sugieren que los mecanismos de RH son similares independientemente del donador de información. Esto, sin embargo, no es

incompatible con la idea de que la elección de molde de reparación pueda influir en algunas de las actividades requeridas. De hecho, la existencia de un homólogo de Rad54 en levaduras, Rdh54/Tid1, que parece actuar en la recombinación alélica en particular (Klein, 1997; Shinohara et al., 1997), ha llevado a la idea de que Rad54 podría actuar específicamente en SCR, dejando otros sucesos de RH para Tid1. Esto podría reflejar distintos requerimientos para la invasión y el intercambio de cadena determinados por características estructurales de la molécula donadora. Usando un ensayo en el que se inducen DSBs meióticos para después devolver las células a condiciones de crecimiento mitótico (return-to-growth assay) se ha mostrado que Rad54 es necesario para SCR pero no para la recombinación alélica, y lo contrario ocurre con Tid1 (Arbel et al., 1999). Esto también es compatible con un papel general en RH de Rad54, sólo que en el caso particular de la recombinación alélica existe redundancia con Tid1.

Por otro lado, de acuerdo con la estrecha relación entre RH y replicación, la SCR espontánea podría reflejar el rescate por BIR de horquillas de replicación colapsadas (ver (Fasullo, 2004; Helleday, 2003) (Figura 7, e-g). Como hemos mencionado anteriormente esta situación puede simularse usando el inhibidor de la topoisomerasa I camptotecina. En células de mamífero se ha demostrado recientemente que la distribución de recombinantes espontáneos es similar a la inducida por camptotecina, y difiere significativamente de la inducida por DSB (Saleh-Gohari et al., 2005). A este respecto cabe mencionar que en levaduras mientras que la SCR desigual espontánea depende de Rad52 (Fasullo and Davis, 1987; Jackson and Fink, 1981; Kadyk and Hartwell, 1992; Kadyk and Hartwell, 1993), es independiente de Rad51, Rad54, Rad55 y Rad57 (Dong and Fasullo, 2003), indicando que, al contrario que la inducida por DSB, la SCR espontánea puede ocurrir en ausencia de intercambio de cadena. Estos resultados estarían de acuerdo con trabajos pasados en los se muestra que agentes que causan DSBs con dos extremos no inducen fuertemente SCEs citológicos (Morgan et al., 1988; Perry and Evans, 1975; Solomon and Bobrow, 1975), lo que contrasta con la fuerte inducción por captotecina (Degrassi et al., 1989). Una posible explicación para esto es que como el CO es un suceso muy poco frecuente en células de mamífero (Johnson and Jasin, 2000; Richardson et al., 1998), la reparación de

DSBs con dos extremos raramente generaría SCE, mientras que la reparación por BIR de DSBs con un solo extremo puede resultar en un SCE citológico aún en la ausencia de resolución con CO (Helleday, 2003). Otra posibilidad más especulativa, de acuerdo a los datos actuales, es que lesiones diferentes de DSBs son responsables de la SCR espontánea (Rothstein et al., 2000). En este sentido se ha observado que células de levadura irradiadas con UV acumulan zonas de ADN de cadena sencilla (gaps) que se reparan en parte por RH (Lopes et al., 2006). Dichas lesiones se repararían por un mecanismo de cambio de molde (template switching) (Higgins et al., 1976) (Figura 7, h-j).

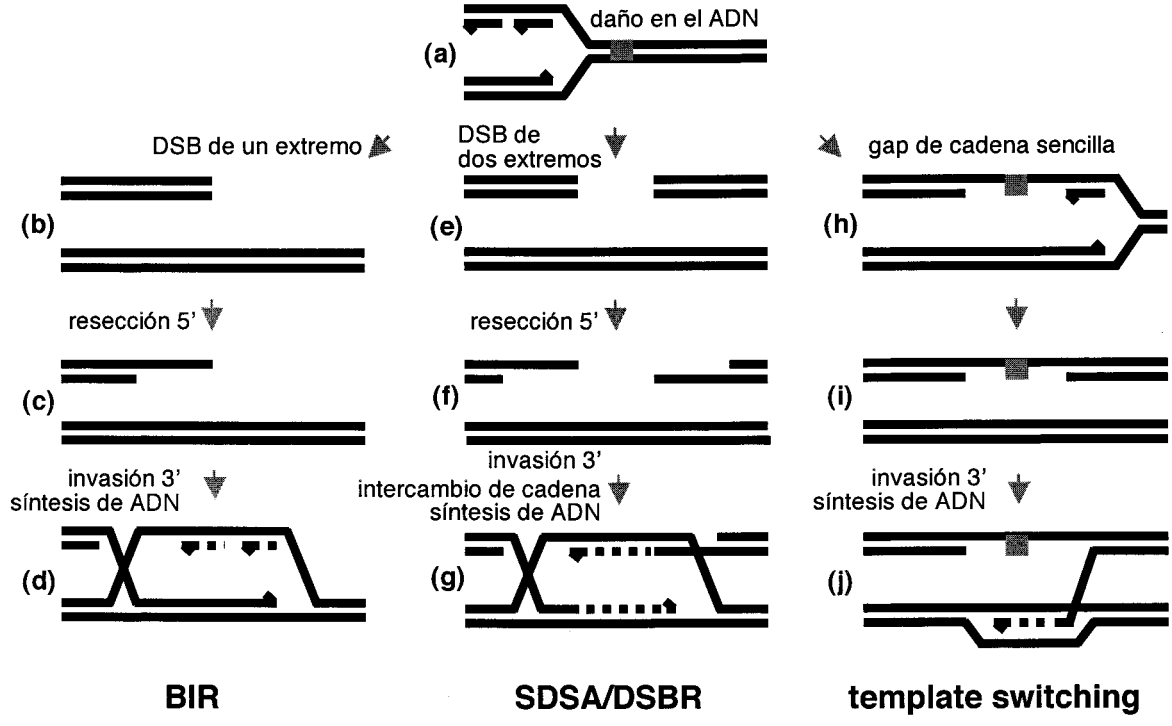


Figura 7. Mecanismos de SCR. Diferentes mecanismos pueden usar la cromátida hermana para reparar daño replicativo. La replicación sobre un ADN dañado **(a)** puede conducir a la formación de un DSB con dos extremos **(b)**, que se pueden reparar por SDSA o DSBR **(c-d)**. Por otro lado la horquilla puede colapsarse, generando un DSB con un solo extremo que por BIR reestablecería la replicación **(e-g)**. Otra posibilidad es que el daño se evite, generando un gap de cadena sencilla, que puede llenarse copiando la información de la cromátida hermana, por un mecanismo de template switching **(h-j)**.

1. 7- Posibles requerimientos específicos de SCR

SCR es el principal mecanismo de RH mitótica en levaduras y células de mamífero (Gonzalez-Barrera et al., 2003; Johnson and Jasin, 2000; Kadyk and Hartwell, 1992) y supone una manera segura de reparar lesiones en el ADN sin afectar la integridad del genoma. Es por lo tanto importante conocer los elementos que determinan la elección de la cromátida hermana como primer donador de información durante la reparación por RH. La proximidad entre cromátidas hermanas podría suponer una ventaja para SCR y, por lo tanto, los factores implicados en mantener las hermanas juntas podrían actuar en la regulación de este proceso. De acuerdo con esta hipótesis numerosos resultados sugieren un papel tanto de las cohesinas como del complejo MRX en SCR.

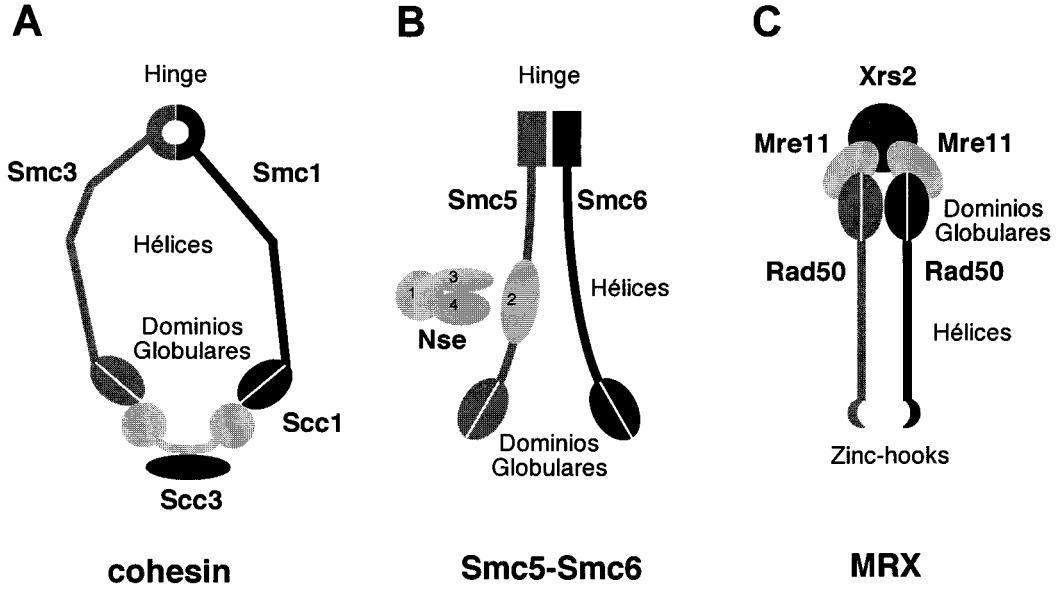


Figura 8. Estructura de los complejos cohesina, Smc5-Smc6 y MRX. Las proteínas SMC comparten una serie de características en cuanto a su estructura. Cada una se dobla sobre sí misma enrollándose de forma antiparalela. Esto deja una zona globular (dominios amino y carboxi-terminales), y un motivo "bisagra" (hinge), separados por una hélice. **A.** Las cohesinas presentan una estructura en forma de anillo. Smc1 y Smc3 interaccionan por su hinge. Scc1 y Scc3 cierran la estructura uniéndose a los dominios globulares. **B.** El complejo Smc5-Smc6 está compuesto por dichas proteínas y una serie de factores llamados Nse (*n*on-*s*mc *e*lement). **C.** En MRX Rad50 presenta una estructura similar a SMC. Dos moléculas de Rad50 interaccionan entre sí y con dos moléculas de Mre11 a través de sus dominios globulares. Esto constituye la zona de unión a ADN dejando en el otro lado un motivo zinc-hook (análogo al hinge) que permite unión a otros complejos MRX. Xrs2 interacciona directamente con Mre11.

A. Cohesinas y complejo Smc5-Smc6.

Las cohesinas son un complejo esencial que mantiene las cromátidas hermanas juntas desde la replicación hasta el inicio de anafase (Nasmyth, 2002). Es un complejo tetramérico formado por dos proteínas SMC (*s*tructural *m*aintenance of *c*hromosomes) (Smc1 y Smc3), y otros dos proteínas (Scc1 y Scc3 en *S. cerevisiae*). Cada componente SMC se dobla sobre sí mismo dejando una "bisagra", por la que las dos proteínas interaccionan, una larga espiral y un dominio globular donde ambas proteínas interaccionan con Scc1, cerrando una estructura en forma de anillo (Figura 8A). Las cohesinas se cargan durante G1, para lo que es necesaria la proteína Scc2, unen las cromátidas hermanas mediante un mecanismo dependiente de replicación y las liberan para la segregación cromosómica tras proteolisis de Scc1 (Haering and Nasmyth, 2003). Interesantemente la unión de las cohesinas a las cromátidas es topológica y no física (Ivanov and Nasmyth, 2005). Todas estas observaciones han conducido a un modelo en el que las cohesinas mantienen juntas las cromátidas hermanas abrazándolas.

Numerosos resultados desde levaduras hasta humanos implican a las cohesinas en reparación de DSBs (revisado en (Lehmann, 2005). Así, mutaciones en *RAD21* (homólogo a *SCC1* en *Schizosaccharomyces pombe*) causan sensibilidad a radiación, UV e inhibidores de replicación (ver (Lehmann, 2005). Mutantes *smc1* de *S. cerevisiae* están afectados en reparar múltiples DSBs (Schar et al., 2004). Además la cohesión es necesaria en *S. cerevisiae* para la recuperación de un cromosoma tras irradiación (Sjogren and Nasmyth, 2001). La represión transcripcional de *SCC1* en células de pollo reduce el número de SCEs citológicos inducidos por daño y aumenta al menos 3 veces las aberraciones cromosómicas inducidas por radiación (Sonoda et al., 2001). Finalmente, en células humanas Smc1 se fosforila tras irradiación (Kim et al., 2002b), y se acumula junto con otros componentes del complejo cohesinas en zonas del genoma irradiadas con laser (Kim et al., 2002a).

El análisis mediante inmunoprecipitación de cromatina (*ch*romatin *i*nmuno*p*recipitation, ChIP) de los factores que interaccionan con el ADN en respuesta a un DSB ha revelado que las cohesinas se cargan a lo largo de una región que se extiende varias kilobases a ambos lados del corte (Strom et al.,

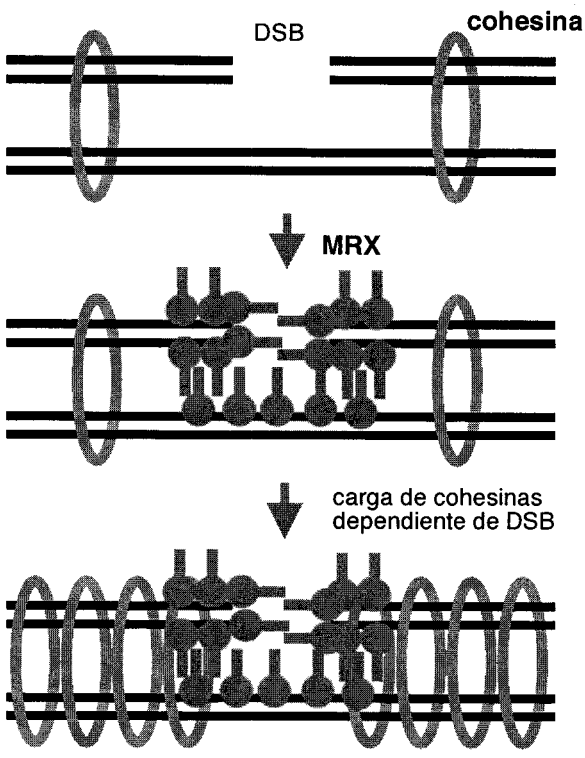


Figura 9. Modelo que explica un posible papel estructural de cohesinas y complejo MRX en SCR. Las cohesinas, que, de forma dependiente de MRX, se cargan abundantemente en respuesta a un DSB, podrían favorecer la interacción de la cromátida rota con su hermana, facilitando así la SCR. MRX se carga en los extremos de un DSB y, mediante interacciones entre los zinc-hooks, puede actuar manteniendo ambos lados unidos, ya sea entre sí o a la cromátida hermana, facilitando la reparación.

2004; Unal et al., 2004). Este fenómeno no se observa durante G1, lo que sugiere que las cohesinas son necesarias para RH y no para NHEJ. Además, la carga de cohesinas promueve cohesión de forma independiente de replicación y es necesaria para recuperar un cromosoma irradiado pero no para recombinación ectópica. Estos resultados sugieren un papel de las cohesinas en SCR favoreciendo la interacción entre la molécula rota y su cromátida hermana (Figura 9). Por otro lado, se ha demostrado que las cohesinas son importantes para mantener el número de repeticiones en el ADNr, posiblemente favoreciendo el SCR igual frente al SCR desigual (Kobayashi et al., 2004).

Además de las cohesinas existen en la célula otros dos complejos con estructura SMC; las condensinas (Smc2-Smc4), necesarias fundamentalmente para la condensación cromosómica, y el complejo Smc5-Smc6, cuyo papel no está aún bien definido (revisado en (Losada and Hirano, 2005), ambos implicados de alguna manera en reparación de ADN (revisado en (Lehmann, 2005). El heterodímero Smc5-Smc6 es el centro de un gran complejo esencial, al que se unen al menos otras cuatro subunidades en *S. pombe* (Nse1-4) y seis en *S. cerevisiae* (Nse1-6) (Figura 8B). Los mutantes en componentes de Smc5-

Smc6 son sensibles a diversos agentes que dañan el ADN, y esta sensibilidad está relacionada con un defecto en RH (Lehmann et al., 1995). Además presentan problemas en la segregación del ADNr, y un defecto de crecimiento que es sinergístico con mutaciones en genes necesarios para la resolución de intermediarios de recombinación, y que se atenúa en *rad52* (Torres-Rosell et al., 2005). Por último, se ha observado que un mutante *smc6* está afectado en SCR desigual inducida por daño (Onoda et al., 2004).

B. El complejo MRX.

Como ya hemos mencionado anteriormente, el complejo MRX se une a los extremos de un DSB poco tiempo después de su formación, y participa en su reparación coordinando las actividades de NHEJ y RH, así como el checkpoint de daño de ADN (revisado en (Haber, 1998a). De acuerdo con un papel general en reparación de DSBs, los mutantes nulos *mre11*, *rad50* y *xrs2* de *S. cerevisiae* son sensibles a radiación y MMS (Ajimura et al., 1993; Game and Mortimer, 1974; Ivanov et al., 1992). Dicha sensibilidad es sólo evidente durante S y G2, lo que sugiere que se debe fundamentalmente a un defecto en RH (Bressan et al., 1999).

A pesar de esta sensibilidad, en mutantes MRX la recombinación alélica y ectópica inducida por daño apenas está afectada y la espontánea incluso aumenta de 5 a 10 veces (Ajimura et al., 1993; Ivanov et al., 1992; Malone and Esposito, 1981; Malone et al., 1990). Este fenotipo de hiper-recombinación podría explicarse si MRX actuara específicamente en SCR, basándose en la idea de que la ausencia de SCR canalizaría la mayor parte de las lesiones en el ADN hacia reparación por recombinación alélica o ectópica (ver (Haber, 1998a)). De hecho se ha observado un papel de MRX en SCR; las frecuencias genéticas de SCR desigual, tanto a nivel espontáneo como inducido por daño, bajan alrededor de 2 veces en mutantes nulos de MRX (Bressan et al., 1999; Dong and Fasullo, 2003); y molecularmente se ha observado una bajada similar en SCE inducido por DSB en mutantes *mre11* (Gonzalez-Barrera et al., 2003).

Un posible papel específico de MRX en SCR podría estar relacionado con la estructura de Rad50, que es similar a las proteínas SMC (Figura 8C), y por lo tanto podría promover la interacción entre cromátidas hermanas facilitando

SCR (Figura 9). *In vitro*, complejos Rad50-Mre11 son capaces de unir dos moléculas de ADN a través de interacciones entre unos motivos "ganchos de zinc" (zinc-hook) (Anderson et al., 2001; de Jager et al., 2001; Hopfner et al., 2002), que sería análogo al hinge de las proteínas SMC. *In vivo*, un mutante *rad50* sin el gancho presenta el mismo defecto de reparación que el mutante nulo, a pesar de que se mantiene la estructura del complejo, y estos defectos se suprimen promoviendo artificialmente interacción entre espirales (Hopfner et al., 2002; Wiltzius et al., 2005). Otra posibilidad no excluyente es que el papel estructural de MRX en reparación sea mantener los dos extremos de los DSBs juntos (Figura 9), como sugiere el hecho de que los dos lados de un DSB permanezcan juntos en células silvestres, pero no así en ausencia de MRX o en mutantes *rad50* sin gancho (Clerici et al., 2006; Lobachev et al., 2004). Finalmente, el efecto de MRX en SCR podría estar, al menos en parte, mediado por las cohesinas, ya que su carga inducida por DSB está afectada en mutantes *mre11* (Strom et al., 2004; Unal et al., 2004).

2- OBJETIVOS

Nos planteamos como objetivo central de esta tesis doctoral el estudio de la SCR como principal mecanismo de reparación postreplicativa. Para ello desarrollamos los siguientes objetivos concretos:

1. Obtener un sistema que nos permita tanto inducir DSBs específicamente durante replicación, como estudiar su posterior reparación por SCR. Decidimos caracterizar en detalle el sistema pRS316-TINV, previamente desarrollado en este laboratorio, que permite la inducción y el análisis molecular de SCE (Gonzalez-Barrera et al., 2003):

   A. En cuanto a la formación de DSBs.

   B. En cuanto a los mecanismos de reparación.

2. Determinar la contribución de RH y NHEJ a la reparación de DSBs replicativos.

3. Determinar los requerimientos genéticos de la reparación de DSBs replicativos, tanto por SCE como por otros posibles mecanismos de RH.

4. Analizar el papel del complejo MRX en SCR. Nos planteamos determinar si MRX actúa específicamente en SCR, lo que estaría de acuerdo con un papel estructural del complejo uniendo la molécula rota a su cromátida hermana; o por el contrario desempeña una función general en RH. Para esto decidimos:

   A. Analizar SCE en comparación con otros mecanismos de RH en mutantes nulos de MRX.

   B. Analizar el papel de la actividad nucleasa de MRX en SCE, así como su relación con Exo1.

5. Determinar otras funciones que actúen específicamente en SCR. Para esto analizamos el papel en SCE, en comparación con otros mecanismos de RH, de:

A. Cohesinas

B. Complejo Smc5-Smc6.

Por último decidimos analizar otro aspecto intrigante de la RH, como es el hecho de que ocurra recombinación en fondo *rad51*Δ, y por lo tanto en ausencia de intercambio de cadena. Para esto caracterizamos a nivel genético y bioquímico un mutante puntual *rad52-L89F*, que se había aislado previamente en nuestro laboratorio como afectado específicamente en RH independiente de Rad51.

3- ARTÍCULOS

Artículo 1

Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange

Felipe Cortés-Ledesma y **Andrés Aguilera**

En revisión, *EMBO Reports*

Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange

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Running Title: Repair of replication-born DSBs by SCE

ABSTRACT

Molecular studies on DSB repair in mitosis are usually performed with enzymatically induced DSBs, but spontaneous DSBs may arise because of replication failures, as could be the case when replication encounters single-strand nicks. To study repair of replication-born DSBs we defined a system in *Saccharomyces cerevisiae* for the induction of single-strand nicks. We show that a minimal 21-bp HO-site is cleaved at only one strand by the HO endonuclease, the resulting nick being converted into a DSB by replication during the S phase. Repair of such replication-born DSBs occurs primarily by *RAD52*-dependent sister-chromatid exchange (SCE), although non-homologous end joining also contributes to this repair. Importantly, we provide molecular evidence that cohesins are required for efficient repair of replication-born DSBs by SCE, as determined in *smc3* mutants, but not for other recombinational repair pathways. This work opens new perspectives to understand the importance of ssDNA nicks as a source of recombination and the relevance of cohesion in the repair of replication-born DSBs.

Key words: Cohesins; DSB repair; Homologous recombination; Sister-chromatid exchange

INTRODUCTION

Double-strand breaks (DSBs) can be repaired by either homologous recombination (HR) or non-homologous end-joining (NHEJ). HR with the sister chromatid (sister-chromatid exchange, SCE), which results in perfect accurate repair, has been shown to be the preferred pathway for the repair of induced DSBs (Gonzalez-Barrera et al., 2003; Johnson and Jasin, 2000; Kadyk and Hartwell, 1992). Nevertheless, the relative incidence of HR and NHEJ varies through the cell cycle. Thus, during G1, HR is practically absent, and DSB-repair occurs primarily by NHEJ, whereas during the S and G2 phases HR is proficient and the efficiency of NHEJ decreases (Ira et al., 2004).

Although *in vivo* studies on DSB-repair in mitosis have been performed with enzymatically induced DSBs, experimental evidence linking HR to replication suggests that DSBs may occur naturally during replication, presumably as a consequence of replication-fork progression impairment (Branzei and Foiani, 2005; Michel et al., 2001). DSBs can originate when replication forks encounter single-strand nicks (Kuzminov, 1999), which are frequently occurring DNA lesions (Lindahl, 1993). It would therefore be important to have a system for the *in vivo* induction of replication-dependent DSBs to understand the role of HR, and in particular SCE, in DSB repair.

Cohesins are responsible for maintaining sister chromatids together after replication, until the anaphase onset, ensuring proper chromosome segregation (Nasmyth, 2002). Interestingly, several observations point to a role of cohesins in DSB repair: the sensitivity of cohesin mutants of different organisms to DNA damaging agents (Lehmann, 2005; Sonoda et al., 2001); the requirement of sister-chromatid cohesion for recovery of chromosome integrity after X-ray irradiation (Sjogren and Nasmyth, 2001), and the loading of cohesins at sites of DNA damage (Kim et al., 2002;

Strom et al., 2004; Unal et al., 2004). However, whether or not the role of cohesins in DNA repair is specific for SCE is not known.

The purpose of this study was to define an *in vivo* system that mimicked a natural scenario for the origin of DSBs in dividing cells, that is, single-strand DNA nicks that are converted into DSBs during replication, and that could permit physical analysis of their repair. We show that, at a 21-bp HO site, the HO endonuclease primarily causes nicks that become DSBs during S phase in a replication-dependent manner. Such DSBs are repaired preferentially by Rad52 dependent SCE, although NHEJ also contributes, and cohesins are necessary for repair of these replication-born DSBs by SCE, but not by other forms of HR.

RESULTS

A system to induce nicks *in vivo*

Using plasmid substrates containing a suboptimal 21-bp long HO-cleavage site, whose cleavage efficiency was below 10%, we recently demonstrated in the yeast *S. cerevisiae* that DSBs are primarily repaired by recombination with the sister chromatid in dividing cells (Gonzalez-Barrera et al., 2003). The question raised here was if the high levels of SCE could indicate that DSBs at the 21-bp HO site appeared specifically during the S-phase of the cell cycle, due to nicks converted into DSBs by replication. To assay whether the 21-bp HO site was nicked at one DNA strand rather than cleaved at both strands, the kinetics of formation of DSBs and ssDNA nicks were analyzed in plasmid pRS316-TINV by native and alkaline gel electrophoresis, respectively. Cleavage products were detected by the specific 1.4 and 2.4-kb fragments obtained in *Xho*I-*Spe*I digested plasmids (Fig 1A). The analysis was made in asynchronous or G1-arrested yeast cultures expressing HO under the control of the *GAL10* promoter. As can be seen in Fig 1B, the number of plasmids containing a nick in asynchronous cultures accumulated at faster rates and to levels approximately double those of DSBs in 2% galactose (above 9% of the plasmids contained a single strand nick at 2h of HO induction whereas only 4% accumulated a DSB). In cells arrested in G1 with a factor, nicks accumulated with the same kinetics and reached the same levels as in asynchronous cultures (above 9% of total DNA molecules contained a nick after 2 h), whereas DSBs accumulated at levels below 1% of the total molecules. These results suggest that the 21-bp HO site was preferentially nicked at one strand. Southern analysis with specific single-stranded DNA probes revealed that both strands were

cleaved indistinctly, although the Watson DNA strand showed a slight cleavage preference (Fig 2).

Replication converts nicks into DSBs

To test the possibility that nicks are converted into DSBs after replication we determined the kinetics of DSB formation in G1-arrested cells after different times of release from arrest, both in the absence and in the presence of hydroxyurea (HU), which delays DNA replication by depleting the intracellular dNTP pools. We confirmed by FACS analysis that 50mM HU was sufficient to inhibit replication significantly, as cells entered S-phase synchronously and much later than in the absence of HU (Fig 3A). Southern analysis after a-factor release revealed that in the absence of HU, DSBs accumulated with the same kinetics and to the same levels of asynchronous cultures (6% of total molecules contained a DSB 2 h after release). However, the kinetics of DSB formation was clearly delayed in the presence of HU (Fig 3B): while in the absence of HU an increase in DSBs was evident 1 h after a-factor release, in the presence of HU induction was observed at 2h, the levels of DSB-containing molecules being below 5% even 4h after release. As expected, no further accumulation of DSBs was observed in G1-arrested cells in which a factor was present all the time. These results indicate that DSBs are formed at the 21-bp HO site after the replication fork encounters the nick, providing a unique system to study the role of SCE in the repair of replication-dependent DSBs that are initiated by a single-strand nick.

Repair of replication-born DSBs by HR and NHEJ

Since this HO site is located in a 0.6-kb *leu2* inverted repeat, the system permits the study of DSB repair efficiency by SCE (Gonzalez-Barrera et al., 2003). As can be seen

in Fig 4A, 2.9-kb and 4.7-kb bands in *Spe*I-*Xho*I digested plasmids appear as the result of unequal SCE events, which can be used as an estimate of total SCE. While other recombination events can result in a 2.9-kb band, the appearance of a 4.7-kb fragment is specific for SCE, and is therefore used as an indicator of SCE events (Gonzalez-Barrera et al., 2003). In order to evaluate the relevance of HR and NHEJ in the repair of replication-born DSBs, we analyzed SCE levels in wild-type, HR-deficient *rad52Δ* and NHEJ-deficient *yku70Δ* cells. As shown in Fig 4B, DSBs accumulated with similar kinetics and at similar levels in wild type, *rad52Δ* and *yku70Δ* strains. In contrast, SCE intermediates, while reaching 2% levels after 6 h of HO induction in 2% galactose, were below detection in *rad52Δ* cells. This result indicates that, as expected, repair of a replication-born DSBs occurs by HR. Interestingly, SCE is modestly increased in *yku70Δ* cells, suggesting that a minor but significant fraction of replication-born DSBs are repaired via NHEJ in wild-type cells, as DSBs produced during G1 and left unrepaired in NHEJ-deficient cells, cannot result in SCE after replication because both chromatids would be cleaved.

Cohesins are required specifically for SCE

Knowing the role of cohesins in holding sister chromatids together after replication (Nasmyth, 2002), we wondered whether cohesins were specifically required for SCE. We determined the effect of a cohesin-subunit thermosensitive allele, *smc3-42*, on the repair by SCE of replication-born DSBs induced at the 21-bp HO site in plasmid pRS316-TINV. As can be seen in Fig 5, the kinetics of DSB accumulation in *smc3-42* cells at the restrictive temperature was the same as in wild type, reaching values of molecules containing DSBs of 6-8% after 6h of HO induction in 2% galactose. Remarkably, the kinetics of accumulation and the overall levels of SCR intermediates,

as indicated by the 4.7-kb band, were severely reduced with respect to the wild type. Thus, whereas almost 1% of total DNA molecules underwent a SCE event in wild-type cells, this value was approximately 0.2% in *smc3* cells. In contrast, the 2.9-kb band, which can also arise by intra-chromatid recombination (F. C.-L. and A. A., unpublished), is still clearly observed in *smc3*, suggesting that cohesins do not act in all types of recombination.

DISCUSSION

We provide an *in vivo* functional assay for the specific production of single-stranded DNA nicks that are converted into DSBs after DNA replication and for the analysis of their repair by SCE. Our 21-bp HO assay provides a useful experimental system for mimicking a natural scenario for the origin of DSBs in dividing cells, in which DSBs and other recombinogenic DNA lesions appear as a consequence of DNA replication failures (Branzei and Foiani, 2005; Michel et al., 2001). We show that these replication-born DSBs are efficiently repaired by Rad52-dependent SCE, even though a fraction may also be repaired by NHEJ. Interestingly, such repair by SCE strongly depends on cohesins, which do not play a role in other types of recombination.

One relevant feature of the 21-bp HO site is that the two ends of the DSB are detected. This is similar to the results obtained with an *E. coli* system in which the single-strand DNA cleavage site of the M13 phage DNA was inserted in the lambda phage chromosome (Kuzminov, 1999). Formation of double-ended DSBs after replication through a nick could be explained in two ways, depending on whether the nick is encountered at the lagging or at the leading strand (Fig 6). In the first case (Fig 6, left), the replication fork could traverse the nick leaving a DSB behind the DNA polymerase in one sister molecule. In the second case (Fig 6, right), a replication fork coming from the other side would lead to a double-ended DSB.

It has been previously shown that induction of a nick in *S. cerevisiae* by the Gene II protein from bacteriophage *f1* stimulates recombination (Strathern et al., 1991). The fact that this increase was only observed in cycling cells (Galli and Schiestl, 1998), together with the pattern of recombination products were consistent with events initiated by DSBs. Nevertheless, neither formation of DSBs nor any repair intermediate has been

analyzed at the molecular level in this system. Nicks can also be produced by the DNA topoisomerase I inhibitor campthotecin. In this case, topoisomerase remains bound to the DNA and could become a physical obstacle for the DNA polymerase. As a consequence, replication through such nicks leads to one-ended DSBs (Avemann et al., 1988; Saleh-Gohari et al., 2005; Strumberg et al., 2000). It is less likely that these one-ended DSBs are converted into two-ended DSBs by a replication fork coming from the opposite direction, because such a fork is likely to encounter another topoisomerase I cleavage complex in its way.

DNA-end resection has been shown to be a major determinant influencing the choice between HR and NHEJ as DSB-repair pathways (Frank-Vaillant and Marcand, 2002; Ira et al., 2004). Thus, unresected DSB ends are primarily repaired by NHEJ, whilst once resected, a DSB can only be repaired by HR (Frank-Vaillant and Marcand, 2002). As expected, HR is involved in the repair of replication-dependent DSBs, which we show that occurs efficiently by SCE in a Rad52-dependent manner. However, SCE increases in a *yku70Δ* mutant, consistent with previous reports (Fasullo et al., 2005), suggesting that NHEJ is not restricted to G1, and contribute to the repair of DSBs formed during replication. Therefore, even in resection proficient stages of the cell cycle (Ira et al., 2004), transient stability of DNA ends allows repair by NHEJ.

Since cohesins hold sister chromatids after replication, their involvement in DNA repair but not in ectopic recombination has led to the idea that their role in DSB repair would be to attach a broken chromatid to its sister to favor repair (Kim et al., 2002; Sjogren and Nasmyth, 2001; Sonoda et al., 2001; Strom et al., 2004; Unal et al., 2004). Molecular evidence for cohesins directly participating in DSB repair comes from the observation that are loaded onto DNA in response to a DSB (Strom et al., 2004; Unal et al., 2004). Our system has allowed us to demonstrate that in a cohesin-subunit

mutant, such as *smc3-42*, repair of replication-born DSBs by SCE is impaired, while no defect in general recombination is observed. This suggests that cohesins loaded at the vicinity of a DSB may ensure that recombinational repair takes place using as donor the sister chromatid. A role for cohesins in preventing instability of the rDNA repeats by favoring SCE has also been proposed (Kobayashi et al., 2004). So far, physical measurement of SCE in linear chromosomes is not possible, but the fact that cohesins also act in circular plasmids (Ivanov and Nasmyth, 2005) and that recombination mechanisms do not depend on the linear or circular nature of the DNA molecule (Paques and Haber, 1999), suggest that our results may be valid for chromosomes.

In summary, in addition to provide a molecular analysis of repair of replication-born DSBs, this study should open new perspectives for understanding how DNA breaks can be formed and repaired during replication, the most natural scenario.

METHODS

Strains, plasmids and oligonucleotides

Strains used were isogenic to W303-1A. The wild-type WS strain (*MAT***a**-*inc ade2-1 his3-11,15 trp1-1 ura3-1 ade3::GAL-HO leu2Δ::SFA1*) was previously described (Gonzalez-Barrera et al., 2003) and *rad52Δ::KanMX4, yku70Δ:: HphMX4* and *bar1Δ:: HphMX4* alleles were obtained by gene replacement following PCR transformation. Deletions were confirmed by PCR and Southern analyses. The *smc3-42*, kindly supplied by K. Nasmyth, was introduced in the WS genetic background by genetic cross. Plasmid pRS316-TINV was described previously (Gonzalez-Barrera et al., 2003). The 25-mer oligonucleotides used to insert the 21-bp HO site into the *LEU2 Eco*RI site were AATTTCAGCTTTCCGCAACAGTATA (HO.1) and AATTTATACTGTTGCGGAAAGCTGA (HO.2).

Analysis of HO cleavage

WS-bar1 (*bar1Δ*) cells, used as wild-type controls, were grown at 30°C in SC-ura with 2% raffinose as carbon source to an OD of 0.4. The *bar1Δ* alelle was used to enhance the effect of a factor. The culture was split in two halves, one of which was supplemented with 0.2 μM a-factor to arrest cells at G1. After 4 hours, 2% galactose was added to induce HO expression. For a-factor release, cells were washed twice with pre-warmed fresh medium without a factor and 50 μg/ml Pronase (SIGMA, USA) was added to remove any trace of a factor. HO cleavage was recurrent in the experimental conditions used, as cells were maintained in 2% galactose after a-factor release. If 2% glucose was added, nicks were quickly repaired and DSBs did not form (data not shown). DNA was isolated from yeast strains, digested, electrophoresed in native (TAE;

40 mM Tris, 20 mM acetic acid, 1 mM EDTA) or alkaline (50 mM NaOH, 1 mM EDTA) conditions, and analyzed by Southern using a *Cla*I-*Eco*RV 600-bp *LEU2* probe. For analysis of strand specificity of nicks, strand-specific probes were obtained by primer extension of a *LEU2* fragment using GTTCCACTTCCAGATGAGGC (Crick) and TAACGGAGGCTTCATCGGAG (Watson) oligos. Southerns were quantified with a Fuji FLA3000 as described (González-Barrera *et al.* 2003). In all cases a representative experiment of 3-4 performed is shown.

Analysis of SCE

It was performed as described (González-Barrera *et al.* 2003).

Acknowledgments

We thank F. Prado and R. Wellinger for reading the manuscript, and D. Haun for style supervision. This work was supported by grants from the Ministry of Science of Education of Spain (SAF2003-00204) and Junta de Andalucía (CVI102). F. C.-L. was the recipient of a pre-doctoral training grant from the Spanish Ministry of Health.

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FIGURE LEGENDS

Fig 1

Breaks at the 21-bp HO site are primarily single-strand nicks. (**A**) Scheme of plasmid pRS316-TINV before and after the HO-induced DSB. Lines with asterisks indicate the *LEU2* probe used for hybridization experiments. (**B**) Kinetics of HO cleavage as assayed by native and alkaline gel electrophoresis of DNA from asynchronous (ASYNC) and G1-arrested cultures of WS-bar1 strains. DNA samples were taken at the indicated times after HO induction and cut with *Xho*I and *Spe*I before electrophoresis. FACS pattern of cultures and quantification data of nicks and DSBs are shown. The percentage of plasmids containing nicks was calculated as twice the difference between the number of broken molecules observed in the alkaline and native gels and normalized with respect to the total DNA fragments. Plasmids containing DSBs were determined directly from the native gels.

Fig 2

Nicks are not specifically produced at one strand. Nicking efficiency at each DNA strand determined by hybridization of DNA from G1-arrested cells with *LEU2* single-stranded specific probes. For other details see Fig 1.

Fig 3

HO-induced DSBs at the 21-bp HO site are formed after DNA replication. (**A**) FACS analysis of the WS-bar1 cells arrested in G1 with a-factor and at different times after release. (**B**) Kinetic of DSB formation after release from G1 arrest as determined by Southern analysis. After 2 h of HO induction in galactose (Gal), WS-bar1 G1-arrested

cells were released from a-factor in the presence or absence of 50 mM HU. Quantification data are plotted at the bottom. For other details see Fig 1.

Fig 4

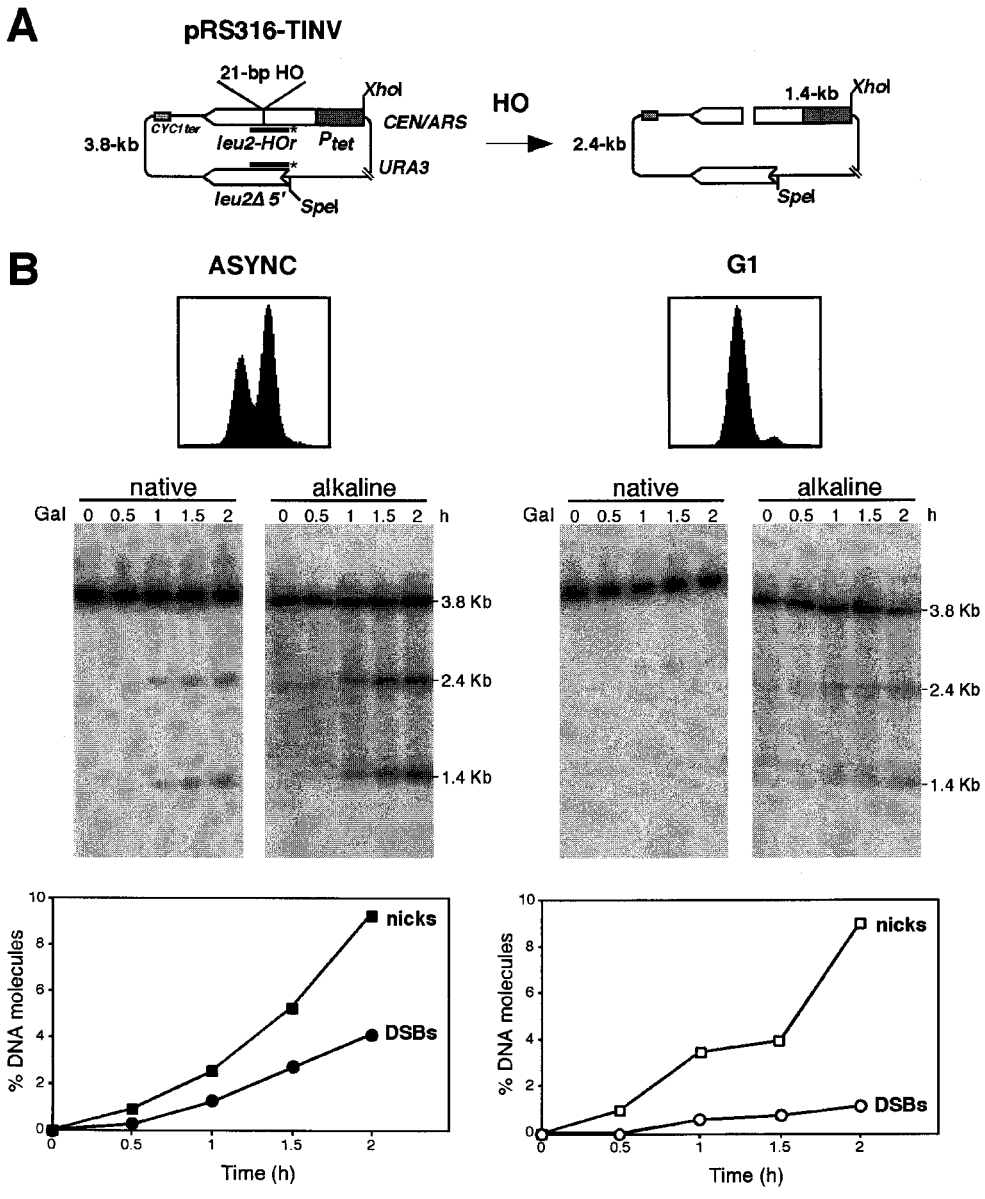
Role of HR and NHEJ in the repair of replication-born DSBs. (**A**) Schemes of plasmid pRS316-TINV and the intermediate produced by unequal SCE. Note that gene conversions are not detected in these assays. (**B**) Kinetics of DSB formation and SCE intermediates in isogenic wild-type, *rad52Δ* and *yku70Δ* strains. The ratio between the 4.7-kb band and the total plasmid DNA determined the levels of SCE. For other details see Fig 1.

Fig 5

Smc3 cohesin subunit is required for efficient SCE. Kinetics of DSB formation and SCE intermediates in isogenic wild-type and *smc3-42* strains. Cultures were incubated at 37°C for 30 minutes prior to HO induction in order to inactivate cohesins. Differences in wild-type overall levels of events from Fig 4 are due to the incubation temperature. For other details see Fig 1.

Fig 6

Model to explain the role of cohesins in the repair of DSBs formed after replication forks encounter single-strand DNA nicks. Double-ended DSBs could be formed after replication through a nick in two ways depending on whether the nick is encountered at the lagging (left) or the leading strand (right). Cohesins (ovals), which are additionally loaded in the proximity of the DSB, favor HR with the sister.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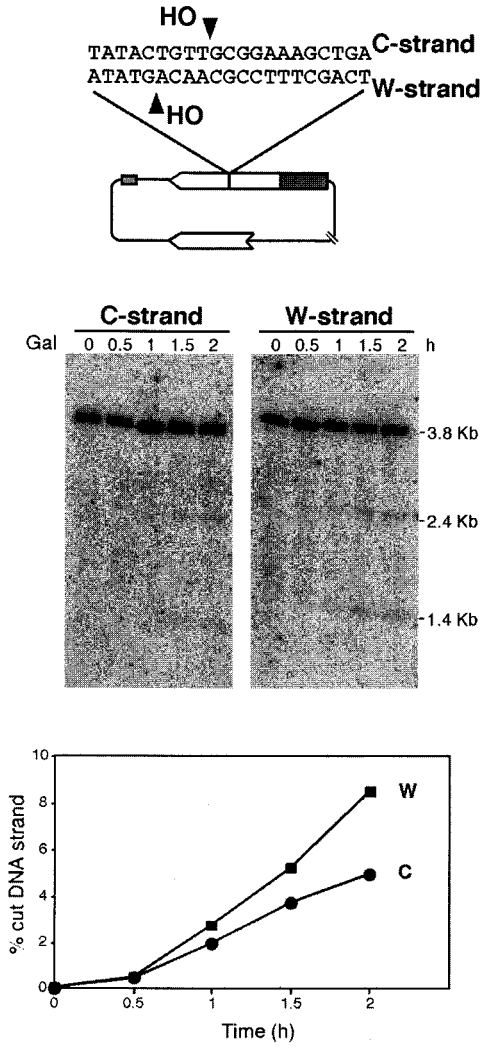
XhoI
CEN/ARS
3.8-kb
CYC1 ter
leu2-HOr
P tet
URA3
leu2Δ 5'
SpeI
HO
1.4-kb
XhoI
2.4-kb
SpeI
B
ASYNC
G1
native
alkaline
native
alkaline
Gal 0 0.5 1 1.5 2 h
0 0.5 1 1.5 2 h
Gal 0 0.5 1 1.5 2 h
0 0.5 1 1.5 2 h
3.8 Kb
2.4 Kb
1.4 Kb
3.8 Kb
2.4 Kb
1.4 Kb
% DNA molecules
nicks
DSBs
Time (h)
% DNA molecules
nicks
DSBs
Time (h)

Figure 1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C-strand
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W-strand
HO
C-strand
W-strand
Gal
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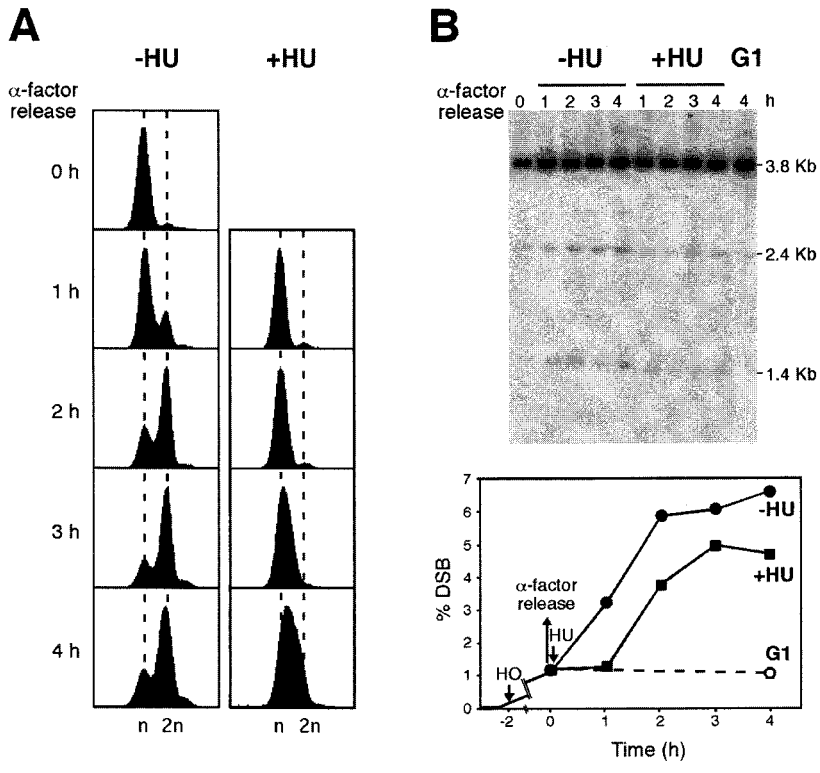
2.4 Kb
1.4 Kb
% cut DNA strand
Time (h)
W
C

Figure 2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+HU
α-factor release
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1 h
2 h
3 h
4 h
n 2n
n 2n
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+HU
G1
α-factor release
0 1 2 3 4 1 2 3 4 4 h
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1.4 Kb
% DSB
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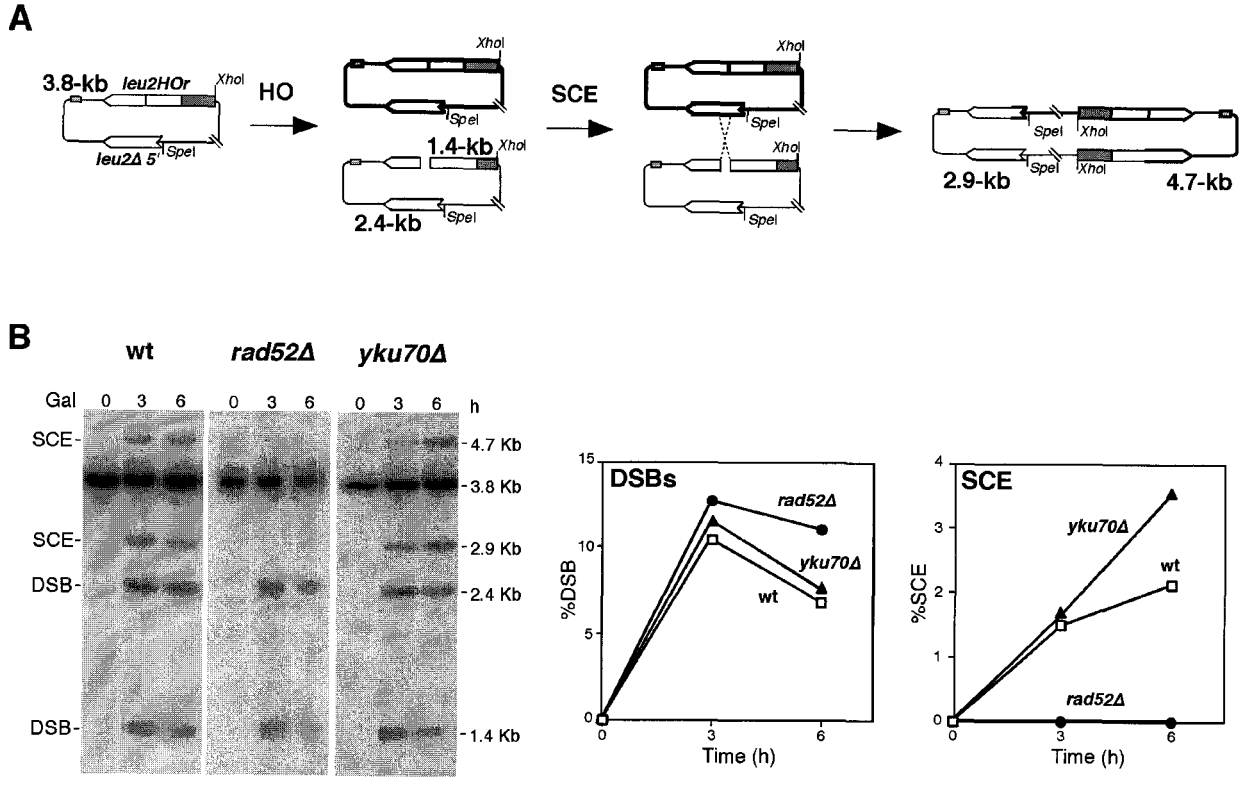
+HU
G1
α-factor release
HU
HO
Time (h)

Figure 3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SpeI
SCE
XhoI
SpeI
XhoI
SpeI
SpeI
XhoI
2.9-kb
4.7-kb
B
wt
rad52Δ
yku70Δ
Gal 0 3 6 0 3 6 0 3 6 h
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SCE-
DSB-
DSB-
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2.9 Kb
2.4 Kb
1.4 Kb
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Time (h)
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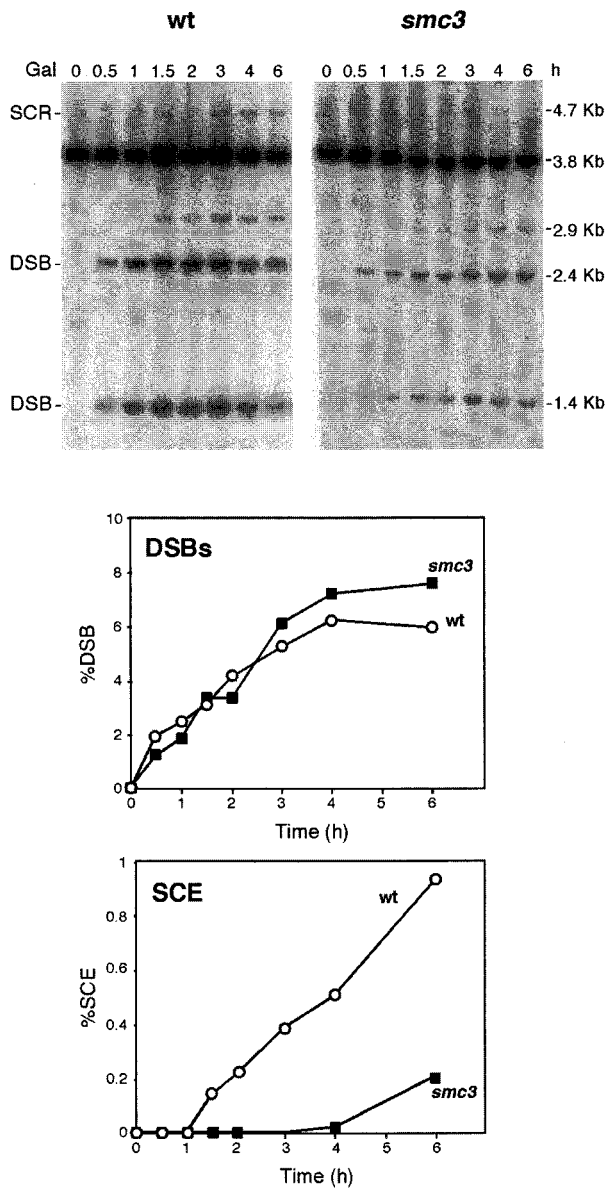
Time (h)

Figure 4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SCR
DSB
DSB
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2.4 Kb
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SCE
wt
smc3
%SCE
Time (h)

Figure 5

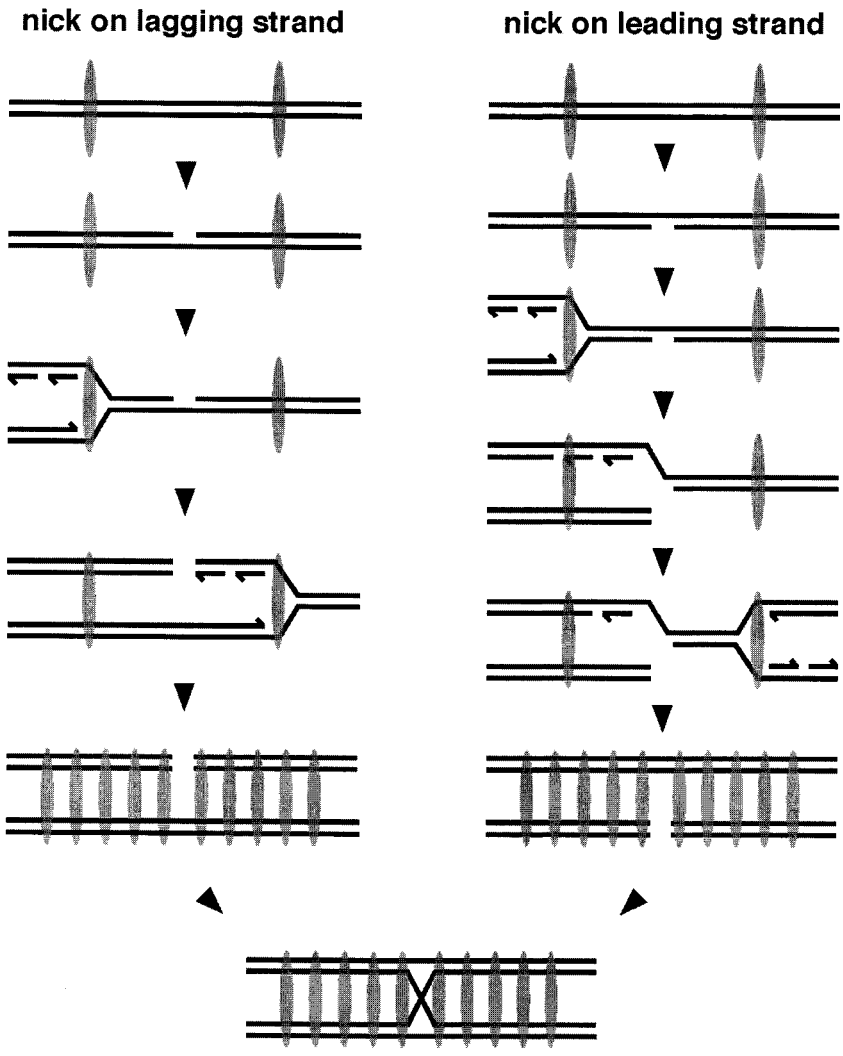

Figure 6

Artículo 2

New roles for Mre11 nuclease activity, Sae 2 and Exo1 in the repair of replication-born double-strand breaks

Felipe Cortés-Ledesma y **Andrés Aguilera**

Enviado, *EMBO Journal*

New roles for Mre11 nuclease activity, Sae2 and Exo1 the repair of replication-born double-strand breaks

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Abstract

Homologous recombination (HR) is the major mechanism used to repair double-strand breaks (DSBs) that result during replication. Even though HR has been extensively investigated, a study of the repair of DSBs specifically induced during S-phase is lacking. Using a minichromosome assay in which DSBs are generated by the encountering of the replication fork with a single-strand DNA nick, we are able to detect repair by sister-chromatid exchange (SCE) and intra-chromatid break-induced replication (IC-BIR) between two inverted repeats. Our study reveals that SCE is strongly dependent on the recombinational repair proteins Rad51, Rad54 and Rad59, whereas the Rad54 paralog Rdh54/Tid1 has no effect. IC-BIR, instead, is stimulated in *rad51*Δ and *rad54*Δ cells, unaffected in *tid1*Δ*/rdh54*Δ and abolished in *rad59*Δ cells. Null mutations of the MRX complex (*mre11*Δ, *rad50*Δ and *xrs2*Δ), significantly increased DSB-end stability and reduced both SCE and IC-BIR. Importantly, *mre11-H125N* and *sae2*Δ, inactivating the nuclease activity of MRX, reduced both SCE and IC-BIR. Instead, *exo1*Δ did not have any effect *per se*, but increased the stability of DNA ends if combined with *mre11-H125N*. Therefore, in addition to providing a detailed molecular analysis of the genetic basis of the repair of replication-born DSBs in yeast, this study provides molecular evidence for an *in vivo* role of the nuclease activity of Mre11, together with Sae2 and Exo1 in mitotic DSB-repair.

Introduction

Double-strand break (DSB) repair must be highly efficient in all organisms; and indeed, a single unrepaired DSB has been shown to be lethal to *Saccharomyces cerevisiae* {Resnick, 1976 #44}. In dividing cells, homologous recombination (HR) is thought to be the main mechanism to repair DNA breaks that arise during replication, as supported by the observations that recombination proteins only form *foci* during S phase {Lisby, 2001 #13} and that in G1 cells HR is practically absent {Aylon, 2005 #52; Ira, 2004 #39}. HR has also been proposed as playing a role in restarting stalled or collapsed forks {Cox, 2000 #35; Michel, 2001 #15}.

HR needs an intact homologous DNA sequence as a template for repair. Depending on the nature and location of the donor used, the outcome can be different {Paques, 1999 #45}. As soon as a sequence is replicated (during S and in G2), any damage in one chromatid can be repaired by sister chromatid recombination (SCR). Since the sister chromatids are identical, repair by SCR is accurate. DSBs can also be repaired using as donor ectopically located homologous sequences (ectopic recombination), or, in diploids, the allelic copy of the homologous chromosome (allelic recombination), in which cases repair can result in loss or reorganization of genetic information. The sister chromatid has been shown to be the preferred substrate for recombinational DSB repair {Gonzalez-Barrera, 2003 #38; Johnson, 2000 #37; Kadyk, 1992 #36}.

SCE can be detected cytologically in mammalian cells {Sonoda, 1999 #46} but not in yeast. Nonetheless, cytological studies are insufficient to provide a mechanistic scenario for SCR. As recombination between sisters occurs between two identical DNA sequences generated by DNA replication, its genetic and molecular analysis is difficult. To overcome this problem, SCE has been generally studied as unequal recombination events occurring between intrachromosomal heteroalleles {Fasullo, 2001 #7; Fasullo, 1987 #47; Johnson, 2000 #37; Kadyk, 1992 #36}. Given the low frequency of spontaneously occurring HR events, many studies have been based on mitotic recombination events induced by DNA damaging agents or by sequence-specific endonucleases. The extensively used endonuclease HO of *S. cerevisiae,* which

cuts at a specific 117-bp sequence (HO site), has become an excellent tool to study DSB recombinational repair {Paques, 1999 #45}. Nonetheless, given the high efficiency of cleavage produced by HO, after replication the two duplicated 117-bp HO sites in both chromatids are cleaved, impeding their repair by SCE. To overcome this problem, we have recently reported that HO-induced DSBs made at a 21-bp HO-cleavage site occur with efficiency below 10%. Based on this mini HO-site we have developed new substrates for the specific *in vivo* analysis of SCE in circular minichromosomes {Gonzalez-Barrera, 2003 #38}.

The steps and proteins involved in HR are well defined in *S. cerevisiae* {Sung, 2000 #95; Symington, 2002 #29}. HR starts with a 5'-resection of the ends of the DSB, a process in which the MRX complex (Mre11, Rad50 and Xrs2) is involved. However, other still unidentified functions must participate at this step, since in null MRX mutants mitotic HR is only slightly affected. Moreover, nuclease deficient *mre11* mutations are MMS and radiation sensitive but show no evident resection defect. Sae2 seems to control the nuclease activity of the complex, as *sae2*Δ and nuclease deficient MRX mutants display the same phenotypes {Prinz, 1997 #87; Rattray, 2001 #88; Lisby, 2004 #89; McKee, 1997 #102}. A candidate protein to function during resection is Exo1, a 5'-3' exonuclease that displays several genetic interactions with MRX {Moreau, 2001 #30; Symington, 2000 #31; Fiorentini, 1997 #34; Tsubouchi, 2000 #93}. Rad52, probably in concert with Rad59, promotes the invasion of the resection-generated 3'-OH single-stranded ends in the homologous duplex DNA. This invasion is stabilized by strand exchange catalyzed by the RecA-homolog Rad51, together with Rad54, a member of the SNF-SWI family of ATPases, Rad55, and Rad57 {Sung, 2000 #95; Symington, 2002 #29}. Instead, the Rad54 paralog, Rdh54/Tid1, seems to act particularly in allelic recombination {Klein, 1997 #56; Shinohara, 1997 #57}. These steps are common to most known HR events, regardless of the pathway and repair template used. Nevertheless, some HR mechanisms may be less dependent on the formation of Rad51-dependent strand-exchange intermediates. Thus, break-induced replication (BIR), in which the invading end primes extensive DNA synthesis, can occur in the absence of Rad51 {Malkova, 1996 #48}, although Rad51 also participates in wild-type events {Davis, 2004 #28}. Also, the single-strand annealing (SSA) mechanism of DSB repair, responsible for most deletions occurring between

direct repeats {Fishman-Lobell, 1992 #50; Lin, 1990 #54} is Rad51 independent {Ivanov, 1996 #59}.

The purpose of this study was to establish the genetic and molecular bases of SCE initiated by replication-born DSBs in comparison with other forms of HR repair, such as intrachromatid recombination by break-induced replication (IC-BIR). Using circular minichromosomes in which DSBs arise by replication through single-strand DNA nicks induced by the HO endonuclease at a 21-bp HO site (F.C.-L. and A.A., submitted), we found that repair of DSBs occurs primarily by SCE and is dependent on Rad51, Rad59 and Rad54, whereas Rdh54/Tid1 has no effect. Instead, IC-BIR is a minor event stimulated in *rad51*Δ and *rad54*Δ cells, unaffected by *tid1*Δ/*rdh54*Δ and decreased in *rad59*Δ cells. In the null MRX mutants *mre11*Δ, *rad50*Δ and *xrs2*Δ, DSBs are stabilized and both SCE and IC-BIR are reduced. Importantly, the nuclease activity of Mre11, as well as Sae2, but not Exo1, is required for both SCE and IC-BIR. Interestingly, *exo1*Δ increased the stability of DNA ends if combined with the nuclease-minus *mre11-H125N* mutation. Altogether, these results provide molecular evidence that inversions between repeats, in addition to reciprocal exchange, may occur by BIR followed by SSA {Bartsch, 2000 #2; Malagon, 2001 #40} and provide experimental evidence for a role of the nuclease activity of MRX, as well as the Sae2 and Exo1 proteins, in mitotic repair of replication-born DSBs.

Results

An *in vivo* molecular assay for the analysis of the repair of replication-born DSBs via SCR and IC-BIR.

For the molecular detection of SCR we used the circular minichromosome pRS316-TINV containing two 1.2-kb *leu2* inverted repeats, one of which carried a 21-bp HO site (Figure 1A) in yeast strains containing the HO endonuclease under the control of the *GAL10* promoter. In this plasmid, cleavage at the HO site can be detected by the appearance of 2.4 and 1.4-kb fragment after *Sce*I-*Xho*I digestion. In a parallel study we provide evidence that at this mini HO-site, the HO endonuclease produces primarily ssDNA nicks that are converted into DSBs during replication (F.C.-L. and A.A, submitted). This produces the possibility of cleavage occurring in only one chromatid, leaving the other intact and competent for repair via SCR. As can be seen in Figure 1B, DSB-containing molecules accumulated at levels above 5% after 2 hours of HO induction in cycling cells, whereas such molecules remained below 1% in G1-arrested cells. Nevertheless, when most cells have completed replication after G1-arrest release, DSBs reached levels similar to those observed in asynchronous cultures (Figure 2B, S/G2).

The use of pRS316-TINV circular minichromosomes containing inverted repeats makes the study of SCE {Gonzalez-Barrera, 2003 #38 } possible as well as that of intrachromatid repeat recombination (ICR). As can be seen in Figure 2A, repair of the replication-born DSB at the mini-HO site in the pRS316-TINV plasmid, occurring with the sister chromatid either by reciprocal exchange SCE or by BIR, leads to 2.9 and 4.7-kb *Sce*I-*Xho*I fragments, whereas repair by intrachromatid BIR only leads to the 2.9-kb band and two type of fragments that can be as large as 1.4 and 3.3-kb if DNA synthesis reaches the end of the invaded template.

Southern analysis of the kinetics of repair after HO-induction could be used to determine the efficiency of SCE versus IC-BIR by determining the relative intensity of each specific fragment. As mentioned above, the 2.4 and 1.4-kb bands indicate DSBs (Figure 1A), the 4.7-kb band is a specific indicator of SCR events, while IC-BIR could be estimated by the difference between the

2.9 and 4.7-kb bands. Following this procedure, we found that the maximum peak of broken molecules was obtained after 3h of HO induction, after which DSBs started to decrease as a consequence of effective repair and processing of DSB ends (Figure 2B). SCR and IC-BIR intermediates were detectable after 1h, the levels of SCR being continuously increasing until reaching a plateau around 2%. Instead, IC-BIR intermediates peaked at 3h to levels of approximately 0.5% to later decrease to undetectable levels. Consistent with previous results, SCR is the major DSB recombinational repair pathway detected in these constructs, even when intrachromatid repeat recombination is possible {Gonzalez-Barrera, 2003 #38}.

It is important to notice that in these molecular assays, both SCR and IC-BIR require additional recombination events to produce a viable recombinant molecule. For SCR, an additional intramolecular reciprocal exchange or a SSA event is required to resolve the dicentric dimer generated by SCR, whereas in IC-BIR an SSA event is needed for healing the two free DNA ends generated by DNA synthesis {Bartsch, 2000 #2; Malagon, 2001 #40}. In this sense, one can see that whereas the SCR events remain stable with time, IC-BIR fragments disappear presumably due to DNA resection occurring during SSA (Figure 2B). The ability of our assay to study repair of replication-born DSBs by HR with either the sister chromatid or an ectopic repeat, makes a possibility of establishing the importance of partner choice (sister versus ectopic sequence), and the specific genetic requirements for each type of HR event.

A distinct and independent role of Rad51 and Rad59 in SCR and IC-BIR

Aimed at establishing the genetic basis of DSB-repair events specifically initiated during replication, we first determined the role that hallmark recombinational repair genes, such Rad51 and Rad59 have on SCR in comparison with IC-BIR. As can be seen in Figure 3A, the accumulation of DSBs after HO induction by galactose was pretty similar in *rad51*Δ versus wild-type cells. Recombination with the sister chromatid was dramatically decreased in *rad51*Δ, consistent with the idea that it occurs by a strand-exchange dependent mechanism, as it is SCE, the term to be used from now on. Instead, IC-BIR intermediates accumulate in *rad51*Δ at frequencies that double those of the wild type, consistent with the original observation that BIR events can also

occur in the absence of Rad51-mediated strand-exchange {Malkova, 1996 #48}. This result could be explained in two ways, either Rad51 is required for the preference for the sister chromatid in DSB repair, SCE events being channeled to ICR in *rad51*Δ mutants, or ICR events become evident in *rad51*Δ, as a higher proportion of them occur by BIR. Keeping in mind that in other DSB-induced recombination systems, BIR events can only be detected in *rad51*Δ background {Malkova, 1996 #48}, we favor the latter hypothesis. In this sense, it is worth noting that intermediates of either BIR (IC-BIR as detected in this study) or the recombination events occurring by the standard DSB-repair model are not different, the diversification of each pathway presumably occurring later if BIR extension is impeded by the barrier formed by Rad51-dependent invasion of the other DSB end {Aguilera, 2001 #58}.

*rad59*Δ had little impact on the overall accumulation of DSBs, but it caused a strong reduction in both SCE and IC-BIR (Figure 3A). The role of Rad59 in recombinational repair was observed previously in *rad51*Δ backgrounds {Bai, 1996 #41; Signon, 2001 #60} and in SSA {Davis, 2001 #61; Jablonovich, 1999 #62; Sugawara, 2000 #63}, in which Rad51 is not essential, in agreement with a role of Rad59 in Rad51-independent recombination. Our study provides evidence for an additional implication of Rad59 in Rad51-dependent DSB recombinational repair. Analysis of *rad51*Δ *rad59*Δ double mutants indicated that, whereas DSBs accumulated at similar levels as in *rad59*Δ cells, SCE was completely abolished (Figure 3B). Instead, *rad51*Δ was epistatic for IC-BIR, the IC-BIR intermediates at 4h of HO induction being only slightly lower in *rad51*Δ *rad59*Δ as compared to *rad51*Δ cells. In contrast, *rad52*Δ completely abolished all HR events (data not shown; González-Barrera et al. 2003). This unexpected result indicates that in the absence of Rad51, Rad59 contribution to IC-BIR is minor, whereas Rad59 is essential for SCE in the absence of Rad51.

Rad54 but not its paralog Tid1/Rdh54 is required for SCE

Two DNA-dependent ATPases of the SNF-SWI family of proteins are involved in DSB repair, although a direct role in Rad51-mediated HR has been only observed for Rad54 {Petukhova, 1998 #65; Rattray, 1995 #64}. We took advantage of our assays to test whether Tid1/Rdh54 had a specific role in

recombinational repair of replication-born DSBs as observed for Rad59. As expected from the known activity of Rad54 in Rad51-dependent strand exchange, identical results to *rad51*Δ were found for DSB accumulation, SCE and IC-BIR events in *rad54*Δ cells (Figure 4). *tid1*Δ/*rdh54*Δ mutants showed a slightly higher accumulation of DSBs than wild-type cells, a modest decrease in SCE and only a delay in IC-BIR events (Figure 4). These results are in agreement with a role of Tid1 in HR different to that of its paralog Rad54, and indicate that Tid1/Rdh54 does not play an essential role by itself in SCE or IC-BIR. Furthermore, in contrast to *rad54*Δ and *rad51*Δ, *tid1*Δ does not cause better efficiency of IC-BIR events, consistent with the idea that Tid1 does not participate in the formation or stabilization of Rad51 nucleoprotein filaments that could interfere with BIR events {Aguilera, 2001 #58}.

Molecular analysis of the role of MRX in SCE and IC-BIR

The MRX complex is required for the initial processing steps of the DSB ends. Although its role in HR *in vivo* is not easily detected (MRX mutants do not diminish the frequency of mitotic heteroallelic recombination) we have recently reported that *mre11*Δ significantly reduces DSB-induced SCE (González-Barrera et al. 2003). Here we extended this study to all components of the MRX complex and compared the effect of each null mutation on SCE with respect to its effect on IC-BIR (Figure 5A). The three mutations, *mre11*Δ, *rad50*Δ and *xrs2*Δ caused a stabilization of the DSBs after induction of the HO endonuclease (Figure 5B). This result is consistent with the role of MRX in the processing of the DSB, the inefficient resection of the DSB being responsible for the permanence of the DSB. Indeed, as can be seen in Figure 5C, the amount of intact HO-induced DSBs in wild-type cells decreased with a fast kinetics following HO repression (DSBs 5% of the initial levels after 12h of glucose addition). Instead, DSBs disappeared with a low rate in *mre11*Δ cells (DSBs 50%).

SCE was significantly reduced to similar levels in the three null MRX mutants, indicating that the three MRX subunits are equally relevant for SCE (Figure 5B). The kinetics of IC-BIR in the three mutants was significantly delayed (above 1,5 h) and the accumulation of IC-BIR products occurred at a rate lower than in the wild type. This result reflects that MRX is also required for

efficient IC-BIR. Interestingly, in contrast to wild-type cells, IC-BIR products remained stable for 24h, which is consistent with a subsequent role of MRX in an expected resection of the DNA ends generated by IC-BIR.

Role of Mre11 nuclease activity, Sae2 and Exo1 in DSB repair via SCE and IC-BIR

To determine the possible role that the nuclease activity of Mre11 may have on SCE and IC-BIR we analyzed the effect of the *mre11-H125N* mutation, which inactivates the nuclease activity {Moreau, 1999 #32}. As can seen in Figure 6, no significant differences were found in the kinetics of accumulation of DSBs in *mre11-H125N* mutants with respect to wild type. However DSB-containing molecules reached higher levels and persisted longer than in *mre11-H125N* than in wild-type cells, although they finally decreased, in contrast to *mre11*Δ. Identical kinetics and overall levels of SCE-product formation were obtained for *mre11*Δ and *mre11-H125N,* SCE being significantly reduced, and to similar levels in both mutants. The levels of IC-BIR events were reduced in the *mre11-H125N*, reaching only 50% of those of wild type, the kinetics of appearance being similar to that of *mre11*Δ. However, in contrast to *mre11*Δ in which such IC-BIR products remained stable with time even at 24 h, IC-BIR events disappear in *mre11-H125N* after the 4h-peak, and become undetectable after 6h, as in wild-type cells (Figure 6). These results indicate that the Mre11 nuclease activity is essential for the role of MRX in the formation of SCE and IC-BIR products. Nonetheless, it is the absence of Mre11, and by extension of the MRX complex, rather than a non-functional MRX complex that is responsible for lack of extensive DNA resection.

Given the similarity of phenotypes between *sae2*Δ and nuclease-deficient MRX mutants {Prinz, 1997 #87; Rattray, 2001 #88}, consistent with a role for Sae2 in the processing of DSB ends by regulating the nuclease activity of Mre11, we analyzed the effect of *sae2*Δ on SCE and IC-BIR in our assays. As can be seen in Figure 6, DSBs accumulated in *sae2*Δ to the same levels and with similar kinetics as *mre11-H125N*. As expected, the effect of *sae2*Δ on the repair of replication-born DSBs by SCE and IC-BIR was identical to the effect of the nuclease-minus *mre11-H125N* mutant.

The incomplete effects of *mre11-H125N* and *sae2*Δ on DSB repair suggest that there must be at least another nuclease responsible for the 5'-end resection reaction. We have assayed whether that nuclease could be the Exo1 exonuclease. As can be seen in Figure 7, *exo1*Δ does not produce a significant effect on DSB accumulation, SCE and IC-BIR as compared to wild type, implying that Exo1 does not play a detectable role in any of the events studied. Double mutants *mre11*Δ *exo1*Δ and *mre11-H125N exo1*Δ show identical patterns of DSB accumulation, SCE and IC-BIR, and quite similar to the *mre11*Δ single mutant. It is noteworthy that whereas the *mre11*Δ mutation is epistatic on *exo1*Δ for all type of events analyzed, this is not the case of *mre11-H125N* for the effect on the accumulation of DSBs and IC-BIR intermediates. These remain intact in *mre11-H125N exo1*Δ mutants as compared to *mre11-H125N* single mutants, in which they decrease after 6h. This suggests that Exo1, although not playing a detectable role in wild-type cells, promotes resection of the DNA ends generated by DSBs and by BIR in the presence of a non-functional MRX complex but not in its absence, suggesting that Exo1 action on extensive DSB-end resection has a structural requirement for the presence of a MRX complex.

Altogether, our results uncover a role for the Mre11 nuclease activity in SCE and IC-BIR, and an active but different functional role of Exo1 in the processing of replication-born DSBs depending on the presence or absence of MRX in the cell.

Discussion

This study provides a molecular characterization of the genetic requirements of DSB repair by SCR, in competition with ICR (Fig. 8A). This is possible because of two features of the plasmid used in this study (pRS316-TINV): a 21-bp HO site, which allows specific formation of DSBs during replication (F.C.-L- and A.A., submitted) in one of the sister chromatids; and the presence of inverted repeats, which allows detection of both SCE and ICR events. This study confirms that SCE induced by replication-born DSBs is dependent on Rad51

and Rad54, which is consistent with the idea that SCE occurs by a standard DSB-repair mechanism. Instead, we show that ICR events measured in this assay occur by BIR. The *rad59*Δ mutant is affected in both SCE and IC-BIR, suggesting that Rad59 also functions in the repair of replication-born DSBs by HR even in the presence of Rad51. In contrast, the *tid1*Δ mutant shows no detectable SCE defects. MRX is required for both SCE and IC-BIR, its main function relying on the nuclease activity of Mre11, as both, a null deletion and a nuclease-minus mutant, as well as *sae2*Δ cells, display the same HR defect. In addition, we provide evidence for Exo1 playing a role in extensive DNA-end resection in the absence of a functional MRX complex.

Molecular evidence that inversions can occur by BIR-SSA in a Rad51, Rad54-independent manner

Although genetic detection of SCE is not trivial, several systems have been developed for its study, based on the analysis of recombination between heteroallelic direct repeats {Fasullo, 1987 #47; Jackson, 1981 #75; Kadyk, 1992 #36; Szostak, 1980 #74}. Some of these direct repeat-based assays permit the study of DSB-induced events by introducing an endonulease-recognition site within the repeats but do not measure SCE at a molecular level {Fasullo, 2001 #7; Johnson, 2000 #37}. Here, we take advantage of the fact that the pRS316-TINV system permits induction and physical detection of SCR {Gonzalez-Barrera, 2003 #38}. Since both chromatids are identical, our system, as well as any other developed so far, can only measure events involving a reciprocal exchange (Figure 2, SCE) or extensive DNA synthesis (Figure 2, SC-BIR). Although we only analyze unequal SCR events, we have shown that it is a valid estimate of total SCR in these assays (data not shown, {Gonzalez-Barrera, 2003 #38}).

SCR is strongly dependent on Rad51 (Fig. 8A), in agreement with previous reports {Fasullo, 2001 #7; Gonzalez-Barrera, 2003 #38}. This suggests that it occurs by crossover, this is SCE, rather than by BIR (Fig. 8B), as crossover requires the strand-exchange activity of Rad51 while BIR can also occur in the absence of Rad51 {Malkova, 1996 #48}. In contrast, IC-BIR kinetics was stimulated in the *rad51*Δ mutant (Fig. 8A). This could be explained if BIR events are favored in *rad51*Δ cells, as previously reported {Malkova, 1996 #48},

even though this does not imply that BIR does not occur via Rad51, as BIR has been shown to depend on Rad51 in one-ended recombination systems {Davis, 2004 #28; Malkova, 2005 #55}. As previously proposed, in two-ended recombination events, the Rad51/DNA nucleoprotein barrier resulting from invasion of the second DSB end could impede progression of DNA synthesis required for BIR {Aguilera, 2001 #58}.

The fact that in contrast to allelic recombination, inversions between inverted repeats are not significantly reduced by *rad51*Δ, suggested that they are not necessarily produced via reciprocal exchange in *rad51*Δ cells, but presumably by a double event consisting of BIR followed by SSA {Bartsch, 2000 #2} (Fig. 8C). Different genetic data support this model {Gonzalez-Barrera, 2002 #107; Ira, 2002 #77; Kang, 2000 #10; Malagon, 2001 #40; Rattray, 2001 #88}. The efficient kinetics of IC-BIR in *rad51*Δ versus wild-type cells observed in this study provides molecular evidence for inversions between repeats occurring by BIR-SSA in *rad51*Δ cells. It might be possible that a DSB will relax one chromatid making the other repeat accessible for a putative Rad51-independent invasion, whereas invasion on the intact sister would still be highly dependent on Rad51.

As expected, our study reveals that Rad54 is required for SCE and not for IC-BIR, in agreement with its role in Rad51-dependent recombination {Dong, 2003 #73; Petukhova, 1998 #65; Rattray, 1995 #64; Signon, 2001 #60}. Instead, Tid1 plays no evident role in either SCE or IC-BIR. It has been previously suggested that Tid1 acts specifically in allelic recombination occurring between homologous chromosomes {Klein, 1997 #56; Shinohara, 1997 #57}. Consistently, in meiotic return-to-growth experiments, Rad54 has been shown to be required for SCE, whereas this is not the case for Tid1 {Arbel, 1999 #1}.

Post-replicative repair of DSBs by SCE and IC-BIR is mediated by Rad59

In contrast to previous reports {Dong, 2003 #73; Signon, 2001 #60}, we find that both SCE and IC-BIR are strongly and equally affected by the *rad59*Δ mutation in a *RAD51* background (Fig. 8A). This indicates that in addition to Rad51-independent HR {Bai, 1996 #41; Jablonovich, 1999 #62; Signon, 2001 #60}, Rad59 participates in Rad51-mediated HR. Our data are consistent with Rad59

acting in all HR events, in the initial annealing step, in concert with Rad52, but with a less predominant role {Bai, 1999 #101; Cortes-Ledesma, 2004 #100; Davis, 2001 #61}. Therefore, although genetically scored inverted-repeat recombination is not strongly affected in the absence of Rad59 {Bai, 1996 #41; González-Barrera, 2002 #107}, this molecular kinetic analysis uncovers a prominent role of Rad59 in HO-induced DSB-repair.

Interestingly, in *rad51*Δ cells, the *rad59*Δ mutation while completely abolishing SCE, reduces the kinetics of IC-BIR only slightly. This effect of *rad59*Δ is much smaller than that previously observed for BIR in the absence of Rad51 {Signon, 2001 #60}. However in those studies, as in ours, BIR in *rad51*Δ *rad59*Δ still remains above wild-type levels. Other genetic studies showed either a synergistic defect of *rad51*Δ and *rad59*Δ or an epistatic effect of *rad59*Δ over *rad51*Δ for inverted repeat recombination {Bai, 1996 #41; Malagon, 2001 #40; Gonzalez-Barrera, 2002 #107}. It is likely that in the absence of Rad51, Rad59 could be required for the SSA step following BIR {Davis, 2001 #61; Jablonovich, 1999 #62; Sugawara, 2000 #63}, rather than to a defect in the BIR reaction itself. Therefore, our data are consistent with previous observations of HR not being completely abolished in *rad51*Δ *rad59*Δ, as compared with *rad52*Δ {Bartsch, 2000 #2; Jablonovich, 1999 #62; Malagon, 2001 #40; Signon, 2001 #60}, and suggest that the residual HR in *rad51 rad59* cells occurs by BIR.

Role of MRX and Exo1 in postreplicative repair of DSBs

A specific role of MRX in SCE rather than other types of HR such as allelic recombination has been largely discussed on the basis of different genetic studies {Bressan, 1999 #3; Hartsuiker, 2001 #80; Ivanov, 1992 #67}, but such specificity has never been shown. We have previously reported that *mre11*Δ mutants are affected in SCE {Gonzalez-Barrera, 2003 #38} and in this study we show that all members of MRX are equally required (Fig. 5). This defect in MRX mutants is also evident in genetically detected spontaneous and UV, 4NQO, MMS and X-ray induced unequal SCR events {Bressan, 1999 #3; Dong, 2003 #73; Kadyk, 1993 #98}, suggesting that our 21-bp HO site-based system, resembles a natural scenario in which DSBs arise during replication (F.C.-L. and A.A., submitted) and, reciprocally, is consistent with the idea that SCE is linked to replication-born DSBs. A lack of MRX-dependency has been observed

when unequal SCR is induced by a DSB at a full 117-bp HO site {Dong, 2003 #73}, in which case it is likely that such unequal events are produced via other mechanisms in addition to SCR {Fasullo, 2001 #7}. Nevertheless, we show that the effect of MRX null-mutations is also observed for IC-BIR, indicating a general rather than sister chromatid-specific role of MRX in HR (Fig. 8A). A previous study also reported a general defect of *mre11*Δ in IR-induced recombination, as both unequal SCR and allelic recombination measured genetically were similarly affected {Bressan, 1999 #3}. Therefore, we can conclude that our assay can uncover defects in HR that are not observed with other enzymatically induced systems.

It is believed that the MRX complex functions in the processing of DSB ends through the nuclease activity of Mre11. However, *mre11* nuclease-deficient mutants do not display a severe DSB-repair defect {Bressan, 1998 #81; Bressan, 1999 #3; Furuse, 1998 #82; Moreau, 1999 #32; Symington, 2000 #31}, suggesting that either the main function of MRX does not rely on its nuclease activity, or that redundant nucleases can substitute for this function. Based on its ability to tether DNA molecules {de Jager, 2001 #84}, a different role has been proposed for MRX in holding both ends of the DSB together, and/or each end to the sister chromatid {Anderson, 2001 #83; de Jager, 2001 #84; Hopfner, 2002 #85; Wiltzius, 2005 #86}. Indeed, it has been shown that after induction of a break, DNA ends flanking the DSB in wild-type cells remain adjacent while loss of a functional MRX complex results in DNA ends being dispersed {Lobachev, 2004 #108}. The nuclease activity of the complex seems to play a role in this DSB-ends bridging, although to a lesser extent {Clerici, 2005 #104}. Here we show that SCE and IC-BIR events measured in our assay depend on the nuclease activity of Mre11. The same result was obtained in *sae2*Δ, proposed to impair the nuclease activity of Mre11, and that behaves as an MRX nuclease-deficient mutant for all phenotypes tested (Fig. 7) {Clerici, 2005 #104; Prinz, 1997 #87; Rattray, 2001 #88}.

Nuclease-deficient *mre11-H125N* and null *mre11*Δ mutants display the same defect in both SCE and IC-BIR, pointing to the primary role of MRX in HR being related to DSB-end processing. A similar defect has been reported recently for ends-in and ends-out recombination after transformation, but such a defect was allele specific, and in particular the *mre11-H125N* mutant displayed

a subtler defect {Lewis, 2004 #53}. In the recombination systems used in this study, the main difference between nuclease-deficient and null *mre11* mutants relies in the stability of DNA ends (Fig. 8A). While DSBs accumulate in *mre11*Δ, consistent with a role of MRX in DNA end resection, they do not accumulate in *mre11-H125N* and *sae2*Δ, although these mutants show a higher persistence of the DSBs, in agreement with the slight resection defect reported for *sae2*Δ mutants {Clerici, 2005 #104}. Consistently, a delay in the formation of Rad52 foci, accompanied by a longer persistence of Mre11 foci, has been observed in these nuclease-deficient mutants {Clerici, 2006 #105; Lisby, 2004 #89}. As mentioned above, the HR defect conferred by both *mre11* mutations is identical, indicating that although DSBs are processed in the absence of a functional MRX, HR does not efficiently repair them.

The persistence of intact DSBs and BIR intermediates in *mre11-H125N exo1*Δ mutants as compared to *mre11-H125N* single mutants, suggests that Exo1 has a structural requirement for the presence of a MRX complex, thus promoting resection of the DNA ends generated by DSBs and by BIR in the presence of a non-functional MRX complex, but not in its absence (Fig. 8A, 8C), and is in agreement with the synergistic resection defect observed in *sae2*Δ *exo1*Δ {Clerici, 2006 #105}. It is possible that MRX could facilitate Exo1-dependent resection, probably via its helicase activity, as previously proposed {Symington, 2000 #31}. Nevertheless, previous observations also suggest a role for Exo1 in the absence of Mre11, as shown by the suppression of *mre11*Δ by Exo1 overexpression, and the synergistic repair defect of *mre11*Δ *exo1*Δ mutants {Lewis, 2002 #91; Moreau, 2001 #30; Nakada, 2004 #94; Tsubouchi, 2000 #93}. It would be interesting to see whether or not this defect is related to the observation that Exo1 participates in the restart of stalled replication forks {Cotta-Ramusino, 2005 #99}.

In summary, the analysis of repair of replication-born DSBs reveals that Rad59 plays a role in Rad51-dependent recombination; that the role of MRX in HR is not specific for SCE, relying on the nuclease activity of Mre11, which is controlled by Sae2; and that Exo1 may play a role in postreplicative DSB repair in a MRX-dependent manner. These results suggest that the functional relevance of HR proteins may vary depending on the nature of DSBs, opening

new perspectives for a more complete understanding of the role of HR in postreplicative repair.

Material and methods

Strains, plasmids and oligonucleotides

All strains used were isogenic to W303-1A. The wild-type WS strain (*MAT***a**-*inc ade2-1 his3-11,15 trp1-1 ura3-1 ade3::GAL-HO leu2*Δ*::SFA1*) was previously described (González-Barrera et al. 2003) and deletion mutants were obtained by gene replacement of the corresponding ORF by the *KanMX4* cassette as described (Malagón and Aguilera 2001). All deletions were confirmed by PCR and Southern analyses (data not shown). The *mre11-H125N* mutation, kindly supplied by L. Symington (Columbia University, NY), was introduced in WS background by genetic cross. Isogenic double mutants were obtained by genetic crosses. Plasmid pRS316-TINV was described in González-Barrera et al. (2003).

Analysis of HO cleavage

WS cells carrying a *bar1*Δ allele (WS-bar1), used as wild-type controls, were grown at 30ºC in SC-ura with 2% raffinose as carbon source to an OD of 0.4. The *bar1*Δ alelle was used to enhance the effect of α factor. The culture was split into two halves, one of which was supplemented with 0.2 µM α-factor to arrest cells at G1. After 4 hours, 2% galactose was added to induce HO expression. For α-factor release, cells were washed twice with pre-warmed fresh medium without α-factor, and 50 µg/ml Pronase (SIGMA, USA) was added to remove any trace of α factor. Yeast samples were taken at different times and DNA was isolated for Southern-blot analysis. In the experimental conditions used, HO cleavage is recurrent, as cells are maintained in 2% galactose after α-factor release.

Physical analysis of recombination

Mid-log phase cultures of yeast cells grown on SC-3% glycerol-2% lactate medium lacking uracil were split. 2% glucose or 2% galactose was added to repress or induce HO-expression, respectively. Yeast samples were taken at different times and DNA was isolated for Southern-blot analysis.

Miscellaneous

DNA isolation was performed as previously described (González-Barrera et al., 2002). Southern analyses, yeast growth conditions, DNA labeling and quantification with a Fuji FLA3000 were performed according to standard procedures (see Prado et al., 1997). In each case a representative experiment of at least 3 performed is shown. Variation was bellow 20% in all cases.

Acknowledgments

We thank F. Prado and R. Wellinger for reading the manuscript, and D. Haun for style supervision. This work was supported by grants from the Ministry of Science of Education of Spain (SAF2003-00204) and Junta de Andalucía (CVI102). F. C.-L. was the recipient of a pre-doctoral training grant from the Spanish Ministry of Health.

Figure Legends

Figure 1. Analysis of cleavage of a 21-bp HO-site located in the circular plasmid pRS316-TINV. **A.** Scheme of plasmid pRS316-TINV (left) and the proposed mechanism by which a 21-bp HO generated nick is converted into a DSB after replication (right). The line marked with an asterisk indicates the 0.7-kb *Cla*I-*EcoRV LEU2* probe used for hybridization experiments. **B.** Southern analysis of HO cleavage in asynchronous cultures (async) in cells arrested in G1 (G1) and after α-factor release (S/G2) in the WS-bar1 wild-type control strain. DNA samples were taken 2 h after HO induction (Gal) or from non-induced controls (Glu), and cut with *Xho*I and *Spe*I before electrophoresis. FACS pattern of cultures (right top) and quantification data of DSBs (right bottom) are shown.

Figure 2. Physical analysis of replication-born HO-induced DSB repair. **A.** Scheme of the two distinguishable recombination events initiated by a replication-born HO-induced DSB in plasmid pRS316-TINV carrying 2 inverted repeats. Fragments generated by HO cleavage and *Xho*I *Spe*I digestion, as detected by the *LEU2* probe (shown in Figure 1), are indicated with their corresponding size. Intra-chromatid recombination (ICR) events occurring by BIR (IC-BIR; bottom) gives rise to a new 2.9-kb band. Note that ICR intermediates contain fragments of undefined length (dashed lines), which depends on the extent of DNA synthesis, if it reaches the end of the invaded template, an additional 3.3-kb band and a 1.4-kb band, which coincides with one of the cleavage products, would appear. Sister chromatid recombination (SCR; top), whether occurring by reciprocal exchange (SCE) or by BIR (SC-BIR), also leads to the formation of the 2.9-kb band, but produces in addition a specific 4.7-kb fragment. Intra-chromatid reciprocal exchange and gene conversions are not detectable in this assay. **B.** Kinetics of HO-induced DSB formation and its repair. Yeast DNA was isolated from cells grown on either SC-2% glucose (Glu) or after different times of growth in SC-2% galactose (Gal), double digested with *Xho*I and *Spe*I, separated by gel electrophoresis and analyzed by Southern. Quantification of DSBs (1.4 plus 2.4-kb bands), SCE

(4.7-kb band) and IC-BIR (2.9 minus 4.7-kb bands) related to total plasmid DNA is shown. The 3.3-kb band expected from IC-BIR events reaching the end of the template is not detected, indicating that either this does not occur, or the intermediate is quickly processed.

Figure 3. Role of Rad51 and Rad59 on the repair of replication-born DSBs via SCE and IC-BIR in *rad51*Δ and *rad59*Δ. **A.** Kinetics and quantification of HO induction and DSB repair in *rad51*Δ and *rad59*Δ strains. **B.** Physical analysis of DSB repair by SCE and IC-BIR in the *rad51*Δ *rad59*Δ double mutant. Only 3 time points after HO induction were analyzed: 0 (control), 4 (maximum for IC-BIR) and 24 (maximum for SCE) hours. Wild-type control data are taken from Figure 2. Other details as in Figure 2.

Figure 4. Role of Rad54 and Tid1/Rdh54 paralogs in DSB repair by SCE and IC-BIR. Kinetics and quantification of HO induction and DSB repair in *rad54*Δ and *tid1*Δ. Other details as in Figure 2.

Figure 5. Role of the MRX complex in DSB repair by SCE and IC-BIR. **A.** Kinetic analysis of DSB formation and SCE and IC-BIR intermediates in *mre11*Δ, *rad50*Δ and *xrs2*Δ mutants. Wild-type data are taken from Figure 2. **B.** Kinetics and quantification of HO-induced DSB stability in wild type and *mre11*Δ. In this case, after 2h of growth in SC-2% galactose, glucose was added to a final concentration of 2% in order to stop HO induction. DSB stability was monitored as the disappearance of the HO-cut specific bands by Southern of DNA samples taken at different time points after glucose addition. The percentage of DSBs related to total plasmid DNA is shown. Other details as in Figure 2.

Figure 6. Role of the nuclease activity of Mre11 in DSB repair by SCE and IC-BIR. Kinetics and quantification of HO-induced recombination in *mre11*Δ and the nuclease deficient *mre11-H125N* and *sae2*Δ mutants. Other details as in Figure 2.

Figure 7. Role of Exo1 in DSB repair by SCE and IC-BIR. Kinetics and quantification of HO-induced SCE and IC-BIR recombination in *exo1*Δ mutants, in wild-type, *mre11*Δ and *mre11-H125N* backgrounds. Only 3 time points after HO induction were analyzed: 0 (control), 4 (maximum for IC-BIR) and 24 (maximum for SCE) hours. Wild-type, *mre11*Δ and *mre11-H125N* control data are taken from Figure 6. Other details as in Figure 2.

Figure 8. Repair by SCE and IC-BIR in pRS316-TINV. **A.** DSB processing and repair by SCE and IC-BIR in different genetic backgrounds. **B.** SCE occurs by DSBR resulting in the formation of a dimer. **C.** IC-BIR followed by SSA can also result in an inversion. Invasion of only one side of the DSB is shown for simplification. In null MRX mutants or in the double *mre11-H125N exo1*Δ the resection step required for SSA would be blocked and, therefore, IC-BIR products accumulate.

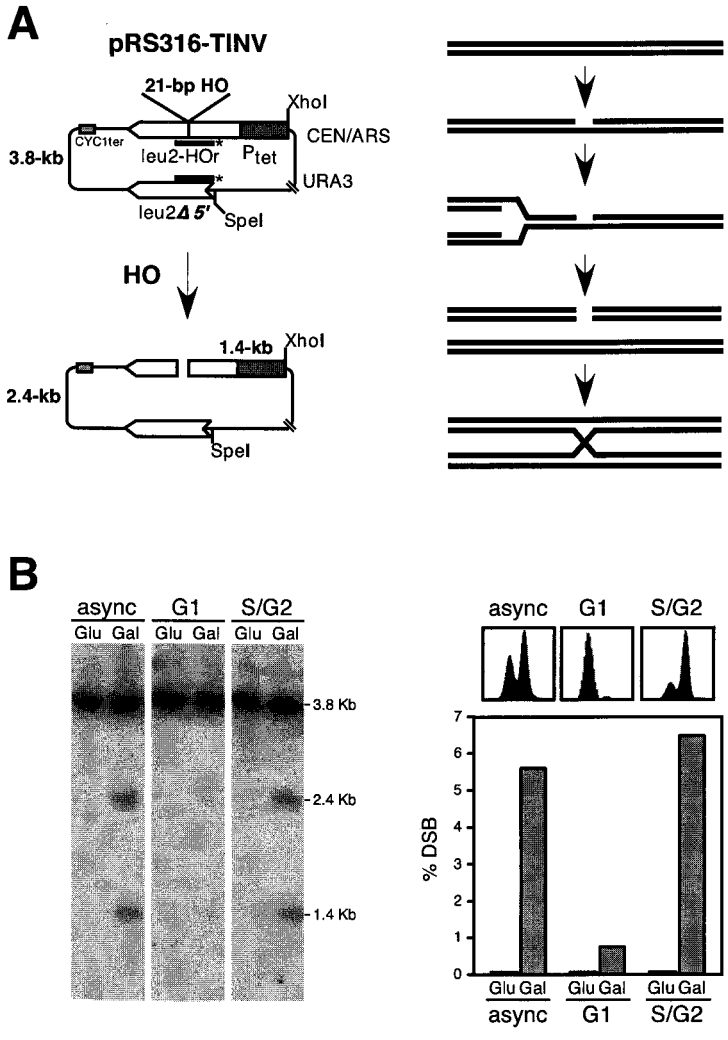

A
pRS316-TINV
21-bp HO
XhoI
CYC1ter
CEN/ARS
3.8-kb
leu2-HOr
Ptet
URA3
leu2Δ 5'
SpeI
HO
1.4-kb
XhoI
2.4-kb
SpeI
B
async
G1
S/G2
Glu Gal
Glu Gal
Glu Gal
3.8 Kb
2.4 Kb
1.4 Kb
async
G1
S/G2
% DSB
7
6
5
4
3
2
1
0
Glu Gal
Glu Gal
Glu Gal
async
G1
S/G2

Figure 1

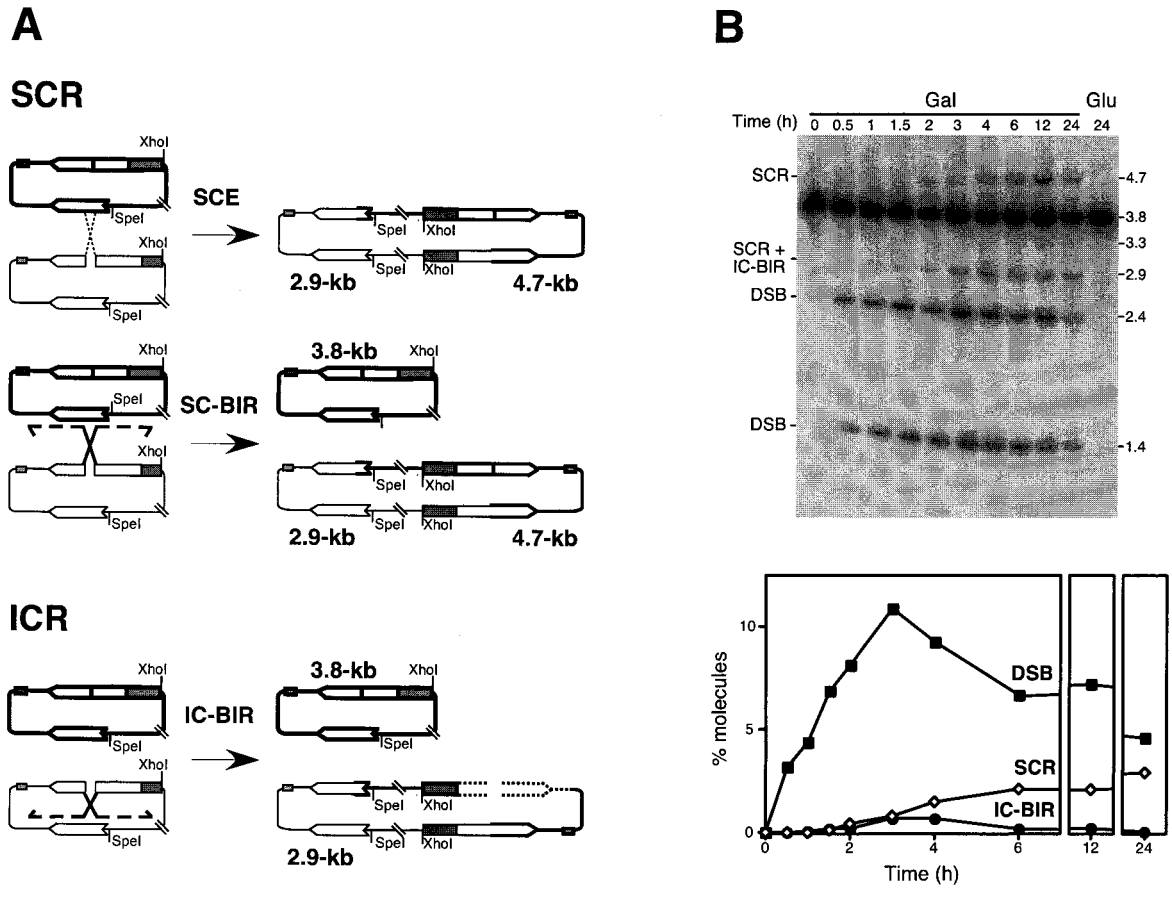

A
SCR
XhoI
SpeI
SCE
2.9-kb
4.7-kb
SC-BIR
3.8-kb
ICR
IC-BIR
B
Gal
Glu
Time (h)
0 0.5 1 1.5 2 3 4 6 12 24 24
SCR
SCR + IC-BIR
DSB
4.7
3.8
3.3
2.9
2.4
1.4
% molecules
DSB
SCR
IC-BIR
Time (h)

Figure 2

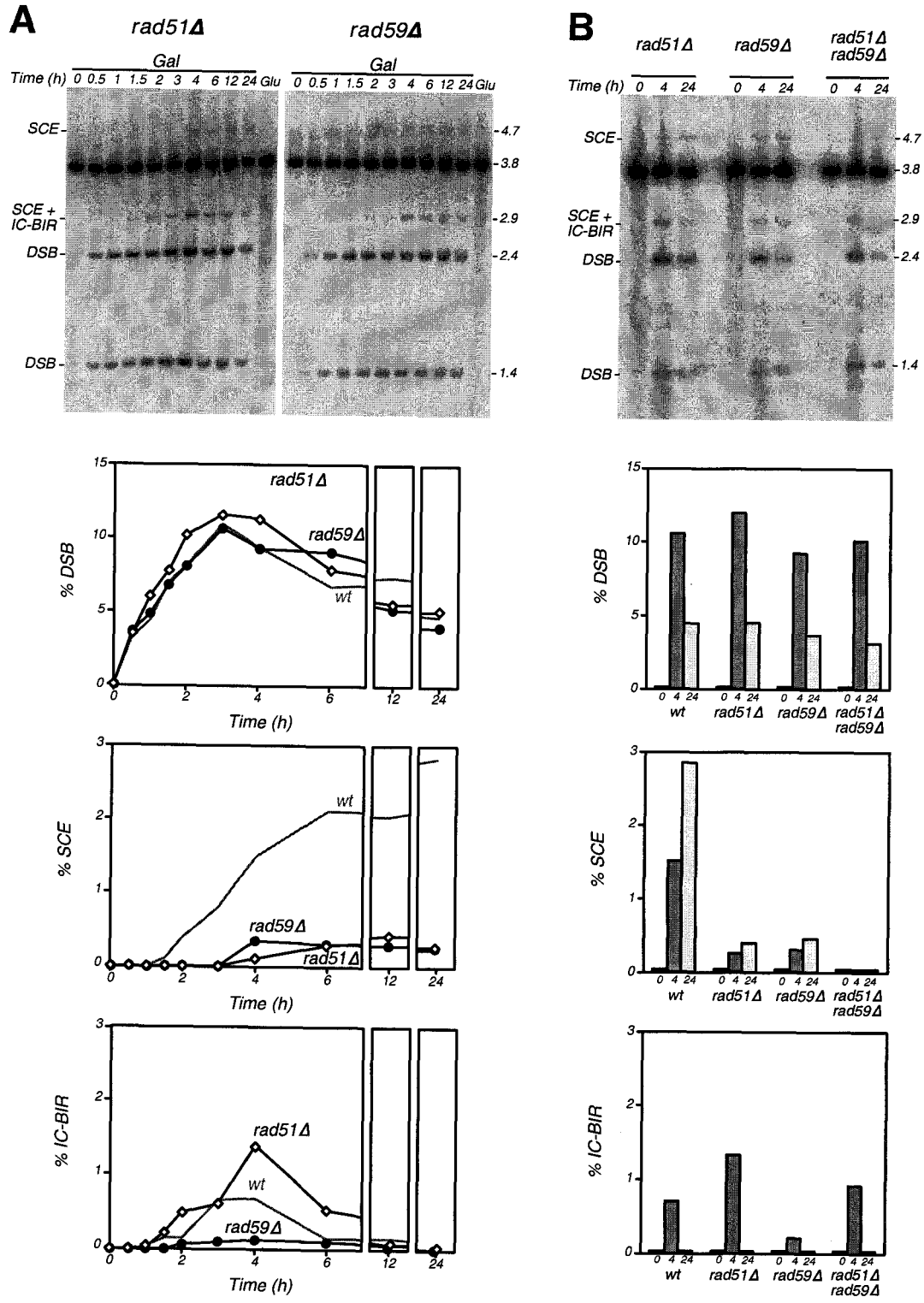

A
rad51Δ
rad59Δ
Gal
Time (h) 0 0.5 1 1.5 2 3 4 6 12 24 Glu
SCE
SCE + IC-BIR
DSB
DSB
4.7
3.8
2.9
2.4
1.4
B
rad51Δ
rad59Δ
rad51Δ rad59Δ
Time (h) 0 4 24
% DSB
% SCE
% IC-BIR
Time (h)
wt
rad51Δ
rad59Δ
rad51Δ rad59Δ

Figure 3

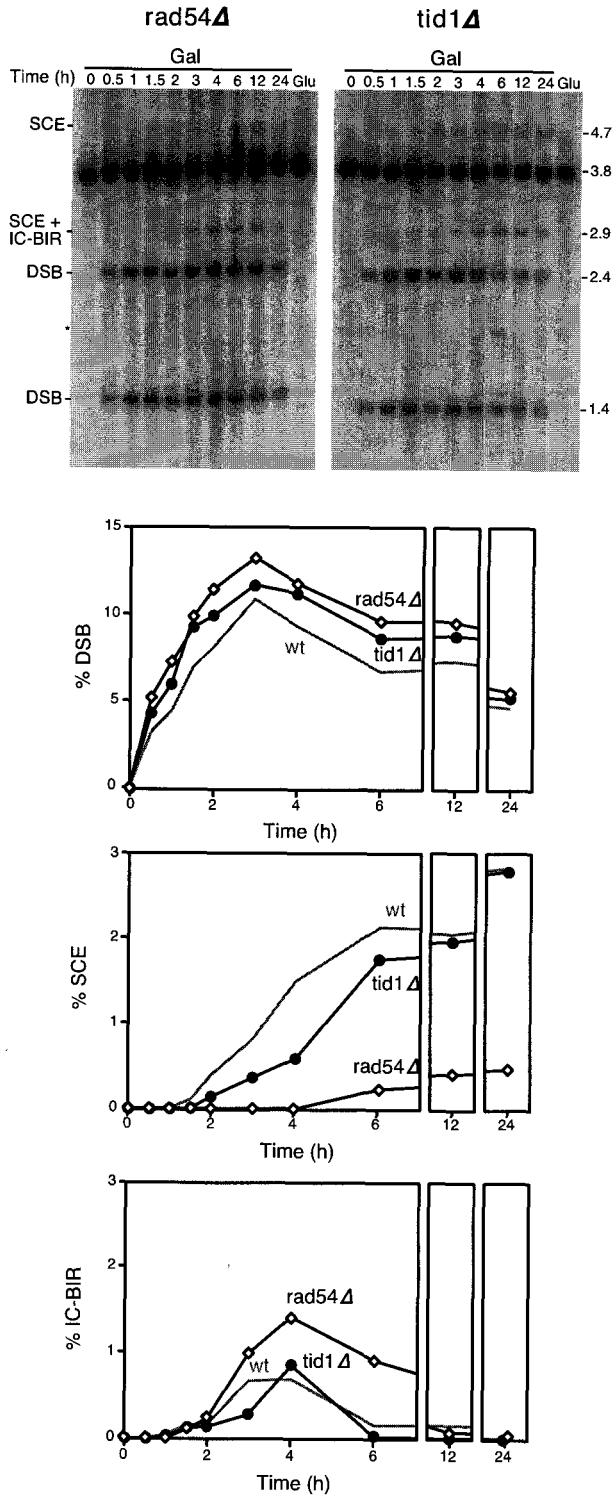

rad54Δ
tid1Δ
Gal
Time (h) 0 0.5 1 1.5 2 3 4 6 12 24 Glu
SCE
SCE + IC-BIR
DSB
DSB
4.7
3.8
2.9
2.4
1.4
% DSB
rad54Δ
wt
tid1Δ
Time (h)
% SCE
wt
tid1Δ
rad54Δ
Time (h)
% IC-BIR
rad54Δ
wt
tid1Δ
Time (h)

Figure 5

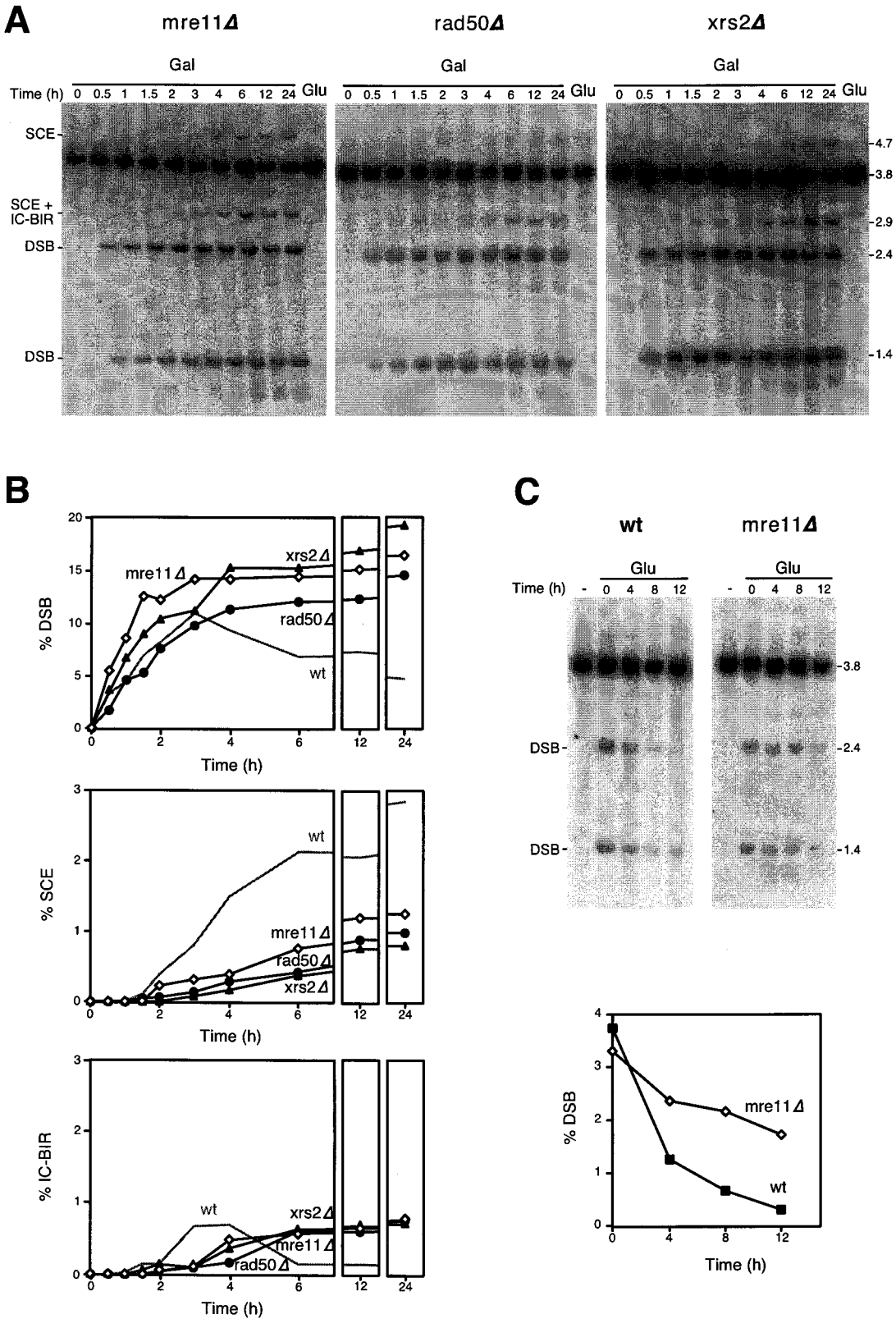

A
mre11Δ
rad50Δ
xrs2Δ
Gal
Glu
Time (h)
0 0.5 1 1.5 2 3 4 6 12 24
SCE
SCE + IC-BIR
DSB
DSB
4.7
3.8
2.9
2.4
1.4
B
% DSB
% SCE
% IC-BIR
Time (h)
mre11Δ
rad50Δ
xrs2Δ
wt
C
wt
mre11Δ
Glu
Time (h)
- 0 4 8 12
DSB
DSB
3.8
2.4
1.4
% DSB
Time (h)
mre11Δ
wt

Figure 5

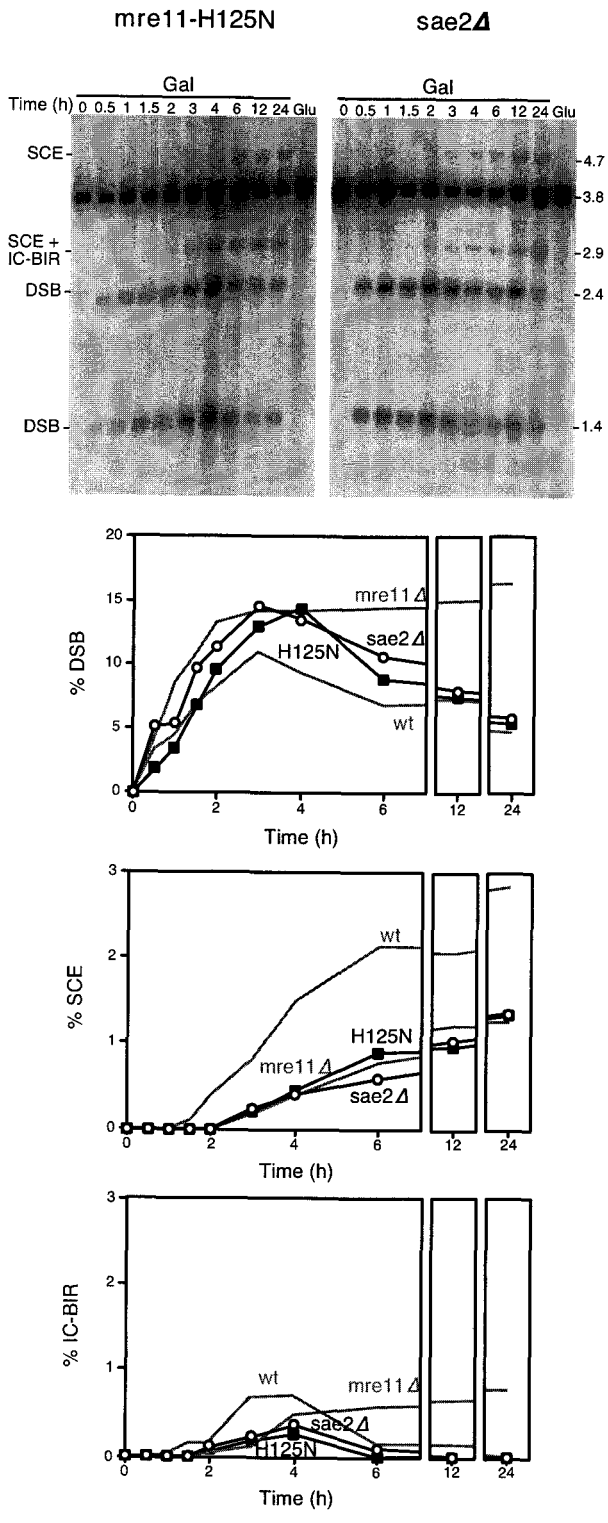

mre11-H125N
sae2Δ
Gal
Time (h) 0 0.5 1 1.5 2 3 4 6 12 24 Glu
SCE
SCE + IC-BIR
DSB
DSB
4.7
3.8
2.9
2.4
1.4
% DSB
mre11Δ
sae2Δ
H125N
wt
Time (h)
% SCE
wt
H125N
mre11Δ
sae2Δ
Time (h)
% IC-BIR
wt
mre11Δ
sae2Δ
H125N
Time (h)

Figure 6

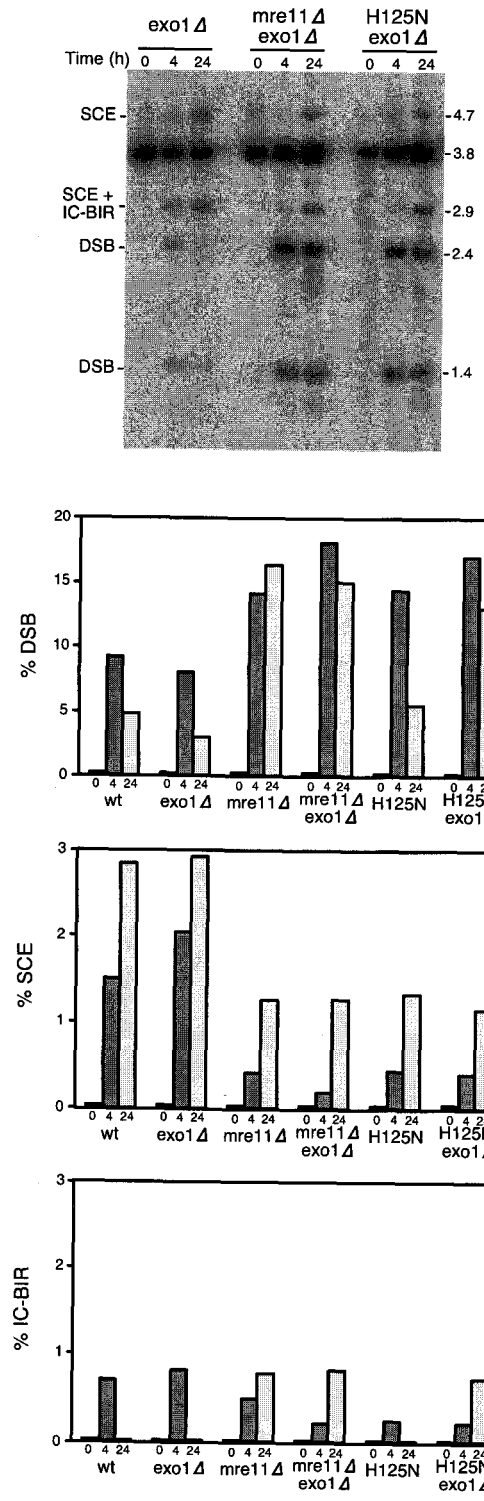
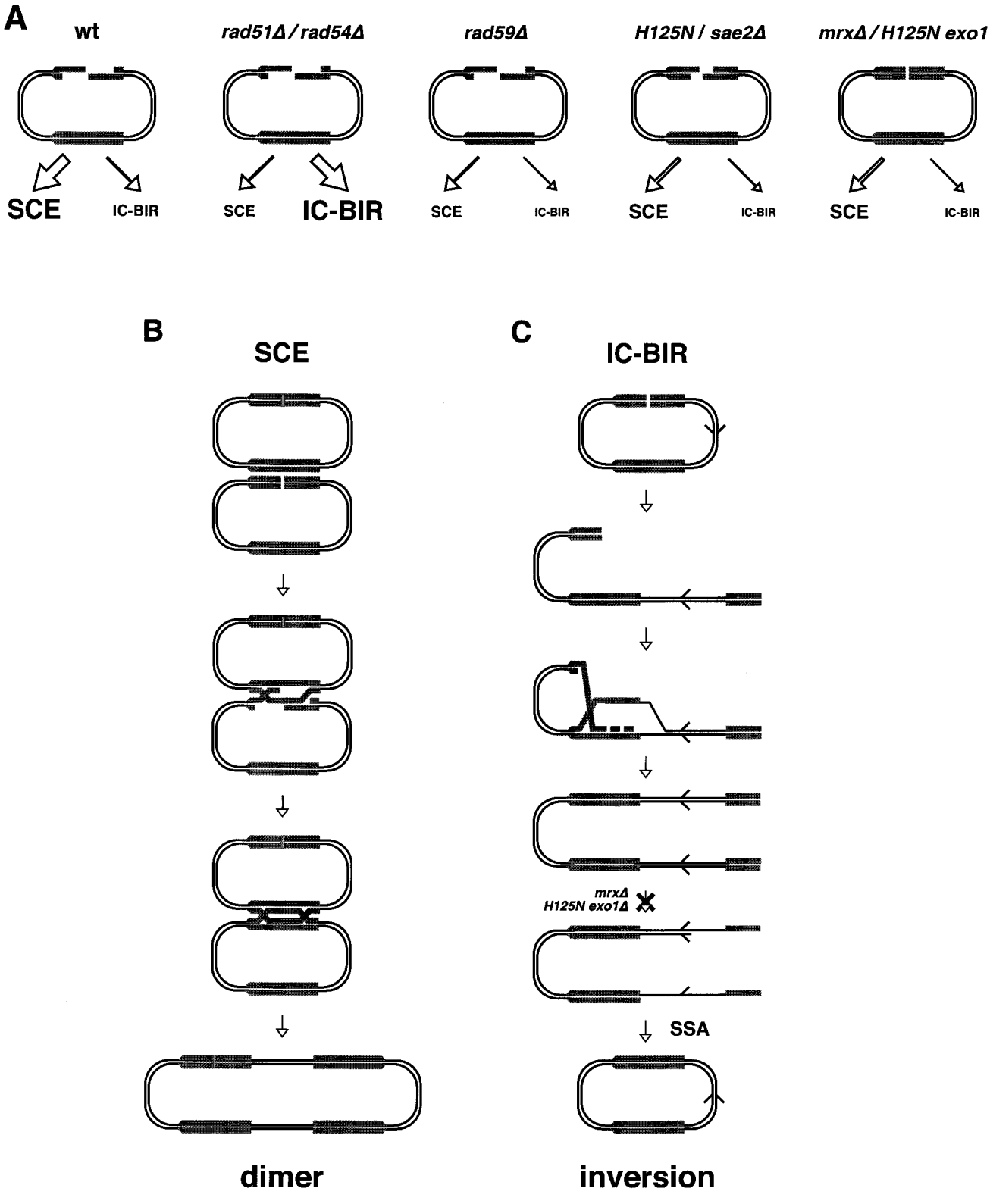

exo1Δ
mre11Δ exo1Δ
H125N exo1Δ
Time (h) 0 4 24 0 4 24 0 4 24
SCE
SCE + IC-BIR
DSB
DSB
4.7
3.8
2.9
2.4
1.4
% DSB
% SCE
% IC-BIR
wt
exo1Δ
mre11Δ
mre11Δ exo1Δ
H125N
H125N exo1Δ

Figure 7


A
wt
rad51Δ / rad54Δ
rad59Δ
H125N / sae2Δ
mrxΔ / H125N exo1
SCE
IC-BIR
SCE
IC-BIR
SCE
IC-BIR
SCE
IC-BIR
SCE
IC-BIR
B
SCE
dimer
C
IC-BIR
mrxΔ
H125N exo1Δ
SSA
inversion

Artículo 3

Smc5-Smc6 mediates DNA double-strand-break repair by promoting sister-chromatid recombination

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En revisión, *Molecular Cell*

Mi contribución a este artículo ha sido determinar los niveles de SCE desigual e igual en los mutantes *nse5-1* y *smc6-9* (Figuras 4-5)

Smc5-Smc6 mediate DNA double-strand-break repair by promoting sister-chromatid recombination

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Summary

DNA double-strand breaks can arise during DNA replication or after exposure to DNA damaging agents and their correct repair is fundamental for cell survival and genomic stability. The Smc5-Smc6 complex is involved in DNA repair and is required for the stability of the repetitive ribosomal gene cluster. Here, we show that following a single DSB induced by the HO endonuclease, Smc5-Smc6 subunits are recruited to the vicinity of the break. Physical analysis of the repair of induced DSBs in *smc5-smc6* mutants reveals that inactivation of the complex reduces sister chromatid recombination. We also show that in the absence of extrinsic DNA damage, Smc5-Smc6 is required to prevent deleterious recombination events in repetitive and single copy regions of the genome thereby suppressing gross chromosomal rearrangements. These findings demonstrate that the Smc5-Smc6 complex is essential for genome stability because it promotes repair of naturally occurring DSBs by sister chromatid recombination thus suppressing inappropriate non-sister recombination events.

Introduction

DNA double-strand breaks (DSB) are one of the most common and threatening alterations of a cell's genetic material. Left unrepaired, DSBs can cause cell death (Bennett et al., 1993) and, if misrepaired, they can lead to genomic instability and the development of cancer in multicellular organisms. Efficient repair of DSBs is thus essential to maintain genome stability and cell viability. Double-strand breaks arise from exposure to a variety of DNA damaging agents, including free radicals, chemicals and radiation, however they can also occur as a consequence of cellular processes, for instance as an intermediate stage in the repair of various DNA lesions or during DNA replication. Paradoxically, DSBs can also have positive consequences for cells. The formation of DSBs in a programmed manner is essential in several developmental programmes, such as meiosis (Keeney, 2001), the rearrangement of immunoglobulin heavy and light chains (Lieber et al., 2004) or mating type switching in budding yeast (Haber, 1998).

Eukaryotic cells have evolved two main mechanisms for the repair of DSBs: non-homologous end-joining (NHEJ) (Daley et al., 2005) and homologous recombination (HR) (Pâques and Haber, 1999; Prado et al., 2003). NHEJ entails the direct rejoining of the broken ends of DNA. In contrast, HR involves a genomic search for similar sequences to be used as a template to repair the break. NHEJ is a relatively inaccurate process often accompanied by insertions or deletions whereas HR is more accurate and ensures repair without any loss of the genetic information (Jackson, 2002; Lewis and Resnick, 2000). The fidelity of the HR process might reflect its use as the prominent DSB repair (DSBR) pathway with 90% of all DSBs in *S. cerevisiae*, and 50% in mammalian cells, being repaired through HR (Johnson and Jasin, 2000; Pâques and Haber, 1999).

Homologous recombination involves the use of an intact donor DNA molecule to act as a template during DSBR. The location of the template in relation to the site

of damage is flexible. HR can take place between sister chromatids (Gonzalez-Barrera et al., 2003), homologous chromosomes or related DNA sequences in the genome, regardless of their chromosomal location (Aylon and Kupiec, 2004; Pâques and Haber, 1999). In yeast and mammalian cells, the cell cycle stage also determines the repair pathway used, HR is favoured in late S and G2 phases and inhibited in G1 because it requires cyclinB-dependent kinase activity (Cdk) (Esashi et al., 2005; Ira et al., 2004); while NHEJ is favoured in G1 phase, when the homologous chromosome is either absent (haploids) or far away (diploids).

In yeast and mammalian cells the preferred template for DSBR by HR is an intact sister chromatid (Johnson and Jasin, 2000; Kadyk and Hartwell, 1992; Moynahan and Jasin, 1997), however due to the fact that sisters are identical in DNA sequence, sister chromatid recombination (SCR) events are more difficult to detect by genetic or physical means than recombination between heteroalleles located in homologous or ectopic chromosome sites. Consequently, despite the importance of DSBR between sister chromatids, this process has been less well studied than repair between homologues. A possible reason for the bias to use sister chromatids as the template for recombinational repair could be the physical proximity between chromatids, thus making DNA repair through sister chromatid exchanges a very fast and efficient process. Consistent with this idea, mutants of the cohesin complex, which holds sister chromatids together (Guacci et al., 1997; Michaelis et al., 1997), are highly sensitive to DNA damage and defective in post-replicative DSB repair (Sjogren and Nasmyth, 2001). In addition, cohesins are recruited *de novo* to the sites of double-strand break damage (Strom et al., 2004; Ünal et al., 2004).

Cohesins are evolutionary conserved complexes containing structural maintenance of chromosome (Smc) proteins, Smc1 and Smc3, at their core (Nasmyth, 2001). Eukaryotic genomes encode for six Smc proteins (Smc1-6)

with a common structure where two globular N- and C-terminal domains, containing Walker A and B ATPase motifs, are joined by two long coiled-coil domains separated by a flexible "hinge" (Hirano, 2005; Jessberger, 2003; Losada and Hirano, 2005). The six Smc proteins form three heterodimers, which are the core components of distinct multiprotein complexes that interact with chromatin and regulate its dynamics. Besides Smc1-Smc3 in cohesin, the Smc2-Smc4 heterodimer forms the core of condensin, a complex required for chromosome condensation and sister chromatid resolution (Swedlow and Hirano, 2003), and the Smc5-Smc6 heterodimer is at the centre of a large essential complex whose molecular function remains unknown (Sergeant et al., 2005; Zhao and Blobel, 2005). Interestingly, mutants in subunits from all three Smc complexes are defective in DNA repair (Aono et al., 2002; Lehmann et al., 1995; Nagao et al., 2004; Sjogren and Nasmyth, 2001).

The function of Smc5-Smc6 is less well understood than that of cohesin and condensin (Jessberger, 2003). The complex was initially discovered through a genetic screen looking for radiation-sensitive mutants in fission yeast. The study identified a hypomorphic allele of *SMC6* (*rad18-X*) that is defective in the repair of diverse types of DNA lesions (Lehmann et al., 1995) and demonstrated that its DNA repair role was epistatic with that of the Rad51 pathway for homologous recombination mediated repair (Lehmann et al., 1995). In addition to Smc5 and Smc6, the complex contains four essential non-Smc subunits in *S. pombe* (Harvey et al., 2004; McDonald et al., 2003; Pebernard et al., 2004; Sergeant et al., 2005) and up to six in *S. cerevisiae* (Fujioka et al., 2002; Hazbun et al., 2003; Zhao and Blobel, 2005), named Nse1-6 (Non-Smc Element) (Fujioka et al., 2002). Interestingly, Nse2 is a SUMO E3 ligase (Andrews et al., 2005; Zhao and Blobel, 2005) whose targets include other subunits of the complex and the DNA repair protein yKu70 (Zhao and Blobel, 2005). Recently, work using temperature sensitive alleles of *SMC6* has shown that the complex is required for the correct disjunction of rDNA (Torres-Rosell et al., 2005). Mutant cells missegregate

regions distal to rDNA but not proximal (Torres-Rosell et al., 2005). The mechanistic reason behind this phenotype has not been fully elucidated. Mutations in *smc5-smc6* are synergistic with the deletion of genes involved in the resolution of recombination intermediates, such as *SGS1*, *TOP3* and *MMS4* and partially suppressed when *RAD52* is deleted (Torres-Rosell et al., 2005). Interestingly, another conditional mutant of Smc6 has been reported to fail in the induction of recombination upon methylmethane sulfonate (MMS) induced damage (Onoda et al., 2004).

Following a single *HO*-induced DSB, the ends of the broken chromosome remain associated (Kaye et al., 2004; Lisby et al., 2003; Lobachev et al., 2004; Melo et al., 2001) and cohesin is recruited to hold sister chromatids together in the regions around the break (Strom et al., 2004; Ünal et al., 2004). On the basis of the capacity of Smc proteins to interact with chromatin and the function of the Smc5-Smc6 complex in DNA repair, it is possible that this complex also plays structural and/or regulatory roles in the vicinity of double-strand breaks. Here we have addressed these questions. First, we examined the binding of Smc5-Smc6 complex subunits in response to a single endonuclease-induced DSB. We show that the complex is enriched around the lesion, demonstrating that these proteins are indeed recruited to sites of DNA damage where DNA has been severed. We show that the function of Smc5-Smc6 at the DSB site is important to regulate its repair. We find that unlike wildtype cells, *smc5-smc6* mutants have a reduced ability to repair the lesion using sister chromatid recombination. The essential role of Smc5-Smc6 prompted us to further investigate its role in the absence of induced DNA damage. We demonstrate that the Smc5-Smc6 complex prevents gross chromosomal rearrangements in repetitive and single copy regions of the yeast genome. These findings reveal that the Smc5-Smc6 complex is essential to maintain genome integrity because it is required to repair DSBs through sister chromatid recombination.

Results

Smc5-Smc6 complex localise around an HO-induced DSB

On the basis of the capacity of Smc proteins to interact with chromatin and the function of the Smc5-Smc6 complex in DNA repair, we tested whether this complex is localized to DSBs. We investigated whether subunits of the complex are recruited to an HO-endonuclease catalyzed DSB formed at a unique site in the *MAT* locus on chromosome III (Strathern et al., 1982). Transcriptional regulation of the HO endonuclease using the galactose inducible promoter allowed us to control the timing of DSB induction (Jensen et al., 1983). DSBs at the *MAT* locus are repaired by homologous recombination with *HML* and *HMR* loci (Haber, 1998). To prevent repair of HO-induced DSBs, both *HM* loci were deleted in our strains (Moore and Haber, 1996), thus maximising the persistence of the break to facilitate possible detection of Smc5-Smc6 subunits at this site.

Cells were engineered to express an epitope tagged version of Smc6 (*SMC6-9xMYC*). Chromatin binding of this protein to sites around the DSB was assayed by chromatin immunoprecipitation (ChIP). We used different primer pairs covering at least 30kb on either side of the DSB site. DNA sequences were amplified from the input chromatin and chromatin immunoprecipitated to calculate the relative percentage of immunoprecipiated material. To control for DSB-independent effects on protein occupancy we also used a primer pair specific for sequences located in a different chromosome (*MET6* locus on chromosome V). First we tested the efficiency of DSB induction and intact DNA damage checkpoint activation. We used the checkpoint protein Ddc2 fused to GFP as an *in vivo* marker of DSB formation in our strains (Melo et al., 2001). Two hours after galactose-mediated HO induction ~80% of the cells arrested as dumbbells with a single Ddc2 focus (Figure 1A-B) demonstrating that the HO break at the *MAT* locus is efficiently induced and not repaired, thus causing G2/M arrest (Lee et al., 1998; Sandell and Zakian, 1993) in our experimental system.

Next we evaluated the binding of Smc6 around the HO site. In the absence of a DSB at *MAT*, we found low Smc6 binding across the region (Figure 1C; uncut). After 2 hours of HO induction, we detected a general increase in binding around the regions flanking the break (Figure 1C; cut). The maximum DSB-induced increase was ~5-fold and localised to regions 4-5 kb away from the DNA break on either side (Figure 1C-D; cut). The relative binding decreased with increasing distance from the break, however the regions immediately adjacent to the DSB site were found to be low binding (Figure 1C-D; cut). Thus the presence of a DSB induces a significant increase in Smc6 binding in a domain at least 25 kb in size. To investigate whether the DSB-associated increase in Smc6 binding reflected an increase in binding of the whole Smc5-Smc6 complex, we used ChIP to monitor the chromatin binding of another subunit of the complex, Nse5. The binding pattern of Nse5 at *MAT* locus in cells with and without a DSB was similar to that found for Smc6 (Supplementary Figure 1) with the peak of binding localised 5kbs away from the break in either direction. This result confirms that the Smc5-Smc6 complex is recruited in a DSB-dependent manner to the *MAT* locus. Next we analysed whether the binding of the complex is limited to breaks occurring at *MAT*. We examined a strain in which the HO recognition site from *MAT* was moved to chromosome VI, on the centromere-proximal side of the *GAT1* locus 150 Kb to the left of *CEN6*. The efficiency of DSB induction on chromosome VI, measured through Ddc2-GFP, was similar to that observed for *MAT* (data not shown). In the absence of a DSB, Smc6 binding across the chromosome VI region was low (Figure 1E; uncut). However, when the DSB was generated, Smc6 association around the DSB increased in a manner comparable to that observed previously for *MAT*, with the peak of localisation residing 5 kb away from the break (Figure 1E; cut). The maximum DSB-induced increase in Smc6 binding around the DSB region was slightly greater than that observed for *MAT* (~7-fold). This result shows that a DSB anywhere in the genome induces the binding of Smc5-Smc6.

Non-Homologous End-Joining is not impaired in *smc5-smc6* mutants

DSB repair occurs either through homologous recombination (HR) or non-homologous end joining (NHEJ). The Smc5-Smc6 complex is required for proper recombinational repair of induced DNA damage (Lehmann et al., 1995). However, recent studies have shown that Nse2 sumoylates yKu70 in response to DNA damage (Zhao and Blobel, 2005). Since yKu70 is central to NHEJ, it is possible that Smc5-Smc6 plays a role in NHEJ repair through sumoylation of yKu70. The recruitment of Smc5-Smc6 to HO-induced DSBs (Figure 1) prompted us to investigate whether the complex affects the NHEJ pathway for DSB repair.

First we looked at the effect of Smc5-Smc6 in NHEJ-mediated repair of a chromosomal DSB at *MAT* in a strain that cannot repair the break by homologous recombination because *HML* and *HMR* have been deleted. Transient expression of HO endonuclease from a galactose-inducible promoter has been shown to produce about 20% survival in wildtype cells and significantly less in NHEJ-impaired strains (*ku70Δ*) (Valencia et al., 2001). In *smc6-9* strains NHEJ was not significantly affected with over 20% cells being able to form colonies following transient HO induction (Figure 2A). We found similar levels of survival in cells deleted for the recombination protein Rad52 (Figure 2A) and the effect of *rad52Δ* and *smc6-9* were not additive (Figure 2A). Consistent with previous reports we also found that cells deleted for yKu70 show a substantial decrease in survival (Figure 2A). Survival in the *smc6-9 ku70Δ* double mutant strain was similar to that observed for the single *ku70Δ* strain (Figure 2A). Next, we employed Southern analysis of restriction fragments (Holmes and Haber, 1999) to monitor DSB repair by NHEJ in another *smc5-smc6* mutant, namely *nse3-12* cells (Figure 2B). Mutant cells grown at the permissive temperature of 23°C were either kept at 23°C or shifted to the non-permissive temperature of 37°C, following which cells were grown for another generation in order to disrupt the protein activity. Galactose was added to a final concentration of 2% for 1 h to induce a DSB, followed by the addition of glucose to repress further HO

expression. DNA collected at 90 and 180 min was analyzed on Southern blots to monitor the kinetics and efficiency of repair by NHEJ (Figure 2B). The efficiency of DSB repair at non-permissive temperatures was the same for *NSE3* and *nse3-12* cells (Figure 2B). These results are consistent with previous reports showing that *nse4* mutants are competent in religation of linearised plasmids (Hu et al., 2005). We conclude that the Smc5-Smc6 complex does not contribute to the religation of a DSB by NHEJ.

Mating type switching in *smc5-smc6* mutants

Next, we evaluated the effect of Smc5-Smc6 in the repair of DSBs by homologous recombination. In the presence of *HML* or *HMR* sequences, HO-induced DSBs in *MAT* are efficiently repaired by a HR mechanism termed gene conversion, which involves the nonreciprocal transfer of genetic information from the donor (in this case *HML* or *HMR*, which, like *MAT*, are located on chromosome III) used as the template for new DNA synthesis. We employed Southern analysis of restriction fragments (Holmes and Haber, 1999) to monitor gene conversion products in *MATa* cells switching to *MAT*α in *smc5-smc6* mutants (Figure 3; data not shown). Mutant cells were grown at the permissive temperature of 23°C and galactose was added to a final concentration of 2% to induce the DSB at *MAT*. The mutants were either shifted to non-permissive temperature (37°C) two hours prior to HO induction or maintained at 23°C and samples were taken at 1, 3 and 6 hrs following DSB induction. DNA was digested with *StyI* and the gel probed with the *MAT* distal fragment (Figure 3A). Percent switching was calculated from the ratio of the amount of HO cleavage at 1h compared to the amount of final product, normalized to the *APA1 gene* DNA in each lane. At both 23°C and 37°C we found a small reduction (<2-fold) in switching efficiency in *nse3-12* and *smc5-smc6* (not shown) mutants compared to wildtype cells (Figure 3B). These results demonstrate that inactivation of Smc5-Smc6 function only slightly reduces DSB-induced *MAT* gene conversion.

Inactivation of Smc5-Smc6 reduces sister chromatid recombination (SCR)

In our chromatin immunoprecipitation experiments, the enrichment of Smc5-Smc6 subunits was detected in cultures at G2/M phases (Figure 1B). DSBs arising during these periods of the cell cycle can be repaired by sister chromatid recombination (SCR) (Gonzalez-Barrera et al., 2003; Johnson and Jasin, 2000; Kadyk and Hartwell, 1992), a HR pathway that uses the neighboring intact sister chromatid as a template for repair. Sister recombination events are difficult to detect by genetic or physical means because sisters are identical in DNA sequence. SCR in mammalian cells has been studied predominantly by cytological identification of crossover events, but these assays do not provide a molecular picture of the repair events (Johnson and Jasin, 2000). To test whether Smc5-Smc6 affect DSBR by SCR, we have used an assay, previously developed by us (Gonzalez-Barrera et al., 2003), that allows the physical monitoring of DSB-induced recombination events occurring in the same chromatid or between sister chromatids.

The assay allows the study of recombination events between inverted repeats. It is based on two *leu2* repeats sharing 1.2 kb of homology that are located in a CEN-based monocopy plasmid. One repeat contains the *leu2-HOr* allele carrying a 21 bp HO site inserted at the internal *LEU2 EcoRI* site and placed under the control of the *tet* promoter. The other repeat is a promoterless truncated *leu2Δ5'* fragment containing the *LEU2* sequence downstream of position +150 from the translation initiation site (Figure 4A). The truncated 21 bp HO cleavage site (instead of the full 117 bp site (Pâques and Haber, 1999)) present in the *leu2-HOr* makes HO endonuclease cutting at this site very inefficient (<10%). In cells growing in galactose (when HO is induced), during or after replication, HO endonuclease cuts the 21 bp HO site of one sister chromatid leaving the other intact in over 90% of the cases (Gonzalez-Barrera et al., 2003). Therefore, the DSB in the *leu2-HOr* site can be repair using the intact chromatid (sister chromatid recombination, SCR). The efficiency of *HO* cleavage in the *leu2-HOr*

allele can be physically monitored by Southern analysis of restriction enzyme-digested DNA (digestion with *XhoI* and *SpeI*). *HO*-induced DSBs accumulate as 1.4kb and 2.4kb bands (Figure 4A-B). The 2.9-kb and 4.7-kb bands appear as the result of unequal SCE events (Figure 4A-B), which can be used as an estimate of total SCR (Gonzalez-Barrera et al., 2003). While other recombination events, such as intra-chromatid recombination, can result in a 2.9-kb band, the appearance of a 4.7-kb fragment is specific for SCE, and is therefore used as an indicator of SCE events (Gonzalez-Barrera et al., 2003). Equal sister chromatid exchange products are not detectable as they result in a 3.8kb fragment also present in the parental plasmid (Gonzalez-Barrera et al., 2003).

In order to evaluate DSB repair of *leu2-HOr* cutting in the *smc6-9* and *nse5-1* strains, mutant cells were first transferred to galactose containing media at 23°C to induce HO for 2hrs and then shifted to 37°C to inactivate Smc5-Smc6 function. We found that at 37°C *smc5-smc6* mutants showed reduced HO cleavage efficiency due to loss of viability as compared to wildtype cells (data not shown), therefore HO expression was interrupted by the addition of 2% glucose to allow us to determine the relative efficiency of repair of similar initial amounts of HO-induced DSBs in all strains. Samples were collected at different times and evaluated by Southern analysis (Figure 4B). The levels of DSB repair in the *smc5-smc6* mutants were similar to wildtype cells after 4hrs (Figure 4C; %DSBs). At this time, over 8% of DSBs in wildtype cells had been repaired by SCR (Figure 4C: %SCR/DSBs) while in the *nse5-1* and *smc6-9* mutants the percentage of DSBs repaired was reduced 4-fold (<2%) (Figure 4C: %SCR/DSBs). Therefore, inactivation of Smc5-Smc6 function significantly reduced the repair of *leu2-HOr* DSB by SCR.

The assay used reports on SCR through detection of unequal SCE events. Direct detection of equal SCE events can be monitored by the formation of dimers in plasmids (Gonzalez-Barrera et al., 2003). Dimeric plasmid formation is a consequence of the reciprocal SCE in circular plasmids (Figure 5A). We used a

plasmid that contains only a single *leu2* (*leu2-HOr*) sequence therefore dimers arise only through equal SCE and not unequal SCE (Gonzalez-Barrera et al., 2003). We used this assay to analyse the kinetics of dimer formation by Southern of undigested plasmids in wildtype and *smc5-smc6* mutant cells (Figure 5B). Cells were grown at 23°C and then shifted to 37°C simultaneously to the induction of HO cleavage. Equal SCE-induced dimers in plasmids accumulated in wildtype cells reaching 1% after 6 hours (Figure 5C; %SCE). In contrast, *nse5-1* and *smc6-9* cells showed significantly reduced levels of plasmid dimer formation (Figure 5C; %SCE). The fact that DSB formation was delayed in *nse5-1* and *smc6-9* mutants (Figure 5C; %DSB) could be partially responsible for this effect. However, even though *nse5-1* and *smc6-9* cells finally reached above 20% of DSBs at 6 hours, SCE levels remained below 3% (Figure 5C; %SCE). These results confirm that the inactivation of the Smc5-Smc6 complex prevents sister chromatid recombination.

Smc5-Smc6 complex increases recombination between different rDNA repeats, but not recombination within individual rRNA genes

Our findings demonstrate that the Smc5-Smc6 complex is required for proper sister chromatid recombination (Figures 4-5). Recently, we showed that *smc6-9* cells have defects in the stability of ribosomal DNA repeats partially dependent on the recombination protein Rad52 (Torres-Rosell et al., 2005). In repetitive regions like the rDNA the function of Smc5-Smc6 would enforce that recombination events occur within the same repeat sequence in the array rather than between different rDNA repeats, which would cause deleterious deletions. To test this hypothesis we analysed the effect of *smc5-smc6* mutants on rDNA recombination. We used a previously described genetic assay that can measure recombination within and between rDNA repeats (Kobayashi et al., 2004). First, an integration plasmid, pNOY705 cleaved with *PflmI* is introduced into rDNA (Figure 6A). The plasmid contains a functional *HIS3* gene and two incomplete fragments of *ura3*, one missing the 5'-end and the other missing the 3'-end,

within a single 35S rRNA gene in rDNA repeats (Figure 6A). Recombination between these two *ura3* fragments to form *URA3* can take place using the 248 bp region shared by both fragments. In the first assay His+ (and Ura–) transformants carrying the plasmid are plated in media lacking uracil, Ura+ recombinant formation can occur through unequal sister-chromatid recombination or alternatively, by an intrachromosomal recombination. In the second assay, Ura+ recombinant colonies are grown to saturation in SC-Ura before being plated in 5-Foa (5-Fluoroorotic acid) to analyse the frequency of Ura- cell formation (Figure 6A). Here, the loss of *URA3* can occur through several mechanisms including; (i) intrachromosomal recombination, (ii) unequal sister-chromatid recombination and, (iii) single strand annealing (SSA) (Ozenberger and Roeder, 1991). Importantly, the difference between the two recombination tests is that in the first assay, the formation of Ura+ cells, the recombination event must occurs within the same rDNA unit, whereas in the second assay, the loss of *URA3*, the recombination occurs between different rDNA units (Figure 6A). Wildtype and *smc6-9* cells carrying the incomplete *ura3* fragments were grown in complete medium to saturation and the frequency of Ura+ recombinants was determined quantitatively and by spot test under permissive conditions. Wildtype and *smc6-9* strains showed similar levels of Ura+ recombinants (Figure 6B-C), demonstrating that recombination within the same rDNA repeat is not altered in this mutant. Wildtype and *smc6-9* Ura+ recombinants were grown in media lacking uracil and plated at permissive conditions in 5-Foa to asses the frequency of Ura- cells (loss of *URA3*) in these strains. We found a ~10-fold increase in *URA3* marker loss in the *smc6-9* mutant (Figure 6B-C). We tested the effect of *rad52Δ* in our assay measuring loss of *URA3* in *smc6-9* cells. We found a reduction in *URA3* loss in *smc6-9 rad52Δ* cells compared to *smc6-9* (Figure 6C-D), since single strand annealing (SSA) is independent of Rad52 (Ozenberger and Roeder, 1991), this result demonstrates that increased loss of *URA3* in *smc6-9* cells does not occur through SSA. The levels of URA3 formation in the first assay were similar in wildtype and smc6-9

cells (Figure 6B-C), therefore, we can rule out that a general increase in recombination causes the increase in *URA3* loss observed in *smc6-9* cells. However, the fact that *smc6-9* cells show wildtype levels of *URA3* formation but increased levels of *URA3* loss demonstrates that the function of the Smc5-Smc6 complex is important to mediate repair in rDNA because it prevents recombination between different repeats in the array.

Inactivation of the replication fork barrier partially suppresses *smc5-smc6* mutants

The rDNA in budding yeast contains a natural and efficient replication fork barrier sequence (RFB) that actively blocks the progression of DNA replication forks when bound tightly by a specific protein called Fob1 (Brewer et al., 1992; Olavarrieta et al., 2002; Takeuchi et al., 2003). Fob1 is also necessary for changes in rDNA repeat copy number and the formation of extrachromosomal ribosomal circles (ERCs) containing one or more rDNA units (Defossez et al., 1999; Kobayashi et al., 1998). These effects have been proposed to arise following the formation of DSBs in forks arrested at the RFB site. Such DSBs have been shown to occur during every S phase (Burkhalter and Sogo, 2004; Weitao et al., 2003), thus their incorrect repair by homologous recombination with another repeat unit could lead to contraction and expansion of the rDNA array as well as to the formation of ERCs.

Despite the occurrence of DSBs every cell cycle (Burkhalter and Sogo, 2004; Weitao et al., 2003) yeast strains maintain a stable rDNA copy number over many generations, suggesting that the majority of these breaks are repaired by error-free mechanisms that do not change the number of repeat units in rDNA, such as SCR. The role of Smc5-Smc6 in SCR (Figure 4-5) and its effect in preventing recombination between different rDNA repeats (Figure 6), prompted us to investigate whether the Smc5-Smc6 complex is present at the RFB site within rDNA. Previously, we showed that Smc6 localises to rDNA regions

cytologically and to the rDNA non-transcribed spacer (NTS) region by ChIP (Torres-Rosell et al., 2005). However, we did not assess the relative binding of this complex within the rDNA sequences. Here we have focused our analysis on the region surrounding the RFB site (Figure 7A). Notably, we found that the strongest binding of Smc6 coincided with the RFB site (Figure 7A: probe 15). Smc6 was also significantly enriched around the ARS region (Figure 7A: probes 21 and 23) and the 35S rRNA gene (Figure 7A: probes 28 and 4). Importantly, deletion of *FOB1* significantly reduced the binding of Smc6 to the RFB site (Figure 7A), demonstrating that fork arrest is important for Smc6 binding to the site. Furthermore, *smc6-9* thermosensitivity is slightly suppressed when *FOB1* is removed (Figure 7B), although to a lesser extent than the effect that we reported earlier for *rad52Δ* (Torres-Rosell et al., 2005). Therefore, localisation of Smc6 to the RFB site might be important for the stability or processing of forks arrested at this site. Interestingly, Smc6 binding to the end of the 35S region (Figure 7A; probe14) was low in *FOB1* cells but high in *fob1Δ* (Figure 7A; probe14).

Increased gross chromosomal rearrangements (GCR) in *smc5-smc6* mutants due to chromosomal translocations

SCR is thought to be an important mechanism for preventing genomic instability. If SCR is not used, a DSB arising at a collapsed fork may recombine or rejoin with a heterologous locus resulting in a chromosomal translocation or deletion. Our results demonstrate that Smc5-Smc6 function is important to enforce appropriate recombinational repair by SCR and that this function is particularly important in repetitive regions. In single copy regions, recombination due to breaks induced by DNA replication is difficult to study in a physiological setting, as stalling of DNA polymerase complexes at sites of abnormal DNA structures or DNA damage is stochastic and rare. We have previously shown that the lethality of *smc6-9* mutants is not suppressed by deleting the rDNA repeats from the genome (Torres-Rosell et al., 2005). Therefore it is likely that the function of Smc5-Smc6 in the regulation of DSBR by SCR is required in stalled/collapsed

forks outside rDNA, and through this function Smc5-Smc6 could play an important role in preventing genomic instability. To test this, we investigated the rates of spontaneous gross chromosomal rearrangements in the absence of exogenous DNA damage in *smc6-9* and *nse3-1* cells grown at permissive temperature. We used an assay that measures the rate of accumulation of GCRs that simultaneously delete a region of chromosome V containing *CAN1* and *URA3* markers integrated in *HXT13* 8.5kb telomeric to *CAN1* resulting in the production of Can^r, $5\text{-}FOA^r$ cells (Myung et al., 2001). *smc6-9* and *nse3-1* mutants showed an increase of ~50-100 fold in GCR rate which was dependent on the homologous recombination machinery (Table 1). To further investigate the rearrangement mechanisms that underlie the increased rates observed, the breakpoint sequences of 14 independent clones were determined and classified. Previous studies using this assay described 3 classes of GCRs: (i) deletion of chromosome V arm combined with addition of a new telomere, referred to as telomere additions, (ii) nonreciprocal translocations with microhomology at the rearrangement breakpoint, and (iii) nonreciprocal translocations with nonhomology at the rearrangement breakpoint (Chen and Kolodner, 1999). Most rearrangements observed in wildtype strains are telomere additions (Table 2). In contrast, the rearrangements observed in *smc6-9* mutants were predominantly nonreciprocal translocations with microhomology (Table 2). *smc6-9* mutants did not show alterations in telomere length compared to wildtype cells (data not shown), thus making unlikely a role in telomere addition pathways. Therefore, our GCR results are consistent with Smc5-Smc6 normally playing a role in mediating error-free recombinational repair between sister chromatids.

Discussion

In the present study we show that the Smc5-Smc6 complex plays a crucial role in promoting recombinational repair with the aligned, identical sister chromatid in order to deliver error-free repair (Figure 8). We have demonstrated that this function is necessary in both repetitive (rDNA) and single copy regions to ensure

genomic stability through generations. We show that Smc5-Smc6 role in SCR is particularly important to ensure that recombination events in tandem repeat loci take place within the same repeat unit and not between different repeats. The Smc5-Smc6 role in promoting SCR is also crucial in sites that have partial homology with regions in other chromosomes. Non-sister recombination at a lesion appearing in a fork while replicating through repetitive sequences can lead to intrachromatid recombination, and the associated deletion of the intervening sequence. On the other hand, recombination between similar sequences in different chromosomes can result in translocations between heterologous chromosomes, or the production of a dicentric chromosome, when the event is accompanied by crossover. We have presented evidence demonstrating that in *smc5-smc6* mutants both deletions between different rDNA repeats (Figure 6) and translocations between different chromosomes through sequences with low homology (translocations with microhomology, Table 2) occurred at a high frequency. These results highlight the importance of the complex in maintaining genomic stability in eukaryotic cells.

Recent data in *S. pombe* has shown that recombination proteins are recruited to forks arrested at replication barriers to promote recombination events that allow cells to survive (Lambert et al., 2005). The study also revealed that recombination in a stalled/collapsed fork is a doubled-edged sword, as it can promote site-specific chromosomal rearrangements thus jeopardizing the integrity of the genome. Further evidence that recombination can be stimulated by fork arrest comes from studies in the budding yeast rDNA showing that the replication fork barrier (RFB) is a hotspot for recombination events that can expand or contract the number of copies in the array (Takeuchi et al., 2003). Here we have shown that the Smc5-Smc6 complex locates to the RFB site in rDNA, where breaks are known to occur in every cell cycle (Burkhalter and Sogo, 2004), and it is required to prevent rDNA instability by preventing recombination events between different rDNA repeat units. The Smc6 enrichment to the rDNA

RFB site and its recruitment to HO-induced DSBs strongly suggest that the complex might promote the correct repair of forks stalled/broken at this site through recombination within the same repeat unit by SCR. Consistent with a general function in the rescue of broken forks, Sjögren and colleagues have shown (accompanying paper) that Smc5-Smc6 is specifically recruited to HU stalled forks only if they suffer collapse.

In the present study we demonstrate that the Smc5-Smc6 role in recombinational repair is initiated by the recruitment of the complex to sites of DSB. We show that different subunits are recruited to DSBs induced by the HO endonuclease during G2/M stages of the cell cycle. Previous reports have demonstrated that cohesin, a related complex, is also loaded around a DSB lesion (Strom et al., 2004; Ünal et al., 2004). Furthermore, these studies showed that postreplicative repair indeed requires DSB-induced cohesin binding to promote local sister chromatid cohesion (Strom et al., 2004; Ünal et al., 2004). The binding pattern of cohesin is similar to that of the Smc5-Smc6 complex. The region of highest cohesin binding upon *MAT* DSB induction locates at around 8kb from the break site while Smc5-Smc6 binding peaks are 5kb away from the break on either side (this study).

Studies using Cre-mediated recombination have demonstrated that the physical distance between LoxP sites has a direct effect on the rate of recombination (Burgess and Kleckner, 1999). Therefore, assuming that the same is true for physiological recombination events, cohesin recruitment to DSB is likely to reinforce the association of the broken end to the sister chromatid thereby providing a bias for SCR and preventing the highly-reactive ends from undergoing aberrant recombination with heterologous sequences. However the functional role of cohesins in SCR remains to be demonstrated. The question arises as to whether the Smc5-Smc6 complex is a passive/structural element in SCR or instead plays an active/inducible role in promoting error-free SCR while suppressing illegitimate recombination. Our study does not rule out either of

these two options. However, there are several characteristics of the Smc5-Smc6 complex that suggest a regulatory role. Unlike cohesin, the complex contains subunits that carry out enzymatic activities specifically in response to DNA damage (Andrews et al., 2005; Zhao and Blobel, 2005) raising the possibility that the complex is recruited to DSB to regulate the activity of target proteins already at the break site. In addition, *smc5-smc6* mutants do not exhibit phenotypes consistent with premature loss of cohesion (Torres-Rosell et al., 2005). Therefore, these observations suggest that if the Smc5-Smc6 complex plays a passive/structural role through a function in sister chromatid cohesion, this would be restricted to local cohesion at damaged sites. Interestingly, the inactivation of cohesin has the same effect on rDNA recombination as that shown here for *smc5-smc6* mutants, namely the increase in recombination rates between different rDNA repeats with no changes in recombination within the same repeat unit (Kobayashi et al., 2004). This raises the possibility that Smc5-Smc6 might enforce SCR and recombination within the same rDNA unit by recruiting cohesin to DSBs occurring at forks stalled at the RFB site.

Replication forks stall or collapse at DNA lesions or problematic genomic regions, and these events have often been associated with recombination and chromosomal rearrangements. Unlike the bacterial models, the role of recombination at stalled forks is poorly understood in eukaryotes. It is presently unclear why recombination at collapsed forks can, under some circumstances, rescue replication while in other cases it generates genomic rearrangements. Here, we have shown that the Smc5-Smc6 complex ensures correct repair by directing DSBs towards SCR repair pathways and consequently suppressing inappropriate non-sister recombination events (Figure 8). We show that this is particularly important for forks blocked at the repetitive RFB site in rDNA, previously shown to be a hotspot for recombination (Takeuchi et al., 2003), due to the presence of surrounding repeats. Our results reveal that this enigmatic

complex is an important factor that regulates repair of DSBs through SCR to minimise genome instability across eukaryotes.

Experimental Procedures

Yeast Strains and Plasmids

All yeast strains were derivatives of the wildtype strain AS499 (S288C) (Torres-Rosell et al., 2005), except for those in Figure 4-5 that are in the W303 background. Yeast strains for the induction of a DSB are derivatives of JKM139 or JKM179 (J. Haber). Epitope tagging for Smc6, Nse5 and Ddc2 at their C-terminus was performed using PCR-based methods (Knop et al., 1999). The *nse5-1, nse3-1* and *nse3-12* temperature sensitive alleles were introduced into strains using a PCR-based allele replacement method.

Generation of temperature sensitive alleles

The *nse5-1*, *nse3-1* and *nse3-12* alleles were generated by random mutagenesis PCR. Briefly, genomic DNA was isolated from strains with an *NSE5* or *NSE3* gene tagged with the *9myc:TRP1* epitope. The genes plus the epitope and the *TRP1* marker was PCR amplified with a high fidelity DNA polymerase. The amplified sequence was re-amplified in 5 parallel reactions using the GeneMorph PCR mutagenesis Kit (Stratagene). The mutagenized PCR products were pooled and used to transform a wild type strain. Transformants were selected for growth in SC medium lacking tryptophan at 25°C, and tested for temperature sensitivity at 37°C. Temperature sensitive colonies were tested for correct integration of the epitope by PCR and western blot, and for rescue of the ts phenotype by a centromeric plasmid carrying the corresponding wild type *NSE* gene.

Growth Conditions and Cell Synchronization

Exponentially dividing cell cultures were grown in YEP media with 2% glucose, 3% glycerol or 2% lactic acid. Cell cycle arrests in G1 were mediated by treatment for 2 hours with 10-8 M α-factor (SIGMA) at 25°C. To release mutant

cells from this block, cells were first shifted to 37°C for 45 min to inactivate temperature sensitive alleles and then washed twice with pre-warmed medium and re-suspended in YPD plus 0.1mg/ml pronase E (SIGMA).

Culture Conditions for *HO* Induction

In strains with a stably integrated *GAL10::HO* sequence, cells were grown in YEP media with 2% lactic acid or 2% raffinose. Cells were then arrested as described above, and subsequently galactose (Sigma, 20% w/v stock) was added to the culture at a final concentration of 2% w/v to induce HO expression.

Analysis of DSB repair, NHEJ, *MAT* switching and SCE

MAT switching was analysed as described before (Holmes and Haber, 1999). Physical analysis of SCR has also been described previously (Gonzalez-Barrera et al., 2003). Purified genomic or plasmid DNA was digested with the appropriate restriction enzymes, separated on a 1.4% native gel, and probed with a 32P-labeled *MAT* distal fragment or the 1.18kb *ClaI-SspI* internal *LEU2* fragment. Southern blots were scanned by PhosphorImager, and the repair efficiency was calculated as described before (Holmes and Haber, 1999). NHEJ experiments were performed as in (Valencia et al., 2001). NHEJ was examined in donorless strains. Re-cutting of *MATa* by HO was prevented by filtering cells out of galactose-containing medium 90 min after DSB induction and diluting cells into YP-dextrose. (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose, pH 5.5). The efficiency of NHEJ was determined as the intensity of the MATa-containing restriction fragment 3 h after HO induction, normalized to the amount of DNA. The SCR assays were performed as described earlier (Gonzalez-Barrera et al., 2003).

Microscopy

Yeast cells with GFP-tagged proteins were analyzed by fluorescence microscopy after DAPI staining. For fluorescence microscopy, series of z-focal plane images

were collected on a Leica IRB using a Hamamatsu C4742-95 digital camera and OpenLab software (Improvision). A tuneable light source (Polychrome IV) with a Xenon lamp was used. Images in different z-axis planes were flattened into a two-dimensional projection and processed in Openlab. DNA was stained using 4, 6,-Diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR) at 1 μg/ml final concentration after short treatment of the cells with 1% Triton.

Chromatin Immunoprecipitation

To fix cells, yeast strains were treated with 1% formaldehyde. Time course analyses to optimize fixation times at 25°C showed the optimal times for Nse5p and Smc6p to be 2 hr. Fixed cells were harvested, washed, and resuspended in ice-cold buffer I (140mM NaCl, 1mM EDTA, 1% (v/v) Triton-X, 0.1% (w/v) sodium deoxycholate, and 50mM HEPES/KOH at pH 7.5) supplemented with protease inhibitors cocktail (Complete, Roche). Cells were broken with a FastPrep machine (Qbiogene) for four rounds of 30s each with intervening incubations on ice for 5min. Cell extracts were separated from the glass beads and sonicated (in a Bioruptor) for twelve rounds of 15s with intervals of 45s. This procedure fragments chromosomal DNA to an average size of ~500bp. Purified anti-HA 12CA5 or anti-MYC 9e10 monoclonal antibodies (Roche) were used at 5μg per 50ml of initial culture. The antibodies were incubated with either protein A/G-agarose beads (Roche) for several hours before use. Sonicated cell extracts were first precleared with protein A/G-agarose beads and then incubated overnight at 4°C with the corresponding antibody beads. The beads were then washed three times for 15 min in buffer I, followed by three washes for 15 min in buffer II (500mM NaCl, 1mM EDTA, 1% (v/v) Triton-X, 0.1% (w/v) sodium deoxycholate, and 50mM HEPES/KOH at pH 7.5) and three further washes also for 15 min in buffer III (250mM LiCl, 1mM EDTA, 0.5% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 10mM Tris-Cl at pH 8.0). All wash buffers were kept at 4°C and supplemented with the above-described protease inhibitors cocktail. The beads were then transferred to a fresh tube and washed with TE

(1mM EDTA and 10mM Tris at pH 7.6). Immunoprecipitated material (IP) was incubated in elution buffer (1% SDS, 10mM EDTA, 50mM Tris-Cl at pH 8.0) at 65°C for 10min. The IP material was then separated from the beads and further incubated overnight at 65°C to reverse the formaldehyde crosslinks. Aliquots of total chromatin solution were similarly heat-treated (whole cell extract). After treatment with proteinase K (Qiagen), material was then treated with RNase (Qiagen) and finally purified through a DNA binding spin mini-column (Qiagen).

PCR Analysis of Co-immunoprecipitated DNA

The whole cell extract (or total input) and IP samples were analyzed by PCR. First, dilutions of the total input were analyzed for every pair of primers to identify the dilution at the linear range and hence determine the initial amount of DNA. PCR products were separated on 2% agarose gels and visualized with ethidium bromide. PCR products were quantified using ImageQuant software. All the primers used for the analysis of *MAT* DSBs have been described before (Shroff et al., 2004). The primers for the rDNA analysis have been described earlier (Huang and Moazed, 2003). Primer sequences for the analysis of the DSB on chromosome VI are available on request. The values are given as a percentage of material immunoprecipitated (ChIP/input).

Acknowledgements

We are thankful to M. Nomura for reagents. We thank C. Sjögren, P.R. Potts and H. Yu for communicating results prior to publication. The Medical Research Council UK supported the Aragon laboratory. Ministerio de Educación y Ciencia, Spain (grant SAF2003-00204) funded the work from Aguilera's lab. Funding for the Haber lab was from NIH grant GM20056. F.C.-L. was a fellow from Fondo de Investigaciones Sanitarias, Spain. G.I. was a Charles A King Medical Foundation of Boston fellow. S. U. was a Marie Curie Fellow from the EU.

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Figure Legends

Figure 1. Smc5-Smc6 complex is enriched around a HO induced DSB. (A-B) A DSB was induced at *MAT* in a strain expressing *DDC2-GFP* to test the efficiency of the cut. Cells were collected at indicated times following transfer to galactose media and scored for the presence of Ddc2 fluorescent foci. The majority of cells arrested in G2/M and exhibited a single focus of fluorescence after 120min. (C) The binding of Smc6 around *MAT* on chrm-III in JKM179 (Smc6-9myc) with (cut) or without (uncut) a DSB at *MAT*. Cells were grown at 30°C, the culture was split, and galactose was added to one half (cut), whereas the other half was grown in the absence of galactose (uncut) before being processed for ChIP. Input DNA and DNA co-immunoprecipitated with anti-myc antibody (IP) were amplified using primer sets corresponding to sequences at different distances from the *MAT* cut site as indicated. Percentage of input chromatin in the immunoprecipitates is plotted on the y axis versus coordinates (in kb) relative to the HO site in chromosome III on the x axis. Smc6 binding to the *MET6* locus is shown as a control for a site located in a different chromosome (chrm-V). The average of 3 independent experiments with its corresponding standard deviation is shown. (D) Representative ethidium bromide stained gel of PCR products (from C). (E) The binding of Smc6 around an HO site located on chrm-VI in JKM179 (*SMC6-9myc*) with (cut) or without (uncut) a DSB. Cells were treated as in C. The average of 3 independent experiments with its corresponding standard deviation is shown.

Figure 2. Inactivation of Smc5-Smc6 does not affect DSB repair by Non-Homologous End Joining. (A) End-joining assays with transient HO induction (2hrs) in wildtype and mutant strains. Percentage cell survival is shown. (B) NHEJ was examined in *NSE3* (wildtype) and *nse3-12* cells. Re-cutting by HO was prevented by filtering cells out of galactose-containing medium (+GAL) 30 min after DSB induction and diluting cells into YP-dextrose (+GLU). The efficiency of NHEJ was determined as the intensity of the *MATa*-containing

restriction fragment 180 min after HO induction, normalized to the amount of DNA

Figure 3. *smc5-smc6* mutants undergo *MAT* switching. (A) *MAT* switching assays in wildtype and *smc5-smc6* mutant strains. DNA extracted at intervals shown after HO cutting was digested with *StyI* and separated by gel electrophoresis. Southern blots were probed with a 32P-labeled *MAT* distal fragment. The 1h time-point represents 1h of galactose induction of the *HO* endonuclease. The parental, switched and HO cleavage products are indicated. (B) Percent switching was calculated from the ratio of the amount of HO cleavage at 1h compared to the amount of final product, normalized to the *MAT* distal DNA in each lane.

Figure 4. Inactivation of Smc5-Smc6 reduces DSB repair by sister chromatid recombination (SCR). (A) Scheme of repair by SCR of an HO-induced DSB in plasmid pRS316-TINV. Fragments generated by HO cleavage and *XhoI SpeI* digestion, as detected by the *LEU2* probe (lines with asterisks), are indicated with their corresponding size. Since other recombination events can also lead to a 2.9-kb fragment, only the 4.7-kb band is used for the measurement of SCR. (B) Southern analysis showing the kinetics of HO-induced SCR in wild-type, *nse5-1* and *smc6-9* cells. DSBs were induced for 2h, cultures were placed at 37°C to inactivate mutant proteins, and 2% glucose was added to repress HO expression. DNA samples were taken at different times in order to monitor DSB repair by SCR. Bands expected by *XhoI SpeI* digestion are shown with their corresponding sizes. (C) Quantification of HO-induced DSB repair by SCR in wild-type, *nse5-1* and *smc6-9* cells. DSB disappearance (2.4kb and 1.4kb bands related to total plasmid DNA; left), and SCR products accumulation (4.7kb band related to initial DSB; right) is shown. The average of 3 independent experiments with its corresponding standard deviation is shown.

Figure 5. Smc5-Smc6 is required for equal SCR. (A) Schematic representation of the monomeric plasmid pCM189-*leu2HOr*, containing no DNA repeats, and the dimer obtained by equal SCR. (B) Southern analysis of the recombination intermediates obtained after HO expression in wild-type, *nse5-1* and *smc6-9* cells. The cultures were placed at 37°C to inactivate mutant protein, and DNA samples were taken at different times after HO induction and analyzed by Southern. Plasmid DNA was not digested prior to electrophoresis. rD indicates relaxed dimers; scD, supercoiled dimers; rM, relaxed monomers; lM, linear monomers; scM, supercoiled monomers. (C) Quantification data of DSBs (lM related to total plasmid DNA; top), and dimeric DNA molecules (scD and rD related to total plasmid DNA; bottom). The average of 3 independent experiments with its corresponding standard deviation is shown.

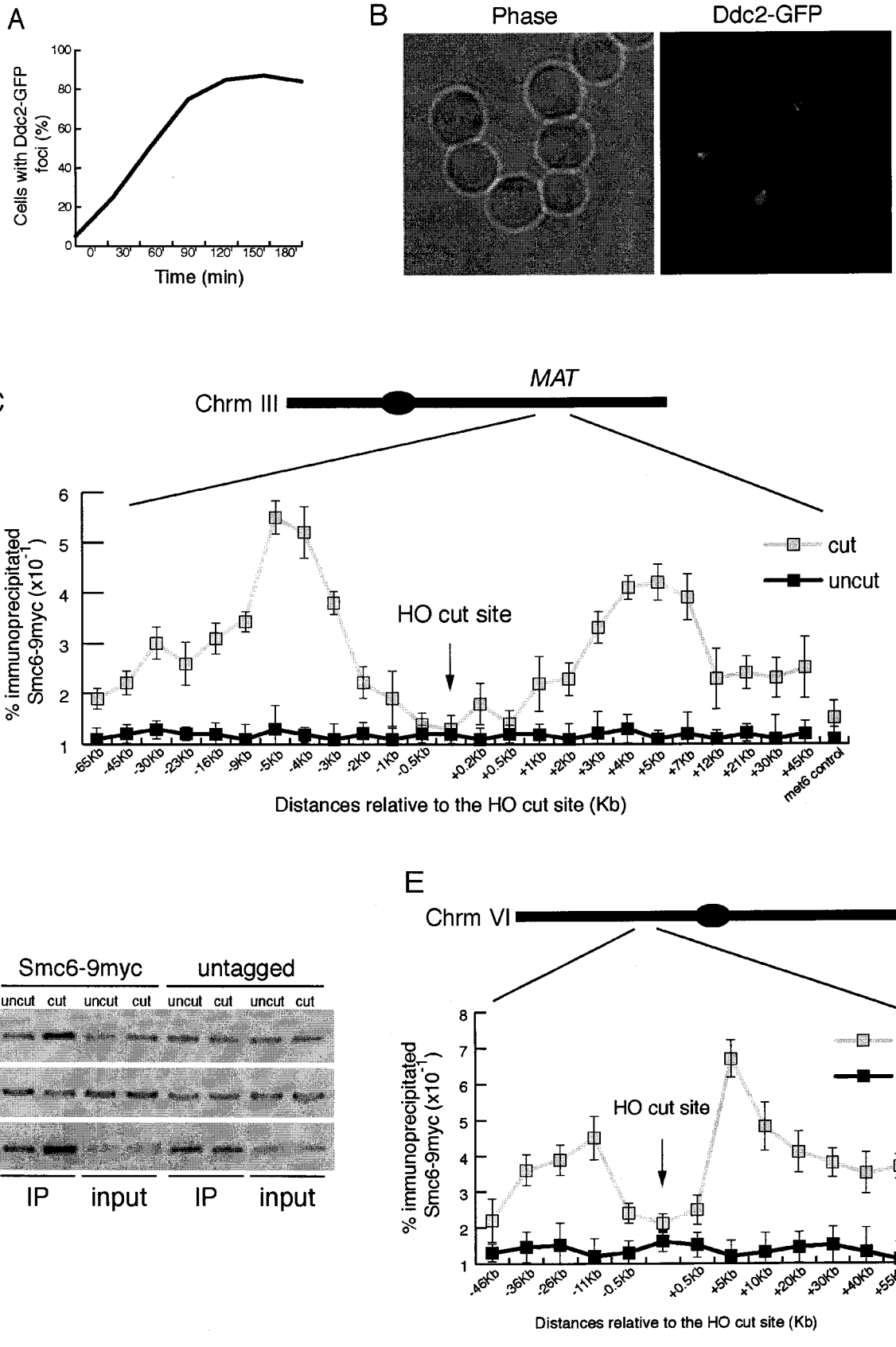
Figure 6. Smc5-Smc6 complex increases recombination between different rDNA repeats, but not recombination within individual rRNA genes. (A) Structure of plasmid pNOY705 and its use for integration into rDNA. The first assay of Ura+ generation measures the level of recombination within a single rDNA repeat, while the second assay, loss of URA+ marker, measures recombination between different rDNA repeat units. (B) Quantification of Ura+ and ura-(FOA^r) colony formation (as in A) in *smc6-9* cells relative to wildtype cells. (C) Wildtype and *smc6-9* strains carrying pNOY705 integrated in the same position within the rDNA array were grown overnight in SC-His medium to saturation. 5fold serial dilutions were spotted onto SC and SC-Ura plates to observe the frequency of Ura+ recombinants. In the second assay, wildtype and *smc6-9* Ura+ recombinant colonies from the first assay were grown overnight in SC-ura medium to saturation. 5-fold serial dilutions were spotted on SC and FOA plates. (D) *rad52Δ* and *smc6-9 rad52Δ* Ura+ recombinants were grown overnight in SC-ura medium to saturation. 10-fold serial dilutions were spotted on SC and FOA plates.

Figure 7. Inactivation of replication fork barrier (RFB) partially suppresses *smc6-9* temperature-sensitive growth. (A) Wildtype and *fob1Δ* cells expressing *SMC6-6HA* or an untagged *SMC6* gene were grown exponentially at 30°C, fixed and processed for ChIP. Input DNA and DNA coimmunoprecipitated with anti-HA antibody (IP) were amplified by using primer sets corresponding to sequences around rDNA as shown. Percentage of input chromatin in the immunoprecipitates is plotted on the y axis versus coordinates in a rDNA unit as shown. The average of 3 independent experiments with its corresponding standard deviation is shown. (B) Wildtype, *smc6-9*, *fob1Δ* and *smc6-9 fob1Δ* cells were grown at 23°C in liquid media. Five-fold serial dilutions were plated and incubated at the indicated temperatures.

Figure 8. Smc5-Smc6 function promotes DSB repair by SCR and suppresses non-sister recombination. DSB can arise during DNA replication as a consequence of breakage or collapse of blocked replication forks. Recruitment of the Smc5-Smc6 complex to such double strand breaks promotes repair of the broken DNA by sister chromatid recombination (SCR), most probably by creating a structure that holds both sister chromatids perfectly aligned or by regulating the function of recombination proteins at the DSB site. *smc5-smc6* mutants are not able to promote recombinational repair of the DSB by error-free sister chromatid recombination and instead other error-prone recombinational repair pathways take place. As a consequence, *smc5-smc6* mutants display a higher frequency of gross chromosomal rearrangements, including chromosomal translocations and deletions.

Figure1

Figure 1



E) Figure2

Figure 2

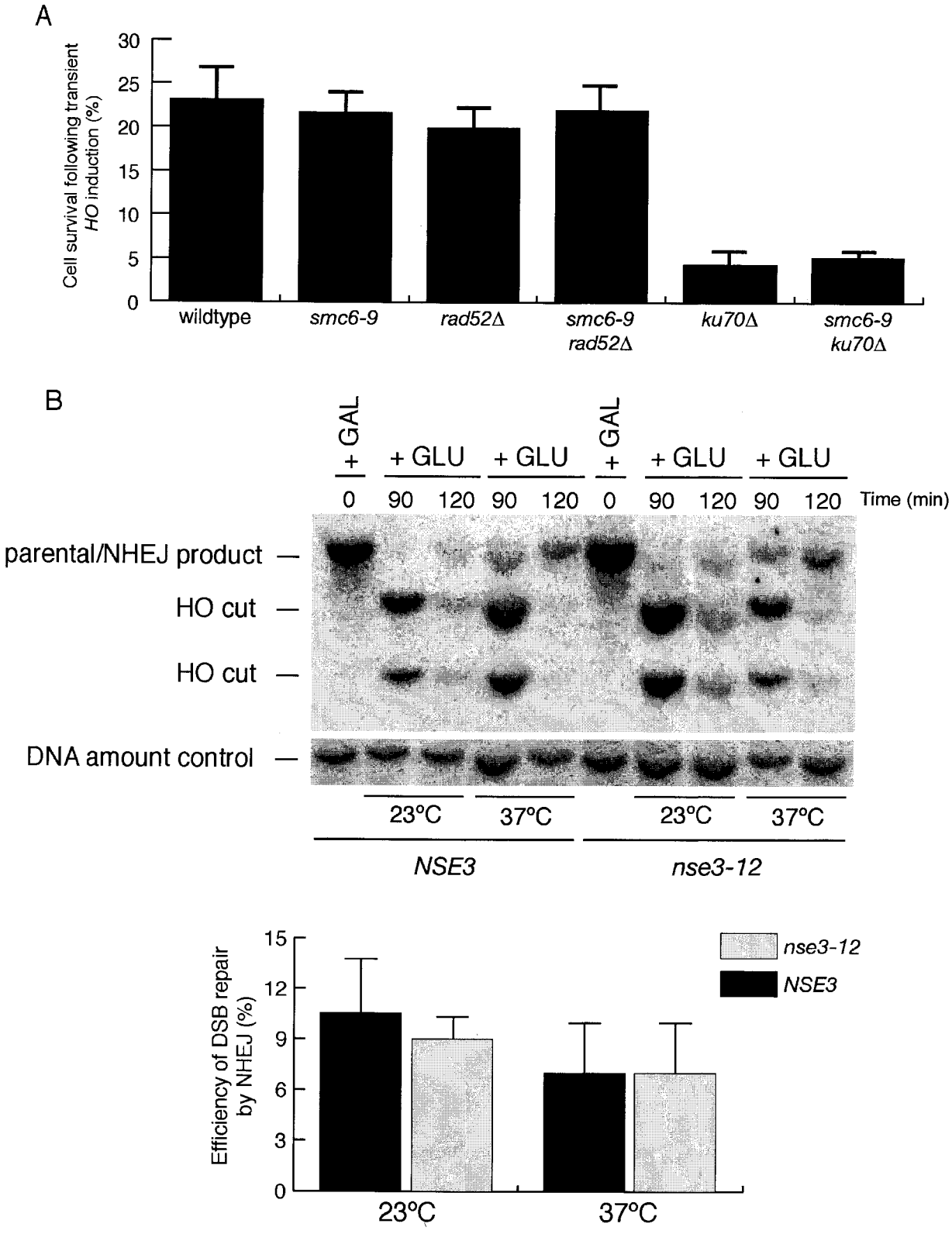
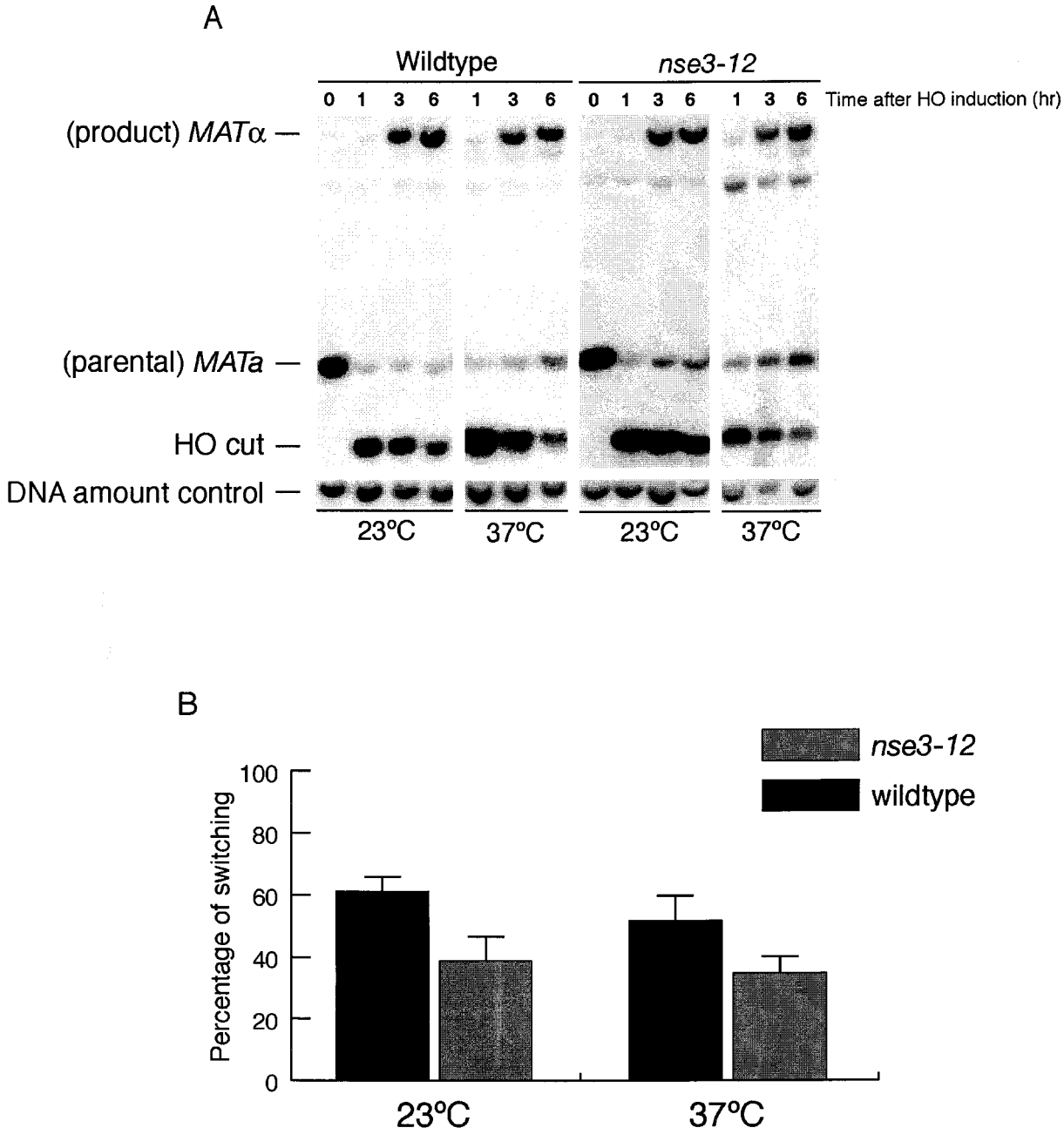


Figure3

Figure 3



E) Figure4

Figure 4

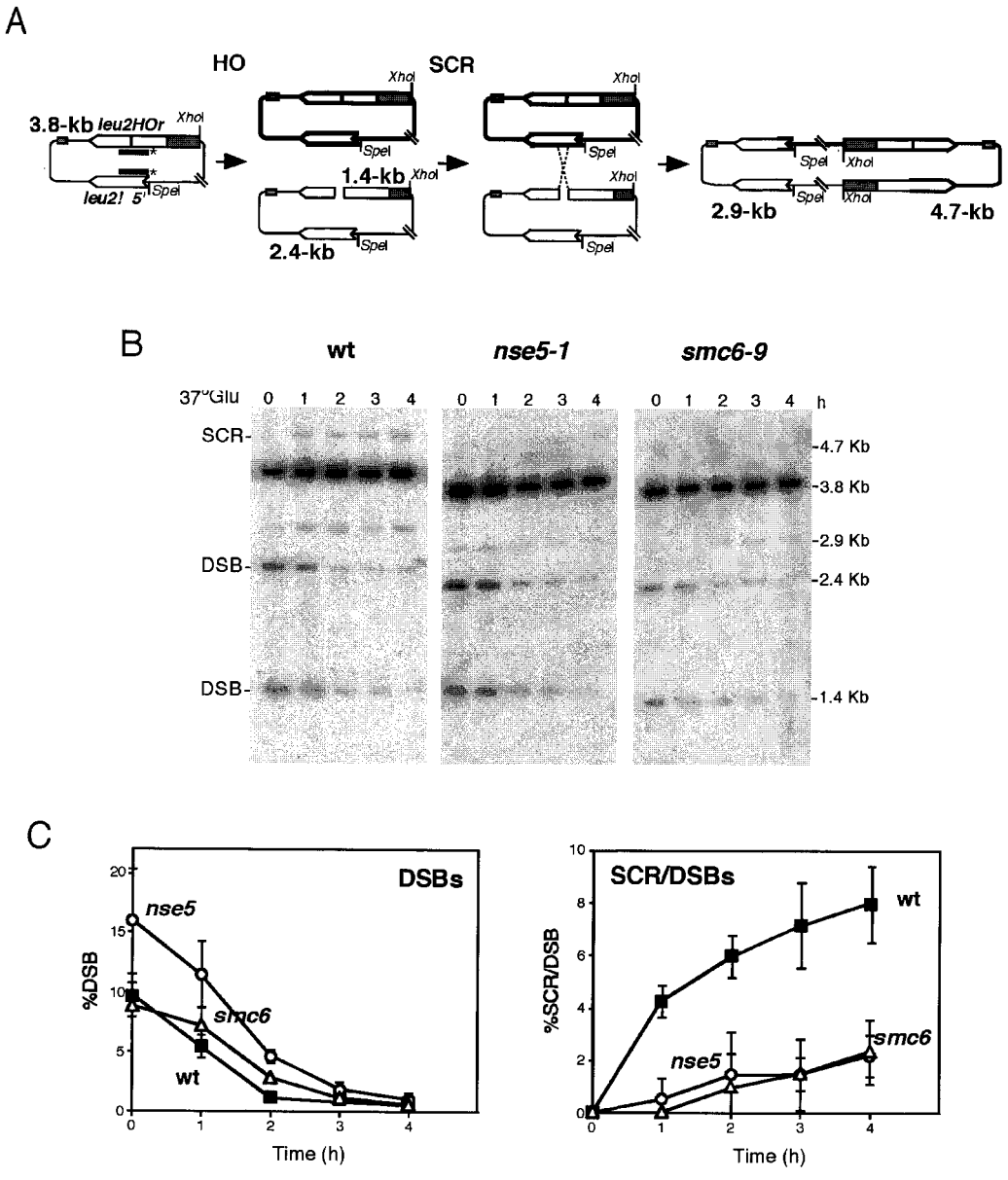
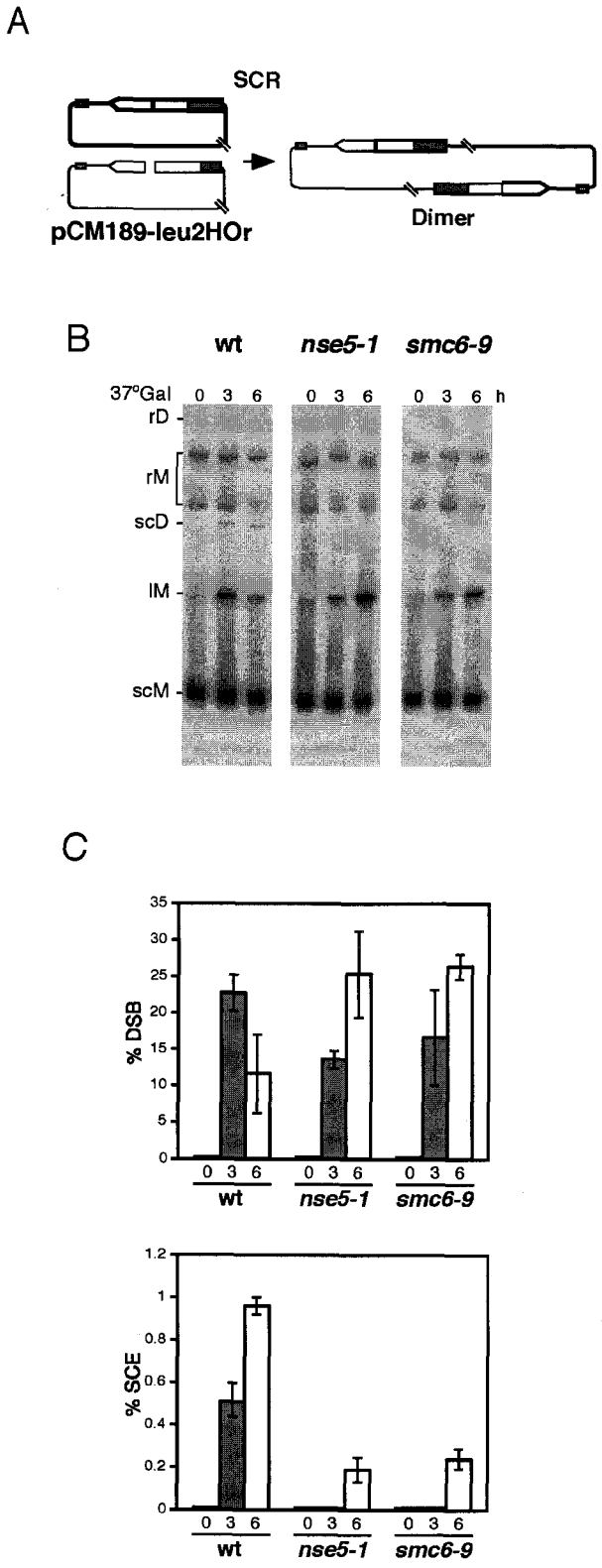


Figure 5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E) Figure6

Figure 6

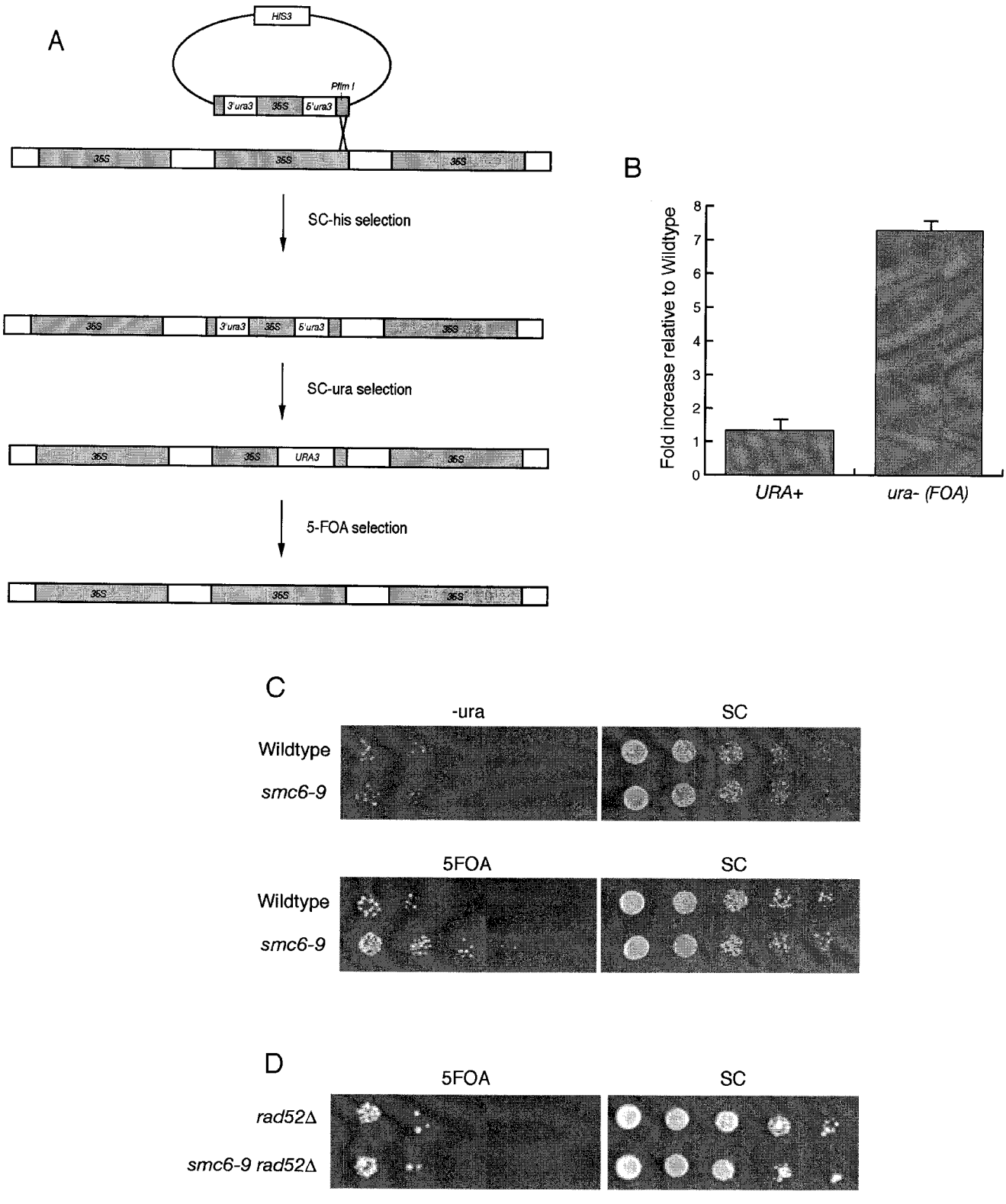


Figure7

Figure 7

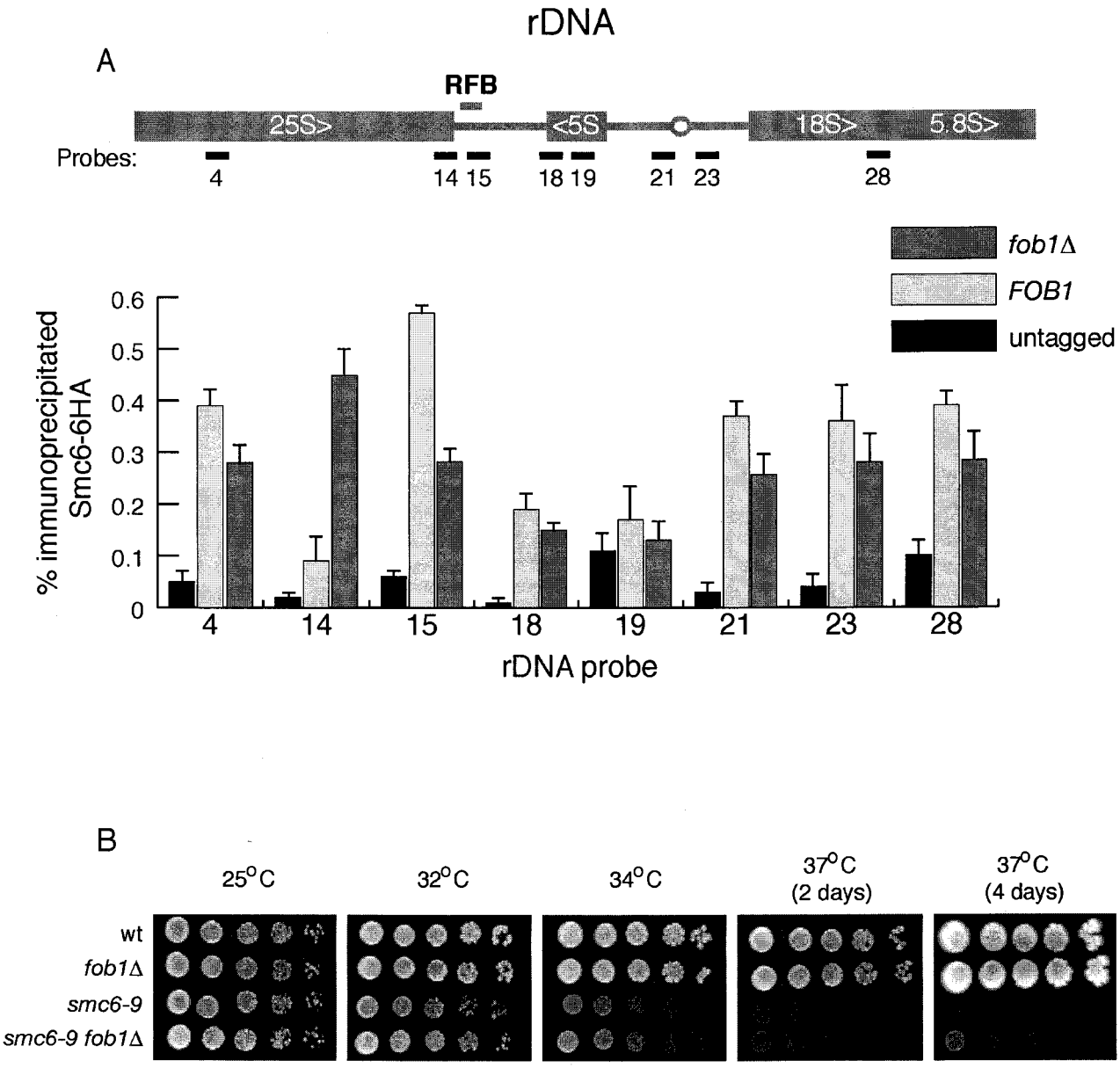


Figure 8

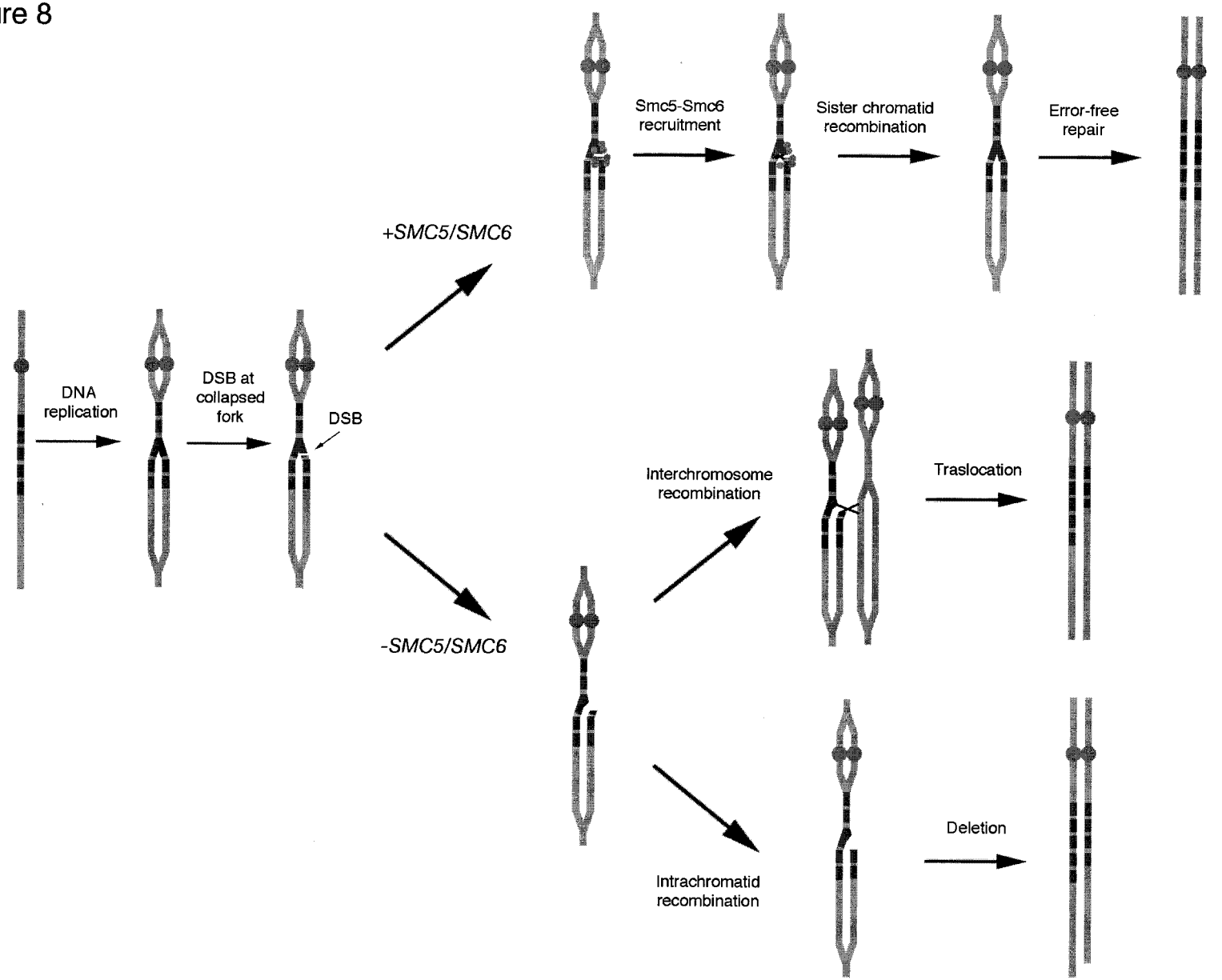

DNA replication
DSB at collapsed fork
DSB
+SMC5/SMC6
-SMC5/SMC6
Smc5-Smc6 recruitment
Sister chromatid recombination
Error-free repair
Interchromosome recombination
Traslocation
Intrachromatid recombination
Deletion

Table 1. The inactivation of the SMC5-6 complex increased GCR formation and the enhanced GCR is dependent on homologous recombination.

Relevant Genotype	Wild type		*pif1-m2*		*rad51Δ*	
	Strain	GCR rate (Can^r 5-FOA^r)	Strain	GCR rate (Can^r 5-FOA^r)	Strain	GCR rate (Can^r 5-FOA^r)
Wild type	RDKY3615	3.5×10^{-10} (1)	RDKY4343	4.8×10^{-8} (137)	RDKY3636	3.5×10^{-9} (10)
smc6-9	YKJM3088	2.7×10^{-8} (76)	YKJM3091	6.3×10^{-7} (1808)	YKJM3141	3.7×10^{-9} (11)
nse3-2	YKJM3539	1.9×10^{-8} (54)	YKJM3758	3.0×10^{-7} (868)		NA

All strains are isogenic with the wild type strain, RDKY3615 [*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069::URA3*] with the exception of the indicated mutations. The numbers in parenthesis indicate the fold induction of GCR rate relative to wild type GCR rate. NA, not available.

Table 2. The *smc6-9* mutation preferentially enhanced the translocation class GCR formation.

Relevant Genotype	Strain	Telomere addition	Translocation		Chromosome fusion
			Nonhomology	Microhomology	
Wild type	RDKY3615	5 (2.9×10^{-10})	1 (6.0×10^{-11})	0	0
smc6-9	YKJM3088	4 (7.2×10^{-9})	1 (1.8×10^{-9})	10* (1.8×10^{-8})	0

de novo telomere addition

GGTGTTACTACTAGGATTTGGCGTGGATGAAGGACCTGCAGTGGAGggtgtggtgtggtgtgggtgtgtgggtgtgtgggtgtggg [V34847]
CTTTTAGTATAACCCTGAAATTTGCCCTATAGAAATCTAGGGTTTCtgtgtggtttccgggtgtgtgtgggtgtgtgggtgtggtg [V33837]
TGCTGCTTCTCATATCCTTTGTATCCTTTTCGGATGTAGGATGAGTtggtgtggtgttactactaggtgtgggtgtggtgtgggtg [V34899]
AATGATGATGATAATGATGATAAGAAGAAGAAGCCTCACCATAAGGgtgtggtgtgtgtgggtgtggtg [V41581]

Microhomology Translocation

V36520 ATGAGGGCTCGAGAAAAATGTTAAAGATTGAATAGTTGATATTGCCTTTC:AGTCGAATATATATTCAAACTAGTGGTTAATAAAAACAAAGTATGTAAA
XIV711857 CCCATTGGTATAGGGCTGGTGGTCAGCACGATAAGTGTCCAAAGACTAGT:GGTAAACTACACAGAATGTGATGCATTGGCACCGGCGAAGCATTTTGAA

V40276 TTTCGAATTGGGCCAGGATTCTTTGACTCCTCAGCAATTGAAAAAGAAGC:TAATTCATTACAGTACAAAGGACATTTTGTTCGATATCCCTGAAGACAC
VI268962 CTCAGCTGAGATAAGTAATATCGTTGATGAATTACAAGGGAACAATGAGC:AGAGGGAAAATGGACAGATCCTTTCGCATTCCTACTTGTTTGATGAGTT

V33212 ATGGCATAGGTGATGAAGATGAAGGAGAAGTACAGAACGCTGAAGTGAAG:AGAGAGCTTAAGCAAAGACATATTGGTATGATTGCCCTTGGTGGTACTA
II637281 ATGCGGCCAGACATAGTTTGTCCCAAATTGGTATTGTTCTACCCACTAAT:TGGTACCCACCAGGACTGTTAGCAGCATATATGCACATATACATACCGC

V32092 TACTGGTGGTGACAAAGTTTTCGAATGGCTATTAAATATCACTGGTGTT:GCAGGCTTTTTTGCATGGTTATTTATCTCAATCTCGCACATCAGATTTAT
IX434219 GTTGACTGGTATTCCAGCTGAACAAGTCACCAGAGTTATCACTGGTGTT:CCATGGTACTCTACCAGATTGAGACCAGCTATCTCCAGTGCTCTATCTAA

V33706 GACGATAAGGTTAAGATAAGTAGATAAGAGAATGATACGAGATAAAGCAC:AAATTAGCAGAAAGAAGAGTGGTTGCGAACAGAGTAAACCGAATCAGGG
VI20700 CCATCTCTTACTTGTGCTCTAAATTCTATCGTTTTGTTTTTGTAATAAAG:AATGAGACTGCCTGCGGCTTAATTGTCTTTTTATTGTAGGCAAGAACAA

V32497 GCTGCAAACCCCAGAAAATCCGTTCCAAGAGCCATCAAAAAAGTTGTTTT:CCGTATCTTAACCTTCTACATTGGCTCTCTATTATTCATTGGACTTTTA
IV432864 TACCTCCTCTTGATACTCCATTAACCAAAAGGTTTGGAACAGTTCCTCTA:CCGGTCACCAACTTGATATATTCTTGCAGCTCTTCCCCATGTCCATGTT

V34991 AATAGTTATTCAGGTCTTGGAAAATTCTCATCAATAAATCTTGTATGGAA:AATGCGGATTGCGGAAGGCCCAAATCCTTGATAAGGTCACTGTATCTGCT
X375924 AAGGCACCTATGGTGAATGTTGTTGCAATCGGCTTAAATAATTGTGAAAA:TATGGAAGATATGTCTTGCAGAGATTAGTTTGAAAAAGTACTACAAAAAT

V38237 AAACGCACTCGTAGATAAAAGCGGCCATATACCTTTCCGTGAATCGAAAT:TGACCCGCCTGCTTCAAGATTCCCTGGGTGGTAATACGAAAACCGCACTA
XII146763 AGATCTTGTTGAAAACGTGGAGATTCTATTTCTTTCAAAACGGTGCTGCC:TGACTGTTTAAGCCTTCTAGCCAAAATCCTTAAAGTTCTTGCTGGCGTAC

V37559 TGTTTTTACAATTAAACACTATGCAACAGGAAAGTATTCTTCAAGAGACAA∇(ΔT):TATCCAACCAAATCTTGATATGATCAAAAATGAAGTACTGACTCTTATG
XVI896402 AGATTGATGAAGCAATCAAAATTAACACATTAACTCGAAAACTAAACATTC:CATTATATGTTGCTGGTTCTAATGGATTGTTCGCTTATGTTTTTATTGA

Nonhomology Translocation (isochromosome type)

V33313 ACGTGGGTCAATACCATTGAAAGATGAGAAAAGTAAAGAATTGTATCCAT:TGCGCTCTTTCCCGACGAGAGTAAATGGCGAGGATACGTTCTCTATGGAG
V273032 AAGGCAATTGAGCGACACAAATAATTTAAATGAGAAAGTGCAAGGCACAC:ATACAGTTCAATCATCCACCCAGGAAGACAAAATACTCGACGGTGACACA

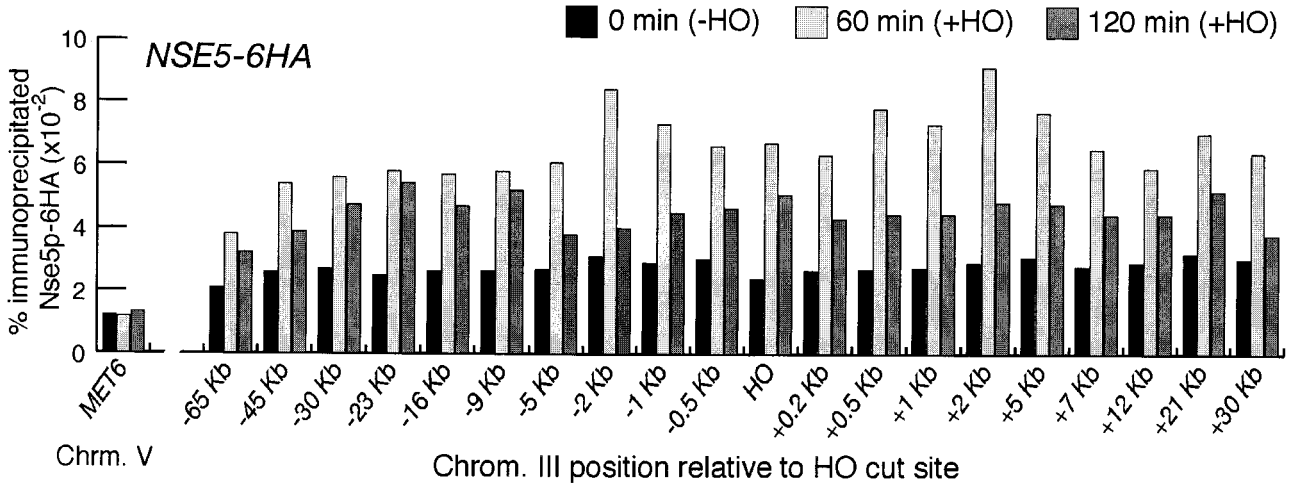
The numbers in parenthesis indicate the GCR rate of different classes of GCRs. New telomere sequences added in the *de novo* telomere addition are presented as lower case. The sequences physically presented on the chromosomes after translocations are underlined. Indicated numbers followed by Roman number, which is chromosome number, are the nucleotide coordinates based on the Stanford SGD. ∇(2T) represents a nucleotide, T deletion where indicated.

Supplementary materials

Figure 1. The binding of Nse5 around *MAT* on chrm-III with (cut) or without (uncut) a DSB. A DSB was induced at *MAT* in strains expressing *NSE5-6HA*, samples were collected just before the induction of the break (0 min) and 1 (60 min) or 2 (120 min) hours following transfer to galactose media. Cells were fixed and processed ChIP. Input DNA and DNA coimmunoprecipitated with anti-HA antibody (IP) were amplified using primer sets corresponding to sequences around *MAT* as indicated. Nse5 binding profiles around the *MAT* site in samples with (light and dark grey) or without (black) a DSB are shown. Percentage of input chromatin in the immunoprecipitates is plotted on the y axis versus coordinates (kb) relative to the HO site (break) in chromosome III on the x axis. Enrichment at the *MET6* locus is shown as a control for a site located in a different chromosome (chrm-V).

F) Supplemental material Fig1

Supplementary Figure 1



Artículo 4

A novel yeast mutation, *rad52-L89F*, causes a specific defect in Rad51-independent recombination that correlates with a reduced ability of Rad52-L89F to interact with Rad59

Felipe Cortés-Ledesma, Paco Malagón y Andrés Aguilera

Genetics (2004), 168: 553-557

DOI: 10.1534/genetics.104.030551

Note

A Novel Yeast Mutation, *rad52-L89F*, Causes a Specific Defect in Rad51-Independent Recombination That Correlates With a Reduced Ability of Rad52-L89F to Interact With Rad59

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Manuscript received April 26, 2004
Accepted for publication May 17, 2004

ABSTRACT

We isolated a novel *rad52* mutation, *rad52-L89F*, which specifically impairs recombination in *rad51Δ* cells. *rad52-L89F* displays phenotypes similar to *rad59Δ* and encodes a mutant protein impaired in its ability to interact with Rad59. These results support the idea that Rad59 acts in homologous recombination via physical interaction with Rad52.

RAD52 is the only gene required for virtually all homologous recombination events in *Saccharomyces cerevisiae*. Null mutations in this gene display the most severe phenotype in many different recombination assays (Paques and Haber 1999). The Rad52 protein shows DNA-binding and strand-annealing activities *in vitro* (Mortensen *et al.* 1996). In addition, Rad52 physically interacts with both the RecA ortholog Rad51 and Rad59 (Symington 2002).

In contrast to *RAD52*, *RAD51* is required for allelic recombination but not for recombination between DNA repeats (Prado *et al.* 2003). One-ended recombination events are *RAD51* dependent (Davis and Symington 2004), but also occur in the absence of Rad51 (Malkova *et al.* 1996). Thus, in the absence of Rad51, recombination may occur by single-strand annealing (SSA), which leads to deletions between direct repeats (Lin *et al.* 1984; Paques and Haber 1999) or break-induced replication, which could give rise to inversions between inverted repeats if followed by SSA (Bartsch *et al.* 2000; Malagon and Aguilera 2001).

Rad59 is homologous to the amino-terminal half of Rad52 and shares several *in vitro* activities with Rad52, such as DNA binding and strand annealing (Petukhova *et al.* 1999; Davis and Symington 2001). It plays an important role in recombination occurring in the absence of Rad51. Thus, *rad59Δ* mutants present only a slight decrease in inverted-repeat recombination, whereas *rad51Δ rad59Δ* double mutants show a strong decrease similar to *rad52Δ* (Bai and Symington 1996).

Isolation of the new *rad52-L89F* mutation: To understand recombination occurring in the absence of Rad51, we searched for mutants with reduced recombination levels in *rad51Δ* background. To facilitate the search we used a *rad51Δ spt6-140* double mutant, which shows high levels of *RAD51*-independent recombination (Malagon and Aguilera 2001). UV-irradiated cells carrying the chromosomic *his3^{b}::INV* inverted repeat system (Aguilera and Klein 1988) were screened for low levels of His^{+} recombinants. This led to the identification of a new *rad52* allele. Sequence analysis showed that the mutant allele carried a single T-to-C substitution at position 165, which results in a Leu-to-Phe change in residue 89 (see Figure 1). This residue is located in the amino terminus of Rad52, which is the most conserved part of the protein, in a domain described as being necessary for DNA binding, self-association, and Rad59 interaction (Symington 2002). Interestingly, the *rad52-1* mutation, which confers a *rad52* null phenotype, is at position 90 (Adzuma *et al.* 1984). The new mutant allele was named *rad52-L89F*.

Homologous recombination in *rad52-L89F*: To understand the types of homologous recombination impaired by *rad52-L89F*, we determined the effect of *rad52-L89F* on the frequency of recombination of the *his3^{b}::INV* system in different *rad* backgrounds (Figure 2A). Recombination frequencies were reduced only 10- and 2-fold below wild-type levels in *rad51Δ* and *rad59Δ* cells, respectively, but 40-fold in the double *rad51Δ rad59Δ*, consistent with previous reports (Bai and Symington 1996; Shinohara *et al.* 1998; Malagon and Aguilera

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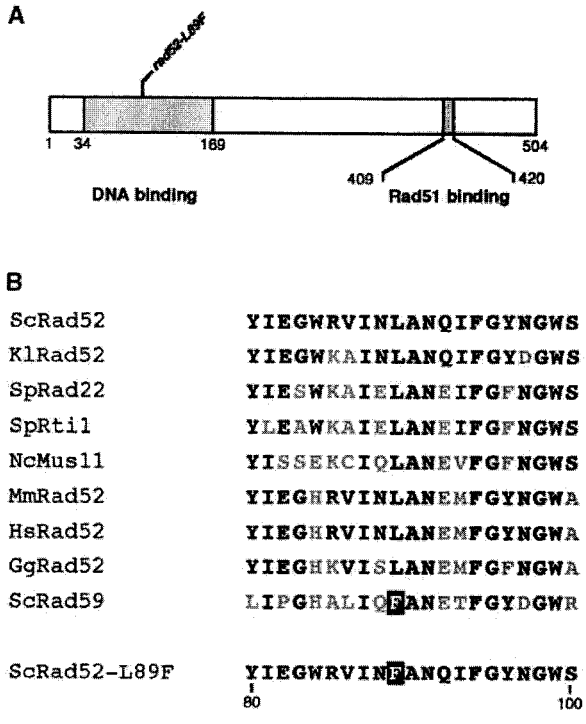

FIGURE 1.—Primary structure of the Rad52-L89F protein. (A) Functional domains of Rad52 and localization of the *rad52-L89F* mutation. (B) Comparative alignment of Rad52 and Rad59 orthologs (Sc, *S. cerevisiae*; Kl, *Kluyveromyces lactis*; Sp, *Schizosaccharomyces pombe*; Nc, *Neurospora crassa*; Gg, chicken; Mm, mouse; Hs, human) and Rad52-L89F. Residues identical to those of ScRad52 are shown in black and the single substitution of Rad52-L89F is indicated as a solid box. The *rad52-L89F* mutant was obtained from the hyperrecombinant strain M137-11Ar51k (*MATα ade2 can1-100 his3^{p}::INV leu2 lys2-128α rad51Δ::KanMX4 spt6-140 trp1 ura3*) by UV mutagenesis. Cells (50 μl) grown in YEPD to an OD_{660nm} of 0.9 were diluted in 6 ml of water and poured into a glass petri dish for irradiation with 45 J/m^2 of UV light (λ = 254 nm). After 5 hr of recovery in YEPD in the dark, cells were plated with the appropriate dilutions. Cell survival was 30% and 10,600 clones were screened for low His$^+$ recombination.

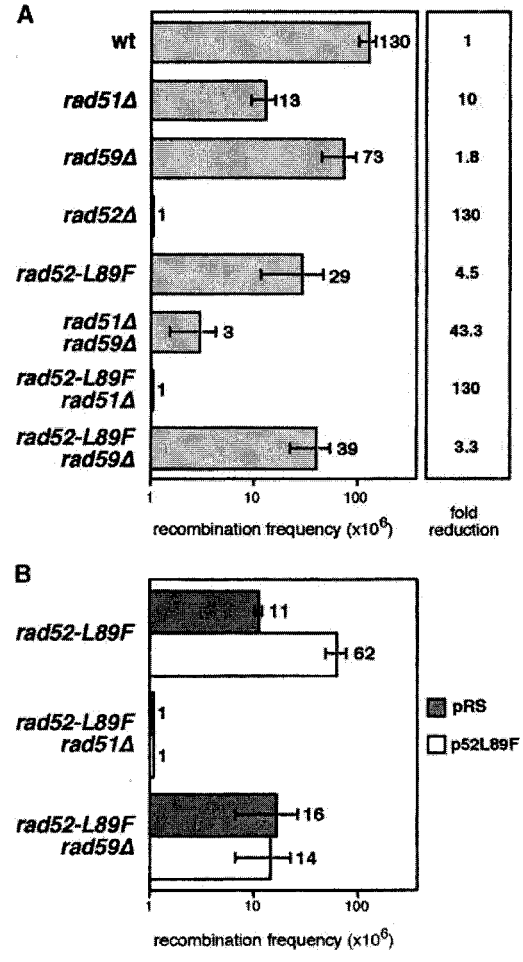

FIGURE 2.—Recombination frequencies of the chromosomal inverted repeat system *his3^{p}::INV* in different *rad* mutants. (A) Recombination in wild-type, *rad51Δ*, *rad59Δ*, *rad52Δ*, *rad52-L89F*, *rad51Δ rad59Δ*, *rad52-L89F rad51Δ*, and *rad52-L89F rad59Δ* strains. (B) Recombination in *rad52-L89F*, *rad52-L89F rad51Δ*, and *rad52-L89F rad59Δ* mutants carrying either pRS424 (pRS, negative control) or pRS424-rad52L89F (p52L89F, overexpressing rad52-L89F) plasmids. Recombination frequencies were determined as the median frequency of six independent colonies isolated from YEPD at 30° (PRADO *et al.* 1997). Frequencies are the average and standard deviation (shown in bars) of two to four median values. The *rad52-L89F* mutation was transferred to other genetic backgrounds by genetic crosses. All *radΔ* strains used for the recombination assays were derived from M137-11A (*MATα ade2 can1-100 his3^{p}::INV leu2 lys2-128α rad51Δ::KanMX4 trp1 ura3*) and were constructed by transformation with the corresponding deletion cassettes or by genetic crosses (MALAGON and AGUILERA 2001).

2001). Nevertheless, whereas in *rad52Δ* cells the reduction was >100-fold, *rad52-L89F* shows, as do *rad51Δ* and *rad59Δ*, only a slight decrease (<5-fold). Interestingly, *rad52-L89F rad51Δ* mutants showed a synergistic decrease and had the same recombination levels as *rad52Δ*. In contrast, deletion of *RAD59* had no effect in *rad52-L89F*.

To determine whether the effect of *rad52-L89F* on recombination was due to a leaky activity of Rad52-L89F, we wondered if its overexpression could reestablish wild-type recombination. As can be seen in Figure 2B, multicopy *rad52-L89F* partially suppressed the recombination defect of *rad52-L89F* up to levels of *rad59Δ*, but had no effect in *rad59Δ* or *rad51Δ* backgrounds (Figure 2B). Therefore, *rad52-L89F* causes the same recombination phenotype as *rad59Δ*, regardless of a putative leakiness of the Rad52-L89F activity.

The recombination phenotypes of *rad52-L89F* are indeed similar to those of the previously characterized *rad52-R70K* allele in *RAD* and *rad51Δ* backgrounds, although they differ when Rad59 is not present (BAI *et al.* 1999). This suggests that a Rad52 amino-terminal domain covering at least the residues from 70 to 89 is essential for recombination in the absence of Rad51. Interestingly, both residues 89 and 70 are conserved in

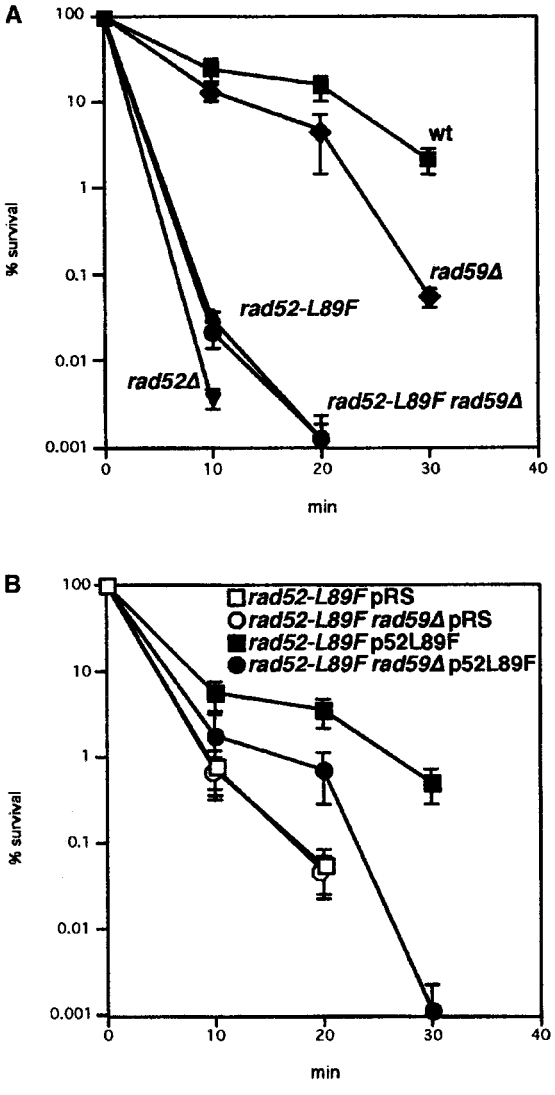

FIGURE 3.—MMS sensitivity of different *rad* strains. (A) Sensitivity of wild-type, *rad59Δ*, *rad52Δ*, *rad52-L89F*, and *rad52-L89F rad59Δ* mutants. (B) Sensitivity of *rad52-L89F* and *rad52-L89F rad59Δ* mutants carrying either pRS424 (pRS, negative control) or pRS424-rad52L89F (p52L89F, overexpressing rad52-L89F) plasmids. Cells were exposed to 0.5% MMS for 0, 10, 20, and 30 min before plating onto YEPD. For each strain, the percentage of survival is referred to the value of cells not exposed to MMS (0 min), taken as 100%. Data are the average and standard deviation (shown in bars) of two to four independent experiments.

all known Rad52 orthologs and the L89F and R70K changes make the terminal domain of the mutant Rad52 proteins more similar to Rad59 (Figure 1B).

Repair of MMS damage in *rad52-L89F*: To further characterize genetic interaction between *rad52-L89F* and *RAD59*, methyl methanesulfonate (MMS) sensitivity was determined by cell survival after different time exposures to 0.5% MMS. The *rad52-L89F* mutant showed a weaker MMS sensitivity than *rad52Δ*, but much stronger than *rad59Δ* (Figure 3A), in contrast to its low recombination defect. Nevertheless, as in recombination, *rad52-L89F* sensitivity was not affected in *rad59Δ* background. Therefore, these data support the idea that *rad52-L89F* behaves like *rad59Δ*. In any case, Rad52-L89F was leaky, as suggested from the observation that overexpression of Rad52-L89F enhanced MMS resistance of *rad52-L89F* strains (Figure 3B).

Rad52-L89F is affected in its ability to interact with Rad59: The similarity of phenotypes between *rad59Δ* and *rad52-L89F* could be explained if in the *rad52-L89F* mutant the levels of Rad59 protein were reduced, as reported for *rad52Δ* mutants (DAVIS and SYMINGTON 2001). Nevertheless, this was not the case because the levels of Rad59 protein in *rad52-L89F* were similar to those of wild-type cells (Figure 4A).

We tested the possibility that Rad52-L89F was impaired in its ability to interact with Rad59. For this purpose, we purified Rad59 fused to the glutathione *S*-transferase (Rad59::GST) from wild-type, *rad52Δ*, and *rad52-L89F* strains overexpressing the GST-fusion protein. Rad59::GST is functional, as it rescues the MMS sensitivity of *rad59Δ*. As negative control we purified GST from a wild-type strain overexpressing GST. As can be seen in Figure 4B, Rad52-L89F protein was present in cell extracts at levels lower than those of Rad52. Indeed, other missense mutations in the amino terminus reduce the levels of Rad52 (ASLESON and LIVINGSTON 2003). As expected, wild-type Rad52 copurified with Rad59::GST, but not with GST. However, no Rad52-L89F protein was detected in the purified Rad59::GST fraction. Since Rad52-L89F protein levels are reduced, we cannot rule out the possibility of a nondetected weak interaction with Rad59 even though our results suggest that Rad52-L89F is affected in its ability to interact with Rad59.

Biological significance of the *in vivo* Rad52-Rad59 interaction: Both human and yeast Rad52 proteins form multimeric ring structures (SHINOHARA *et al.* 1998; STASIAK *et al.* 2000; RANATUNGA *et al.* 2001), and Rad59 has also been reported to self-associate (DAVIS and SYMINGTON 2003). It would be interesting to know whether Rad52 and Rad59 could form heteromeric ring structures (SYMINGTON 2002). This is supported by the fact that the Rad52 regions necessary and sufficient for self-interaction and Rad59 binding coincide (DAVIS and SYMINGTON 2003).

Our study confirms that the amino terminus of Rad52 is important for its interaction with Rad59. The reduced ability of Rad52-L89F to interact with Rad59 could at least partially explain the *rad59Δ*-like recombination phenotype of the *rad52-L89F* mutant. It could cause a reduction of the presence of Rad59 at recombination centers, leading to a *rad59* phenocopy. As Rad59 is essential in recombination occurring in the absence of Rad51-dependent strand exchange, this would explain why the recombination phenotype of *rad52-L89F* is specifically observed in a *rad51* background.

The MMS sensitivity of *rad52-L89F* is much more severe than its recombination defect. Other mutations in

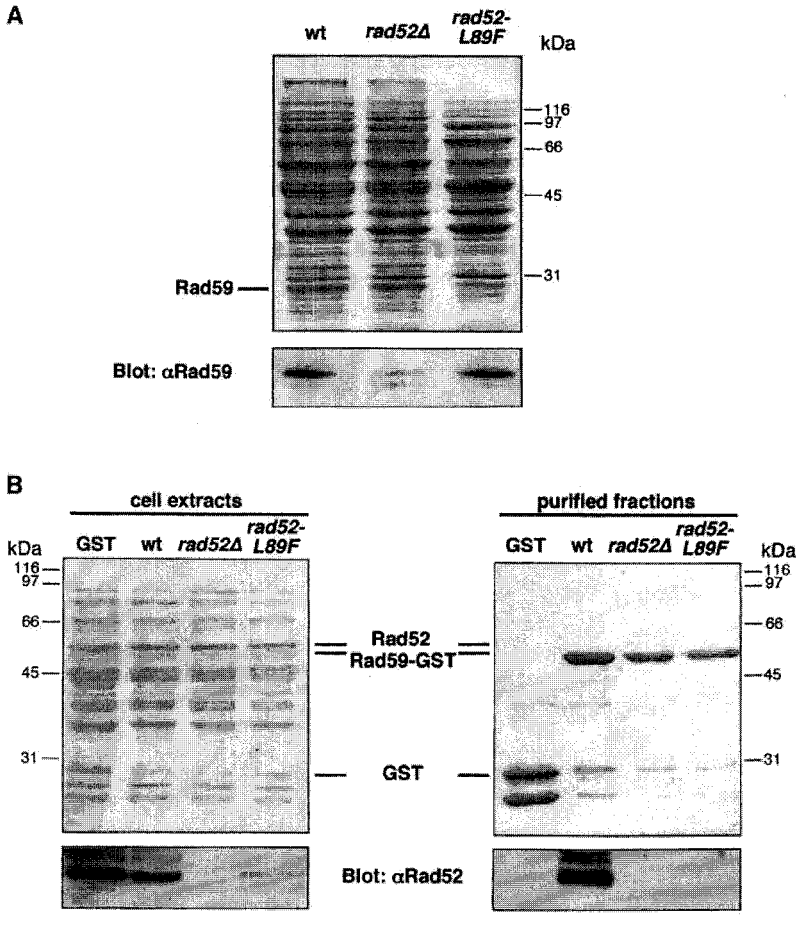

FIGURE 4.—Rad52-L89F-Rad59 interaction. (A) SDS-PAGE analysis of Rad59 protein in wild-type, *rad52Δ*, and *rad52-L89F* strains. Total protein extract (5 μg) was loaded for each strain. Coomassie staining (top) and Western blot using αRad59 polyclonal antibody (bottom) are shown. The position in the gel at which Rad59 migrates is indicated. (B) Purification of Rad59-GST fusion protein in wild-type, *rad52Δ*, and *rad52-L89F* strains. Purification of GST in the wild type was included as a negative control. Protein expression was under the control of the *CUP1* promoter and was induced by addition of $CuSO_4$ to a final concentration of 0.5 mM. Coomassie staining (top) and Western blot using αRad52 polyclonal antibody (bottom) of total cell extracts (left) and purified fractions (right) are shown. The positions in the gel at which Rad59-GST, Rad52, and GST migrate are indicated. For the preparation of extracts, cells were grown in 500 ml of SC-Leu to an OD_{660nm} of 0.8. Cells were harvested, washed with water, and resuspended in one pellet volume of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 5 mM DTT, 10% glycerol, 1 M NaCl. Leupeptin and pepstatin A were added to a final concentration of 2 μg/ml and 1 μg/ml, respectively. Extracts were made with glass beads (McCRAITH and PHIZICKY 1990), followed by supplementation with 1 mM PMSF and 30 min centrifugation at 14,000 rpm in a JA-20 Beckman rotor. For Rad59::GST protein purification, supernatant was incubated with 1/100 volume of glutathione-Sepharose 4B (A. P. Biotech) previously equilibrated in 50 mM potassium phosphate pH 7.2, 1 mM DTT, 0.5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 M NaCl. Samples were washed twice with 500 bead volume of 50 mM potassium phosphate pH 7.2, 1 mM DTT, 0.5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5 M NaCl. Proteins were eluted by boiling in 1× loading buffer for 5 min.

RAD52 and other *RAD* genes have been reported to separate recombinational and DNA repair phenotypes (MORTENSEN *et al.* 2002; SYMINGTON 2002). In our case, the lower amount of stable Rad52-L89F protein present in the cell (Figure 4B) is sufficient for spontaneous recombination, but not for the repair of MMS-induced damage. Consistently, overexpression of Rad52-L89F significantly suppresses the MMS sensitivity phenotype (Figure 3B).

Concluding remarks: A novel *rad52* mutation (*rad52-L89F*), identified by its specific effect in recombination occurring in the absence of Rad51, encodes a mutant Rad52 protein impaired in its ability to interact with Rad59. This, together with the strong similarity of recombination and repair phenotypes of *rad52-L89F* and *rad59Δ*, suggests that Rad59-Rad52 interaction is essential for the role of Rad59 in recombination in the absence of Rad51-mediated strand exchange.

We are grateful to L. Symington for yeast strain gifts, P. Sung and W. Heyer for antibodies, F. Prado for critical reading of the manuscript, and D. Haun for style supervision. This work was supported by grants from the Spanish Ministry of Science and Technology (BMC2000-0439 and SAF2003-00204) and the Regional Government of Andalusia (CVI0102). F.C.-L. was the recipient of predoctoral training grants from the Spanish Ministry of Education and Ministry of Health.

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Communicating editor: L. S. SYMINGTON

4- DISCUSIÓN GENERAL

La RH se ha medido tradicionalmente por métodos genéticos (revisado en (Paques and Haber, 1999; Prado et al., 2003), pero esto no es suficiente para determinar con exactitud todos los mecanismo y funciones implicados, ya que estos ensayos sólo permiten analizar productos finales viables. Por lo tanto el estudio de la RH a nivel molecular, que permite observar intermediarios y productos de recombinación no recuperables genéticamente, ha siso de gran ayuda en el desarrollo de los modelos de RH. Dado que los niveles de recombinación espontáneos son demasiado bajos para ser detectados molecularmente, muchos estudios se han basado en la inducción con endonucleasas específicas de sitio, como HO de *S. cerevisiae* (revisado en (Paques and Haber, 1999). Pero ésta no es una situación natural; las evidencias experimentales que asocian RH a replicación sugieren que la principal fuente de DSBs durante el crecimiento celular sea la replicación, y, a su vez la RH parece ser el principal mecanismo de reparación de dichas lesiones postreplicativas (revisado en (Branzei and Foiani, 2005; Cox et al., 2000; Haber, 1999; Kogoma, 1997; Kuzminov, 2001a; Michel et al., 2001; Rothstein et al., 2000). En esta tesis doctoral hemos analizado el corte en una diana reducida (21 pb) de la endonucleasa HO en el sistema pRS316-TINV (Artículo 1), encontrando que los DSBs ocurren fundamentalmente durante la replicación. Esto supone la oportunidad de inducir DSBs de una manera similar a como ocurren naturalmente en la célula, y por lo tanto de analizar a nivel molecular unos sucesos de RH similares a los espontáneos. Además demostramos que surgen como consecuencia de replicación sobre SSBs, algo que se ha propuesto como responsable de la mayor parte de la RH espontánea (Saleh-Gohari et al., 2005). Este sistema permitirá en un futuro analizar en detalle el encuentro de la horquilla de replicación con un SSB, así como su colapso y posterior reparación.

De entre los diferentes sustratos homólogos, la cromátida hermana es el preferido por la RH (Gonzalez-Barrera et al., 2003; Johnson and Jasin, 2000; Kadyk and Hartwell, 1992), de acuerdo con el hecho de que en levaduras la RH sólo está activa en fases del ciclo celular en las que la cromátida hermana está presente, S y G2 (Aylon et al., 2004; Ira et al., 2004; Lisby et al., 2001). En pRS316-TINV la conversión de un SSB en DSB por la replicación deja una cromátida intacta para ser usada como molde de reparación (Artículo 1). Este

sistema permite así la detección mediante Southern-blot de SCE en cinéticas de inducción por la aparición de dímeros (Gonzalez-Barrera et al., 2003) (Artículo 1). El SCE es totalmente dependiente de Rad52, indicando que, como se espera, ocurre por RH. Sin embargo, sube en *yku70*Δ, lo que sugiere que parte de estos DSB replicativos se reparan por NHEJ (Artículo 1). Esto indicaría que, si bien RH está activa sólo en S y G2, NHEJ está activo durante todo el ciclo, aunque su eficiencia puede disminuir en S y G2 por competencia con RH, de acuerdo con resultados previos (Aylon et al., 2004; Helleday, 2003; Ira et al., 2004; Rothkamm et al., 2003; Saleh-Gohari and Helleday, 2004; Takata et al., 1998). Una característica importante del sistema pRS316-TINV es que, al contener repeticiones invertidas, además de SCE se puede medir recombinación intracromatídica mediada por BIR (IC-BIR) (Artículo 2), permitiendo la competición, dentro del mismo sistema, de dos sustratos de recombinación diferentes, la cromátida hermana y la repetición intracromatídica. Esto supone un avance, ya que en estudios comparativos anteriores se usaban dos sistemas (Bressan et al., 1999; Kadyk and Hartwell, 1992). Así, en un solo ensayo se puede determinar si un posible defecto en SCR es específico, evitando además posibles artefactos derivados de comparar sistemas diferentes. Sin embargo, el sistema pRS316-TINV presenta la principal limitación de no detectar todos los sucesos de SCR sino tan solo SCE. De todas formas, hasta la fecha no se ha desarrollado un sistema con esas características.

Encontramos que SCE es el suceso más frecuente detectado con el sistema pRS316-TINV en células silvestres (Artículo 2). Sin embargo, en *rad51*Δ sube el IC-BIR a costa de una bajada del SCE (Artículo 2), lo que sugiere una mayor dependencia de Rad51 para la invasión en la cromátida hermana, a la vez que confirma que, en sistemas con dos extremos del DSB, Rad51 actúa impidiendo los sucesos de BIR (Malkova et al., 1996), posiblemente actuando como barrera a la replicación (Aguilera, 2001). Interesantemente, el aumento de IC-BIR en *rad51*Δ supone una prueba molecular de que las inversiones entre repeticiones invertidas pueden ocurrir en ausencia de intercambio de cadena mediante BIR seguido de SSA (Bartsch et al., 2000; Figura 5). Hemos encontrado una implicación de Rad59 en SCE mayor de la esperada (Artículo 2), teniendo en cuenta resultados anteriores

(Dong and Fasullo, 2003). Al contrario que en *rad51*Δ, en *rad59*Δ también se observa una bajada en IC-BIR. Estos datos, junto con la observación de que la acción de Rad59 está mediada por su interacción con Rad52 (Artículo 5), son consistentes con un papel general de Rad59, junto con Rad52, en RH , más que con una función en la recombinación independiente de Rad51. Como se espera *rad54*Δ se comporta igual que *rad51*Δ, mientras que *tid1*Δ no tiene efecto (Artículo 2), de acuerdo con Rad54 facilitando el intercambio de cadena (Rattray and Symington, 1995), y Tid1 actuando específicamente en recombinación alélica (Klein, 1997; Shinohara et al., 1997).

Hemos determinado que mutantes nulos de MRX están afectados en SCE, pero el efecto no es específico, ya que también lo están IC-BIR (Artículo 2). Esto sugiere una función de MRX general en RH, que puede ser funcional, y estar relacionada con la resección, o estructural, manteniendo unidos los extremos del DSB. Además, en los mutantes se observa una acumulación de DSBs, así como de intermediarios de IC-BIR, indicando un defecto en el procesamiento de extremos de ADN. Al eliminar la actividad nucleasa del complejo, con las mutaciones *mre11-H125N* o *sae2*Δ, afectamos la RH de la misma forma que en los mutantes nulos (Artículo 2), lo que sugiere que la función principal de MRX es enzimática más que estructural. Sin embargo, la acumulación de DSBs e intermediarios de IC-BIR sólo se observa si además se elimina Exo1. Así, Exo1 puede procesar los extremos sólo en presencia de MRX, posiblemente debido a que la actividad helicasa del complejo podría facilitar la acción de Exo1. De cualquier forma, estos extremos aberrantemente procesados por Exo1 no pueden repararse eficientemente por SCE o IC-BIR.

El análisis a nivel molecular nos ha permitido determinar la acción en SCE de proteínas esenciales como son las cohesinas y el complejo Smc5-Smc6. En ausencia de cohesinas funcionales el SCE baja y el IC-BIR sube (Artículo 1), indicando un papel específico de las cohesinas en SCR. Esto implica que las cohesinas son importantes para mantener la estabilidad genómica favoreciendo que los DSBs replicativos se reparen por SCR. Si no funcionan bien, otras secuencias homólogas (la repetición intracromatídica en nuestro caso) pueden usarse preferentemente, pudiendo dar lugar a deleciones o translocaciones. En mutantes del complejo Smc5-Smc6 hemos obtenido una bajada en SCE similar (Artículo 3). Por dificultades técnicas debidas a una muy

baja inducción de DSBs a temperatura restrictiva, no hemos podido realizar el experimento en las mismas condiciones que con las cohesinas, por lo que no hemos podido analizar IC-BIR. Sin embargo, otros datos, como la carga en respuesta a un DSB, la recombinación eficiente en el cambio de sexo, la inestabilidad en el ADNr y el aumento de reordenaciones cromosmicas (Artículo 3), sugieren un papel de Smc5-Smc6 en el mantenimiento de la estabilidad genómica favoreciendo la SCR. Estas observaciones a cerca del papel de proteínas SMC en SCR ponen de manifiesto una importancia de la estructura cromosómica en facilitar la reparación con la cromátida hermana.

En resumen hemos desarrollado un sistema para la inducción de SSBs que se convierten en DSBs al paso de la replicación. Hemos determinado la dependencia de los principales genes de RH para la reparación de dichos DSBs replicativos mediante SCR, y en competencia con IC-BIR. Hemos prestado especial atención al complejo MRX y a su posible efecto específico en SCR, encontrando que desempeña una función general en RH, dependiente en gran parte de su actividad nucleasa. Por último hemos encontrado factores estructurales, como son las cohesinas y el complejo Smc5-Smc6, que facilitan la SCR, colaborando así en el mantenimiento de la estabilidad genómica.

5- CONCLUSIONES

1. Los DSBs inducidos en la diana HO de 21 pb del sistema pRS316-TINV ocurren fundamentalmente durante la replicación, por la conversión de SSBs preexistentes.

2. El principal producto de reparación detectado en dicho sistema es SCE desigual dependiente de Rad52.

3. NHEJ es responsable de una parte minoritaria de la reparación de estos DSBs replicativos.

4. En pRS316-TINV, además de SCE desigual, podemos detectar sucesos de IC-BIR. También podemos detectar SCE igual por la formación de dímeros en el sistema pCM189-leu2HOr, en el que no hay repeticiones.

5. En fondo *rad51*Δ la reparación de DSBs replicativos se desvía a IC-BIR, lo que supone una prueba molecular del modelo BIR-SSA.

6. Rad59 juega un papel general en RH, incluso en presencia de Rad51, que depende fundamentalmente de su interacción con Rad52.

7. *rad54*Δ se comporta como *rad51*Δ, mientras que *tid1*Δ no tiene efecto en ninguno de los sucesos de RH analizados.

8. MRX desempeña una función general en RH, relacionada principalmente con su actividad nucleasa.

9. Exo1 es redundante con la actividad nucleasa de MRX en cuanto al procesamiento de extremos, pero sólo en presencia del complejo.

10. Las cohesinas favorecen que la reparación de DSBs replicativos ocurra por SCR, asegurando el mantenimiento de la estabilidad genómica.

11. El complejo Smc5-Smc6 actúa de forma similar a las cohesinas en la reparación de DSBs, y en particular en SCR.

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UNIVERSIDAD DE SEVILLA

Reunido el tribunal en el día de la fecha, integrado por los abajo firmantes, para evaluar la tesis doctoral de D. Felipe Cortés Ledesma
titulada Reparación Postreplicativa de Cortes de Doble Cadena en Saccharomyces cerevisiae,
acordó otorgarle la calificación de Sobresaliente "Cum Laude" por unanimidad

Sevilla, a 21 de julio de 2006.

Vocal,

Vocal,

Vocal,

Presidente,

Secretario,

Doctorando,