Tesis doctoral

Characterization of the Cdc14 phosphatase in the DNA damage response

Facundo Nehuén Ramos Ochoa

Director: Andrés Clemente Blanco

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Dr. Andrés Clemente Blanco, científico titular del Consejo Superior de Investigaciones Científicas (CSIC)

CERTIFICA:

Que el graduado **Facundo Nehuén Ramos Ochoa** ha realizado el trabajo titulado "***Characterization of the Cdc14 phosphatase in the DNA damage response***", bajo mi dirección en el Instituto de Biología Funcional y Genómica, centro mixto de la Universidad de Salamanca (Departamento de Microbiología y Genética) y el Consejo Superior de Investigaciones Científicas, para optar al grado de Doctor. Además, certifico que el autor de dicho trabajo cumple con los requisitos necesarios para optar a la mención de "*Doctor Internacional*".

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Fdo.: Andrés Clemente Blanco

Director de la Tesis Doctoral

Tesis doctoral

Caracterización de la fosfatasa Cdc14 en la respuesta a daño en el ADN

Facundo Nehuén Ramos Ochoa

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Introducción

Importancia de la respuesta a daño en el ADN.

El material genético de todos los seres vivos está expuesto a diferentes factores externos e internos que pueden generar daño en el ADN y por lo tanto derivar en inestabilidad genómica. Uno de los daños más perjudiciales para las células son las roturas de doble cadena o DSB (del inglés *Double strand break*). Estas roturas desatan la activación de una serie de procesos incluidos en lo que se denomina como respuesta a daño en el ADN, o DDR (*DNA damage response*). Estos mecanismos están constantemente patrullando el genoma en busca de lesiones con el fin de identificarlas y repararlas, por lo que se convierten en esenciales para la viabilidad celular. El DDR tiene como principal objetivo coordinar la parada en el ciclo celular con la reparación del daño en el ADN. Para ello tiene que ejecutar de manera ordenada y altamente regulada dos procesos celulares fundamentales: 1) la activación del punto de control de daño, mediante el cual se inhibe la segregación cromosómica hasta que el daño haya sido reparado; y 2) la activación del programa transcripcional que permitirá la reparación de la molécula de ADN.

Activación del punto de control de daño.

Uno de los primeros complejos que detectan los DSBs es el MRX (MRN en mamíferos), ya que es el responsable de iniciar la cascada de señalización que rige la parada del ciclo celular, y además interviene el procesamiento inicial de la molécula de ADN dañada. Para ello, MRX permite el reclutamiento de las quinasas transductoras Mec1/Tel1 que serán las encargadas que detener la progresión en el ciclo celular y de activar la transcripción de genes implicados en reparación de lesiones en el material genético. Una vez detectada la señal y, dependiendo del tipo de daño, se llevará a cabo un tipo de reparación u otra.

En la levadura de gemación *Saccharomyces cerevisiae,* la activación del punto de control frente a roturas de doble cadena ocurre principalmente antes de anafase, en lo que se denomina comúnmente como G2/M. Este bloqueo en la progresión del ciclo se consigue mediante la estabilización de la securina (Pds1), que inhibe la proteína encargada de la eliminación de la cohesión entre cromátidas hermanas y, por lo tanto, la segregación de las mismas. Numerosos estudios han puesto de manifiesto que este bloqueo se lleva a cabo a partir de la activación de dos vías que se ejecutan en paralelo y que están gobernadas por las

quinasas efectoras Rad53 y Chk1 en *S. cerevisiae*. Estas dos rutas también regulan la activación del programa transcripcional implicado en la reparación de lesiones en el material genético. De hecho, uno de los ejemplos más estudiados es el de la familia de genes de la ribonucleótido reductasa (RNR).

Activación de los mecanismos de reparación del ADN.

Como hemos mencionado en el apartado anterior, la activación del punto de control de daño en el ADN en la levadura de gemación ocurre mayoritariamente en G2/M. Cabe destacar que en esta etapa del ciclo celular, el mecanismo preferente para la reparación del DSB es la recombinación homóloga (HR, *Homologous recombination*). Esta preferencia por HR está determinada por la activación de la resección del ADN. La resección consiste en la eliminación en sentido 5'-3' de una de las hebras de la molécula dañada (la que presenta el extremo 5' libre). Este proceso tiene como objetivo la generación una larga molécula de ADN de cadena sencilla (ssDNA, *single-stranded DNA*) que será la encargada de llevar a cabo la búsqueda de homología y la invasión de la molécula que será utilizada como molde para recuperar la información perdida. La resección consta de dos pasos y está altamente regulada por la actividad de la quinasa dependiente de ciclina (Cdk), y es por ello que su activación está limitada a las etapas del ciclo donde existe una alta actividad de dicha quinasa. El primer paso de la resección del ADN depende del procesamiento llevado a cabo por MRX, mientras que la segunda etapa implica la participación de dos complejos de nucleasas: la exonucleasa Exo1 y el complejo Sgs1-Dna2. Si bien ambas maquinarias de resección son independientes y están reguladas de manera diferente, la ausencia de una de ellas puede ser compensada por la otra. El hecho de activar la resección predispone a las células a ejecutar la vía de reparación por HR en lugar de la unión de extremos no homólogos (NHEJ). Este último sistema de reparación es esencial para las células cuando no es posible llevar a cabo HR, pero es susceptible de generar cambios en la secuencia de ADN debido a inserciones/deleciones. Es por ello que las células han desarrollado mecanismos moleculares que permiten derivar la reparación del ADN a un sistema fidedigno como la HR cuando existe una secuencia homóloga en la cromátida hermana.

Dentro de la reparación por recombinación homóloga encontramos distintas formas de resolver la estructura que se forma durante la invasión de la secuencia que actúa como donadora. En este trabajo nos hemos centrado en dos de estas rutas:

1) **El anillamiento de cadena dependiente síntesis (SDSA):** durante este proceso, la cadena que invade la molécula donadora se desplaza y anilla con la cadena complementaria del otro extremo del corte. De esta manera, actúa como cebador para la síntesis de la hebra previamente reseccionada.
2) **Reparación de roturas de doble cadena (DSBR):** es el sistema clásico de reparación por HR e implica la formación de intermediarios complejos que, según como se resuelvan, pueden dar lugar a entrecruzamientos o no. Este tipo de reparación va asociado a la incorporación de la información del donador a ambos lados de la posición en la que se ha producido la rotura.

Como hemos mencionado anteriormente, la Cdk juega un papel importante en la activación de la resección y, en consecuencia, de la reparación por HR. También se sabe que la activación y la presencia de la nucleasa Dna2 en el núcleo en presencia de daño se debe a la fosforilación impuesta por la Cdk. Dado que la activación/desactivación de estos procesos está altamente regulada durante el DDR, es lógico pensar que tiene que haber alguna fosfatasa implicada en la regulación del balance entre la fosforilación/defosforilación de diferentes componentes de la respuesta a daño.

La fosfatasa Cdc14.

La serina/treonina fosfatasa Cdc14 fue inicialmente descubierta en *S. cerevisiae* por su papel esencial en la salida de mitosis. A lo largo de los años se han descrito numerosas funciones de Cdc14 en distintos procesos celulares, incluyendo la respuesta a daño en el ADN. Respecto a su regulación en un ciclo celular en ausencia de daño en *S. cerevisiae*, se sabe que Cdc14 permanece en un estado "inactivo" en el nucléolo interaccionando con Net1 durante interfase. Según las células progresan por anafase, Cdc14 se libera a través de dos vía se señalización inter-conectadas: FEAR (*Fourteen early anaphase release*) y MEN (*Mitotic exit network*). La liberación de Cdc14 dependiente de FEAR ocurre al principio de anafase, coincide con la fosforilación de Net1, y está limitada al núcleo. Por el contrario, la liberación dependiente de MEN es tardía y está asociada a la translocación de la fosfatasa al citoplasma. Dicho cambio en la localización de la fosfatasa es imprescindible para la defosforilación de las diana de Cdc14 y la salida de mitosis.

Respecto a la función de la fosfatasa en el contexto de daño, existe cierto grado de controversia. Por un lado se cree que la función de la fosfatasa está limitada a la activación del punto de control de daño, pero por otro se ha demostrado que se necesita para la

reparación de lesiones generadas por distintos agentes genotóxicos. Cabe destacar que tanto en la levadura de fisión como en células de mamífero la fosfatasa se libera al nucleoplasma en respuesta a daño, sugiriendo que podría haber cierto grado de conservación entre los distintos organismos a nivel funcional y en el contexto del DDR. No obstante, los mecanismos moleculares a través de los cuales Cdc14 controla un proceso u otro y la regulación de la activación de la fosfatasa en presencia de lesiones en el ADN se desconocen.

Resumen

Las células de todos los organismos vivos están expuestas constantemente a numerosos factores intrínsecos y extrínsecos que pueden generar daños en el material genético. Con el fin de hacer frente a dichas amenazas, las células han desarrollado una serie de mecanismos que tienen como objetivo final el mantenimiento de la integridad del genoma. Estos procesos se engloban dentro de lo que se conoce como respuesta a daño en el ADN o DDR (del inglés *DNA damage response*). Cabe destacar, que el correcto funcionamiento de las vías implicadas en dicha respuesta así como su regulación son fundamentales para que las células puedan mantener su viabilidad.

A lo largo de los años se han descrito numerosas proteínas clave para la coordinación y la ejecución de la respuesta a daño en el ADN. Basándonos en los conocimientos previos que ponen de manifiesto un posible papel de la fosfatasa Cdc14 en el DDR, decidimos caracterizar su función en el organismo modelo *Saccharomyces cerevisiae*. Estudios previos, realizados tanto en mamíferos como en levaduras, demostraron que la fosfatasa Cdc14 estaría implicada tanto en la activación del punto de control de daño como en la regulación de los propios mecanismos de reparación. No obstante, los mecanismos moleculares por los cuáles ejerce su función aún se desconocen. Es por ello que nos planteamos estudiar a nivel bioquímico y molecular cuáles son las diana de la fosfatasa en el contexto de daño en el ADN con el fin de profundizar en la caracterización de su función.

Uno de los grandes pilares del DDR es la activación del punto de control del daño que tiene como objetivo estimular una serie efectores que, por un lado, impedirán la progresión en el ciclo celular, y por otro, activarán los procesos de reparación del daño. Una vez que la lesión haya sido reparada, se desactivará el punto de control para permitir la reentrada en el ciclo celular. Teniendo en cuenta que estos mecanismos están altamente regulados por la quinasa dependiente de ciclina (Cdk), y que Cdc14 tiene predilección por defosforilar residuos que han sido previamente fosforilados por dicha quinasa, es lógico pensar que la fosfatasa podría jugar un papel importante en la regulación de dichos fenómenos. De hecho, nuestros primeros resultados obtenidos a partir ensayos de viabilidad en gota demostraron que mutantes *cdc14-1* sensibles a temperatura eran incapaces de crecer en presencia de distintos agentes genotóxicos cuando la función de la fosfatasa estaba comprometida. Esto sugería

que la función de Cdc14 era esencial para responder frente a estos agentes y mantener la viabilidad celular.

Teniendo en cuenta estas observaciones lo primero que hicimos fue estudiar el papel de Cdc14 en la activación del punto de control de daño. Para ello utilizamos uno de los mejores sistemas descritos para el estudio de los procesos de reparación de un daño en el ADN: el sistema de la endonucleasa HO (*homothallic*). En este sistema, el promotor endógeno del gen que codifica para la enzima HO ha sido sustituido por el promotor inducible por galactosa (*GAL1*), facilitando el control de su expresión. La particularidad de esta endonucleasa es que detecta una única secuencia específica en el genoma, en el locus *MAT* de la levadura, y genera único daño de doble cadena (DSB, *double strand break*) en el ADN. El corte generado puede ser reparado por recombinación homóloga (HR, *homologous recombination*) mediante el uso de dos secuencias homólogas conocidas como *HMR* y *HML*, ubicadas a ambos lados de la lesión en el mismo cromosoma. Estas secuencias son utilizadas por la célula de manera natural para copiar la información genética perdida y así regenerar la secuencia de ADN durante el cambio del tipo sexual de la levadura. Utilizando variantes de este sistema podemos estudiar diferentes aspectos de la activación/desactivación del punto de control y de los mecanismos de reparación. De hecho, a lo largo de este trabajo hemos utilizado diversas modificaciones de este sistema que se irán detallando a medida que se requiera.

Con el fin de determinar si Cdc14 está implicada en la activación del punto de control de daño utilizamos una variante del sistema HO en el que las secuencias *HMR* y *HML* han sido previamente eliminadas. De esta manera, una vez que añadimos galactosa al medio, se induce la expresión de la enzima y la consecuente generación de una rotura de doble cadena no reparable. Una vez que se genere la lesión, las células activarán el punto de control y permanecerán paradas en el ciclo celular. Se sabe, por otros trabajos presentes en la literatura, que el bloqueo de la progresión del ciclo celular en *S. cerevisiae* ocurre antes de anafase y se denomina G2/M. Cuando analizamos la activación del punto de control no encontramos diferencias entre células silvestres y mutantes *cdc14-1*, indicando que la función de la fosfatasa no se requiere para activar el bloqueo característico de células sometidas a daño en el ADN. Además, medimos los niveles de fosforilación de la quinasa efectora Rad53, comúnmente utilizada como indicador de la activación del punto de control de daño, en varios sistemas y en contextos de daño diferentes. No obstante, no encontramos diferencias significativas entre cepas silvestres y mutantes de Cdc14 sensibles a temperatura,

confirmando que la función de la fosfatasa es dispensable para la activación del bloqueo en G2/M en presencia de lesiones en el ADN.

Seguidamente, estudiamos la localización de la fosfatasa en presencia de uno (HO) o múltiples DSBs (fleomicina). Se sabe que en un ciclo mitótico en ausencia de daño en *S. cerevisiae*, Cdc14 permanece retenida e inhibida en el nucléolo interaccionando con Net1 durante interfase. También se ha demostrado que la liberación de la fosfatasa ocurre gracias a la activación consecutiva de dos vías de señalización, FEAR (*Fourteen early anaphase release*) y MEN (*Mitotic exit network*). Mientras que FEAR controla la activación y restringe la liberación de Cdc14 al núcleo durante el comienzo de anafase, MEN es la responsable de la salida masiva de la fosfatasa al citoplasma para promover la salida de mitosis. Dentro de las modificaciones que gobiernan la ejecución de estas dos vías, cabe destacar que la fosforilación de Net1 es uno de los eventos determinantes para promover la salida de la fosfatasa al nucleoplasma durante FEAR. En este trabajo, demostramos que Cdc14 se libera de manera transitoria del nucléolo al nucleoplasma en presencia de uno o varios DSBs. Es importante destacar que esta liberación al núcleo no es suficiente para promover la salida de mitosis ya que las células permanecen detenidas en G2/M una vez que la señal de la fosfatasa se localiza en el nucléolo. Estas observaciones nos indican dos cosas: 1) confirman nuestros previos resultados de que Cdc14 no se necesita para la activación del punto de control de daño, 2) la liberación de la fosfatasa debe de estar altamente controlada para evitar su masiva liberación y, por consiguiente, la salida de mitosis. De hecho, es probable que la liberación de Cdc14 en daño esté controlada por mecanismos diferentes a FEAR/MEN. En nuestras condiciones experimentales de daño en el ADN, vimos que Net1 se fosforila en presencia de daño, y dicha modificación coincide temporalmente con la liberación de la fosfatasa al nucleoplasma. No obstante, cuando la señal de Cdc14 desaparece del núcleo, Net1 permanece fosforilado. Aunque los niveles de fosforilación de Net1 sean comparables a aquellos observados en una situación en la que Cdc14 se libera por MEN en un ciclo en ausencia de daño, no podemos descartar que existan otros mecanismos que regulen la liberación de la fosfatasa en el contexto de daño.

Habiendo demostrado que la fosfatasa Cdc14 no se necesita para la activación del punto de control pero sí para mantener la viabilidad de células expuestas a distintos tipos de daño, y teniendo en cuenta que la fosfatasa cambia su patrón de localización en respuesta a lesiones en el ADN, nos planteamos estudiar su papel en distintos sistemas de reparación por recombinación homóloga. Para ello utilizamos dos variantes del sistema HO mencionado

anteriormente y analizamos la reparación mediante Southern blot. Mediante estos sistemas es posible estudiar la reparación de una lesión mediante dos tipos de HR: 1) anillamiento de cadena dependiente de síntesis (SDSA), el cual utiliza una secuencia donadora del mismo cromosoma; 2) reparación de roturas de doble cadena (DSBR), el cual presenta la secuencia donadora en otro cromosoma. Sorprendentemente, encontramos que Cdc14 se necesita para reparar de manera eficiente el daño producido por la endonucleasa HO. En este punto nos planteamos si la fosfatasa podría tener un papel en la decisión de utilizar HR o unión de extremos no homólogos (NHEJ). Esta toma de decisión es uno de los primeros sucesos que tiene lugar durante la reparación de una rotura en el ADN, y por ello cualquier alteración en este paso puede desencadenar en un gran problema a la hora de reparar. Para validar esta hipótesis, utilizamos otra variante del sistema HO que permite distinguir entre un sistema de reparación u otro. Observamos que células que carecen de la actividad de la fosfatasa reparan de manera ineficiente el daño producido por la enzima, confirmando nuestros resultados obtenidos a partir de los otros sistemas HO. No obstante, este fenotipo no va asociado a un cambio en el ratio NHEJ/HR. Por lo tanto, la fosfatasa Cdc14 no participa en la decisión de utilizar un sistema de reparación u otro en presencia de una lesión en el ADN.

Lo siguiente que hicimos fue analizar el proceso de resección, uno de los primeros pasos para reparar una lesión en el ADN mediante HR. Este proceso consiste en la degradación de una de las cadenas del ADN en sentido 5'-3' con el fin de generar ADN de cadena sencilla (ssDNA) necesario para la búsqueda de homología y la invasión del donador. Durante la resección intervienen dos complejos: la exonucleasa Exo1, capaz de procesar la molécula de ADN por sí sola, y el complejo Sgs1-Dna2-STR. Este segundo grupo de proteínas se compone principalmente de la helicasa Sgs1 y de la nucleasa Dna2. Dna2 es una nucleasa esencial que no es capaz de actuar como tal por sí sola, y por ello requiere de la actividad de Sgs1 para separar las dos hebras del ADN. Utilizando otro sistema HO en el que generamos una única rotura no reparable, estudiamos la resección del ADN mediante Southern blot diseñando una serie de sondas que hibridan a distintas distancias del punto de corte de la enzima HO. Vimos que mutantes *cdc14-1* no presentaron problemas en la activación y en la ejecución de la resección bajo estas circunstancias. Este resultado sugiere que la fosfatasa no se necesita para iniciar el proceso de la reparación por HR. Dado que los mutantes carentes de la función de Cdc14 presentan defectos en reparación, podríamos suponer que la fosfatasa estaría actuando en algún paso siguiente de la recombinación homóloga.

No obstante, decidimos estudiar el papel de Cdc14 en la resección en un fondo genético capaz de reparar el daño. Para ello, utilizamos la variante del sistema HO mencionado anteriormente que presenta una secuencia donadora en otro cromosoma. Gracias a la capacidad de estas células de reparar la rotura somos capaces de analizar la resección y la reparación en un mismo experimento mediante Souhern blot. Curiosamente, observamos que mutantes *cdc14-1* que no tienen problemas a la hora de activar la resección son incapaces de desactivar dicho proceso. Concretamente, vimos que células que carecen de la función de la fosfatasa siguen reseccionando a largas distancias y de manera descontrolada, generando así, largas cadenas de ADN monocatenario. Es importante resaltar que este fenotipo al que hemos denominado de "hiper-resección" explica los problemas de reparación observados anteriormente en mutantes *cdc14-1*. Con el fin de determinar cuál es la vía de resección sobre la que actúa Cdc14, construimos una batería de mutantes de depleción de Exo1 y Sgs1, y estudiamos la contribución de ambas vías en la resección del ADN en mutantes *cdc14-1*. Para ello utilizamos la misma aproximación mencionada anteriormente. Descubrimos que la vía de Sgs1-Dna2 es la responsable del fenotipo de hiper-resección de los mutante *cdc14-1* ya que la ausencia de Sgs1 revierte dichos defectos de resección.

Otro de los experimentos destacables de este trabajo es la identificación de nuevas diana de la fosfatasa Cdc14 en respuesta a un daño en el ADN. Una de las herramientas más eficientes en este contexto es la espectrometría de masas combinada con enriquecimiento de péptidos fosforilados por dióxido de titanio. Esto permite determinar variaciones en los niveles de fosforilación de proteínas en distintas condiciones. En nuestro caso en concreto, hemos estudiado las variaciones de dichos niveles en presencia y en ausencia de Cdc14 en daño. Aquellas proteínas que presenten una reducción de los niveles de fosforilación tras la inducción de una lesión y que ocurran exclusivamente en cepas silvestres, pero no en mutantes *cdc14-1*, serán candidatos ideales a tener en cuenta como posibles diana de la fosfatasa. Dentro de la diversidad de dianas identificadas encontramos proteínas implicadas en transcripción, organización de la cromatina, replicación, señalización, reparación y puntos de control. Curiosamente, dentro de las dianas identificamos a la nucleasa Dna2 que, como hemos destacado previamente, junto con Sgs1 forma parte de una de las dos maquinarias implicadas en la resección del ADN. Con el fin de determinar si Cdc14 estaba actuando sobre esta proteína, llevamos a cabo ensayos de localización y de Western blot. Determinamos que Cdc14 defosforila a Dna2 en presencia de un daño reparable y cuando las células han activado de manera eficiente el punto de control. Suponemos que esta defosforilación es necesaria

para la inhibición de su función y la consecuente reparación, ya que se ha descrito que la activación y la translocación de Dna2 al núcleo dependen de su fosforilación por Cdk.

Finalmente, para confirmar que Dna2 es la diana de Cdc14 implicada en el fenotipo de hiper-resección, construimos una cepa portadora del *degron* inducible por auxina. Mediante este sistema, es posible eliminar una proteína esencial (en este caso Dna2) vía proteasoma, simplemente añadiendo auxina al medio de cultivo en el momento deseado. El sistema es muy rápido y efectivo y, además, la auxina no es tóxica para el crecimiento normal de las levaduras por lo que supone una gran herramienta en el estudio de la función de proteínas esenciales. A favor de nuestros resultados previos, cuando eliminamos Dna2 revertimos los defectos de resección y reparación anteriormente descritos en los mutantes *cdc14-1*. De esta manera, concluimos que la función de la fosfatasa Cdc14 en el proceso de resección y reparación cursa, al menos en parte, a través de la inhibición de Dna2. No podemos descartar que Cdc14 esté participando en otras etapas del proceso de reparación. De hecho, se ha descrito que la fosfatasa promueve la activación de la resolvasa Yen1, implicada en la resolución de intermediarios de reparación. Además, en nuestro experimento de espectrometría de masas, hemos identificado otras proteínas directamente implicadas en reparación. Por lo tanto, sería interesante validar estas posibles diana de Cdc14 con el fin de conocer aún mejor la importancia de la fosfatasa en el DDR.

Como idea general del trabajo realizado en esta tesis doctoral podemos destacar que la fosfatasa Cdc14 participa en el apagado de la resección actuando directamente sobre una de las vías que participan en dicho proceso. Además, la inhibición de este proceso es fundamental para que las células puedan llevar a cabo la reparación de la lesión en el ADN. Para ello, los mecanismos de activación/desactivación tienen que estar altamente regulados a nivel temporal y espacial. En este contexto proponemos un modelo en el que Cdc14 se libera para desfosforilar a Dna2 una vez que la resección ha tenido lugar. Esta defosforilación inactivaría la función de la nucleasa, probablemente a través de cambios en su localización subcelular, y permitiría la regeneración de la molécula de ADN previamente dañada. Estos resultados tienen especial relevancia en el campo de las fosfatasas y el DDR ya que ponen de manifiesto la importancia de dichas proteínas en la regulación de los procesos de reparación que tienen lugar durante la ejecución de la respuesta a daño.

Conclusiones

1. La actividad de la fosfatasa Cdc14 es esencial para la viabilidad celular cuando las células están expuestas a estrés genotóxico en *Saccharomyces cerevisiae*.
2. La función de Cdc14 es dispensable para la correcta activación del punto de control de daño.
3. La reparación del ADN analizada mediante SDSA y DSBR es ineficiente en células que carecen de la función de Cdc14. Por el contrario, la elección del sistema de reparación ocurre sin problemas en ausencia de la fosfatasa.
4. Cdc14 se libera de manera transitoria del nucléolo al nucleoplasma en respuesta a una o más roturas de doble cadena o DSBs.
5. Cdc14 no se necesita para la activación ni la ejecución de la resección del ADN a corta y a largas distancias de la rotura.
6. El sistema de reparación del ADN del fondo genético AC1595 supone una herramienta muy potente para el estudio de la resección y de la reparación del ADN mediante SDSA, combinando métodos convencionales de análisis físico y secuenciación masiva.
7. El uso de la secuenciación masiva en el estudio de la reparación del ADN en AC1595 aporta información detallada acerca de la producción del DSB, la resección, la direccionalidad de la síntesis del ADN, la fidelidad de copia, y la longitud de la cadena copiada.
8. La actividad de Cdc14 se necesita para inhibir la resección actuando sobre la vía de Sgs1-Dna2 una vez que las células han llevado a cabo la búsqueda de homología y la invasión de la secuencia donadora.
9. Cdc14 defosforila e inactiva a Dna2 durante la respuesta a daño para apagar la resección del ADN y, así, prevenir la formación de largas cadenas de ADN de cadena sencilla (ssDNA) que pueden interferir en la recuperación de la molécula.
10. La defosforilación de Dna2 por parte de Cdc14 es necesaria para la correcta ejecución de los mecanismos de reparación del ADN.

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A mi madre, a mi padre y a mi hermano.

Index

Abbreviations

Δ	Deletion
5-FOA	5-Fluoro Orotic Acid
4-NQO	4-Nitroquinoline 1-oxide
AID	Auxin-inducible degron
APC/C	Anaphase promoting complex or cyclosome
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BIR	Break induced replication
bp	Basepairs
Cdk	Cyclin-dependent kinase
CFP	Cyan fluorescent protein
cloNAT	Nourseothricin sulfate
CO	Crossover
CORE	Counterselectable reporter
CSM	Complete supplement
C-terminal	Carboxy-terminal
DAPI	4’,6-diamidino-2-phenylindole
DDA	Data-dependent acquisition
DDR	DNA damage response
dHJ	Double Holliday junction
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-Pk	DNA-dependent protein kinase
DSB	Double-strand break
DSBR	Double-strand break repair
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
DUSP	Dual-specificity phosphatase family
EDTA	Ethylenediaminetetra-acetic acid
ETOH	Ethanol
FACS	Fluorescence-activated cell sorting
FEAR	Fourteen early anaphase release
GAP	GTPase-activating proteins
GC	Gene conversion
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTPase	Guanosine triphosphatase
h	Hours
HA	Hemagglutinin
HO	Homothallic
HPH	Hygromycin B
HR	Homologous recombination
HU	Hydroxyurea
IAA	Indole-3-acetic acid
IR	Ionizing radiation
kb	Kilobases
LB	Luria Bertani
lncRNA	Long non-coding RNA
LOH	Loss of heterozygosity
LTR	Long terminal repeat
MAT	Mating-type

Min	Minutes
MEF	Mouse embryonic fibroblast
MEN	Mitotic exit network
MMS	Methyl methanesulfonate
MRN	Mre11-Rad50-Nbs1
MRX	Mre11-Rad50-Xrs2
MS	Mass spectrometry
MSA	Multistage activation
NER	Nucleotide excision repair
NHEJ	Non homologous end-joining
NLS	Nuclear localization signal
NoLS	Nucleolar localization sequence
N-terminal	Amino-terminal
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCNA	Proliferating nuclear antigen
PEG	Polyethylene glycol
PIKK	Phosphatidylinositol 3’ kinase-like kinase
RENT	Regulator of the nucleolar silencing and telophase exit
RFP	Red fluorescent protein
RNase	Ribonuclease
RNR	Ribonucleotide reductase
RPA	Replication protein A
r.p.m.	Revolutions per minute
SD	Synthetic minimal medium
SDS	Sodium dodecyl sulfate
SDSA	Synthesis-dependent strand annealing
Sec	Seconds
SMM	Supplemented minimal medium
SPB	Spindle pole body
SSA	Single strand annealing
ssDNA	Single-stranded DNA
STR	Sgs1-Top3-Rmi1
SUMO	Small ubiquitin-like modifier
TCA	Trichloroacetic acid
TE	Tris-EDTA
TFA	Trifluoroacetic acid
TIR	Transport inhibitor response
tRNA	Transfer ribonucleic acid
UV	Ultraviolet
v	Volume
YFP	Yellow fluorescent protein
YNB	Yeast nitrogen base
YPD	Yeast extract-peptone-dextrose

1. The DNA damage response

1.1. Importance and goals of the DNA damage response.

Mammalian genomes are constantly exposed to different endogenous and exogenous stresses that can cause genome instability. Endogenous DNA damage can be generated as failures in the normal functioning of the cell, such as errors committed by the DNA replication machinery, or accumulation of undesired metabolites like reactive oxygen species. On the other hand, different exogenous factors including viral infections, ultraviolet and ionizing radiation and chemicals products can also generate DNA lesions. In order to quantify the susceptibility of cells when dealing with this variety of DNA-damaging agents, it has been estimated that every single cell in the human body is exposed to about 10^5 spontaneous DNA lesions per day [1]. Considering that one of the main goals for cells is to transfer an intact copy of the genetic material to daughter cells they have to preserve the integrity of the genome. To achieve this aim cells have developed a coordinated set of actions, collectively known as the DNA damage response (DDR) that counteracts the huge amount of DNA damage they may suffer. These mechanisms are constantly surveying the genome in order to identify errors and fix them, thus becoming essential for cell viability [2, 3]. Indeed, when the DDR fails or the rate of DNA damage overwhelm the capacity of the cell to deal with it, the increased accumulation of genetic alterations can lead to senescence, death or malignant transformation [4-6]. In this regard, multiple congenital human disorders have been directly linked to failures in executing these essential mechanisms, mirroring the importance of the DDR and its components in the maintenance of genome stability and normal development [7-10].

The DDR is highly conserved and several studies have characterized a series of mechanisms that are executed within this sophisticated response. Once the DNA lesion occurred, the main objective of the damage response is to couple cell cycle progression with DNA repair. This is attained by the fine tune orchestration of two inter-connected pathways: 1) the DNA damage checkpoint, a molecular process that promotes a cell cycle arrest by inhibiting the segregation of the duplicated chromosomes until the DNA molecule is restored; and 2) the activation of a transcriptional program that allows the achievement of the DNA repair process. Mirroring the complexity of these pathways, the execution of the DDR involves proteins that have been

classified as sensors, signal transducing proteins, effector kinases, mediators, and effector proteins.

1.2. The initial steps of the DNA damage response.

The initiation of the DDR activation involves the recognition of the DNA damage by sensor proteins that are recruited to chromatin. Within these proteins we find the highly conserved MRX/MRN complex (Mre11-Rad50-Xrs2 in the budding yeast, Mre11-Rad50-Nbs1 in mammals) and the 9-1-1 heterotrimeric complex (Rad17-Ddc1-Mec3 in *Saccharomyces cerevisiae*, Rad9-Rad1-Hus1 in mammalian cells). The MRX complex has several roles at the damaged site: 1) is responsible for the initial 3' to 5' processing of one DNA strand, 2) maintains the broken DNA ends in close proximity, and 3) recruits signal transducing kinases to the break site, thus mediating DDR signaling. In addition, the MRX complex is essential to generate and resect meiotic double-strand breaks (DSBs) generated by the topoisomerase-like protein Spo11 which forms a covalent linkage between a tyrosine residue and the 5' end of the cleaved strand [11]. Regarding the 9-1-1 complex, its structure resembles the proliferating nuclear antigen (PCNA). Its loading around a DNA lesion depends on Rad24 (Rad17 in mammals)-containing clamp loader complex. Once loaded onto DNA, the 9-1-1 clamp complex has three functions: 1) activation of Mec1, 2) recruitment of Dpb11 to the DNA break site, and 3) promotion of resection at telomeres. Both the MRX and the 9-1-1 complexes activate the signal of several transducing kinases included in the phosphatidylinositol 3' kinase-like kinase family (PIKKs): the Ataxia-telangiectasia mutated (Tel1, ATM in mammals), the ATM and Rad3-related (Mec1, ATR in mammals), and the DNA-dependent protein kinase (DNA-PK). Mec1/Tel1 activation transduces phosphorylation to a high number of adaptors that finally will activate the effector kinases implicated in the DNA damage checkpoint activation and the execution of the DNA repair [12, 13]. It has been shown that Mec1/Tel1-dependent phosphorylation of repair and checkpoint substrates occurs mainly on an evolutionarily conserved (S/T)Q consensus sequence [14]. One clear example is the phosphorylation of the SQ motif located at the C-terminal of histone H2A in the surroundings of the DSB [15], a phosphorylation that is required for the recruitment of multiple DNA repair factors to the lesion. Regarding the role of DNA-PKs, it has been shown that they are essential for DNA repair by non homologous end-joining (NHEJ), telomere maintenance and induction of apoptosis [16, 17].

2. The DNA damage checkpoint

2.1. The DNA damage checkpoint in *S. cerevisiae*.

One of the most hazardous types of DNA damage is the DSB since it affects the two complementary DNA strands. Importantly, if they are not properly repaired they can lead to the generation of mutations and chromosomal rearrangements. However, DSBs play also important roles in the cellular physiology since programmed DNA breaks are generated during meiotic recombination [11, 18], normal rearrangement of immunoglobulin genes in mammalian lymphocytes (V-(D)-J recombination) [19] and mating-type switching in yeast [20].

The generation of DSBs in *S. cerevisiae* triggers the activation of a checkpoint response characterized by a cell cycle arrest prior to anaphase (termed as G2/M) with the sister centromeres of replicated chromosomes attached to the mitotic spindle and under tension [21]. Once a DSB is generated, it is recognized by the MRX complex (Fig.1) which recruits and activates Tel1. Tel1 stabilizes the MRX retention at the damaged site [22] and promotes the removal of the heterodimer Ku (Ku70/Ku80), a protein involved in NHEJ [23-25]. Importantly, Ku released from the break site allows the activation of the DNA end resection [24]. Tel1 also recruits the adaptor Rad9 to the DSB through the generation of Ser129-phosphorylated histone H2A (ɣH2A). Taking into account that Rad9 is required for the activation of the checkpoint kinase Rad53, we can consider Tel1 as one of the first factors involved in the activation of this kinase. On the other side, the activation of the resection machinery generates ssDNA thus resulting in the attenuation of the Tel1 signaling and promoting the activation of Mec1.

One of the first modifications that take place at a DSB that is going to be repaired by homologous recombination is the resection process. DNA end resection is a two-step process that involves the nucleolytic degradation of the 5'-ended strand at both sides of the DSB in order to generate ssDNA [26, 27]. The initial processing of the DNA molecule depends on the MRX complex and consists in the endonucleolytic cleavage of the DNA near the DSB ends [28, 29] (Fig.1). This reaction is highly stimulated by Sae2 (CtIP in mammals) previously phosphorylated by the cyclin-dependent kinase (Cdk) [30] and is followed by Mre11-dependent 3' to 5' resection toward the break site to generate a 5'-ended substrate (Fig.1). The nick created by the MRX serves as an entry site for nucleases that are able to process the DNA molecule in 5'-3' direction. The second step of the resection process consists on an

extensive processing of the DNA molecule triggered by two redundant pathways: the exonuclease 1 (Exo1) and the Sgs1/Dna2-Top3-Rmi1 complex [31, 32] (Fig.1). The generated long ssDNA tracks are then coated by the replication protein A (RPA) and serve as the signal for Mec1 recognition of the lesion. Additionally, It has been shown that Mec1 binding to DNA also depends on Ddc2/ATRIP, an essential protein that stimulates the interaction of Mec1 with its substrates [33-35].

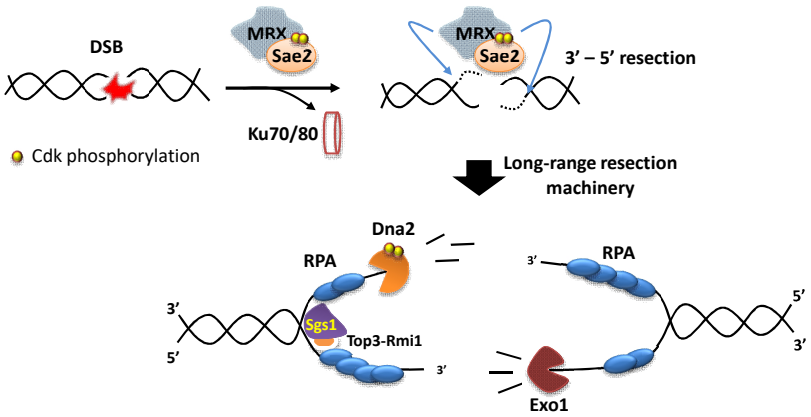


Fig.1. MRX recruitment to the DSBs initiates the signaling for the activation of the DNA damage checkpoint. In the presence of a double-strand break (DSB) the MRX complex and Sae2 are recruited to the damage site and signal its presence. Importantly, Cdk-dependent phosphorylation of Sae2 enhances MRX endonucleolytic activity thus allowing a cleavage at the vicinity of the break and a subsequent short resection in 3'- 5' direction. This processing provides a physical substrate required for Exo1 and Sgs1-Dna2 to initiate the long-range resection and generate single-stranded DNA (ssDNA). In a following step, RPA is recruited to protect the ssDNA generated and creating the structure needed for Mec1 recognition of the DNA lesion.

Once recruited to the damaged site, Mec1 channels the signal to the effector kinases Chk1 and Rad53 establishing two parallel branches of the damage checkpoint (Fig.2). Rad53 is involved in numerous functions within the DDR including replisome stabilization, expression of repair-related genes, inhibition of late origin firing, inhibition of recombination at stalled forks, fork resumption and repair [36]. Rad53 phosphorylation and activation require the Rad9 adaptor (equivalent to 53BP1, MDC1 and BRCA1 in mammals) which binds chromatin containing phosphorylated γH2A [37]. Once positioned at the DSB vicinity, Rad9 is phosphorylated by the Cdk [38] and Mec1 [39] and serves as a scaffold for Rad53 binding. Accumulation of Rad53 at the vicinity of the DSB enhances its autophosphorylation and consequently, its full activation [40]. Upon activated, Rad53 is released from the Rad9

complex, amplifying the checkpoint signal throughout the cell [41]. The Chk1 branch of the damage checkpoint also requires Rad9 for its activation (Fig.2). In addition, it has been proposed that Chk1 oligomerizes and probably undergoes autophosphorylation for its fully activation [42].

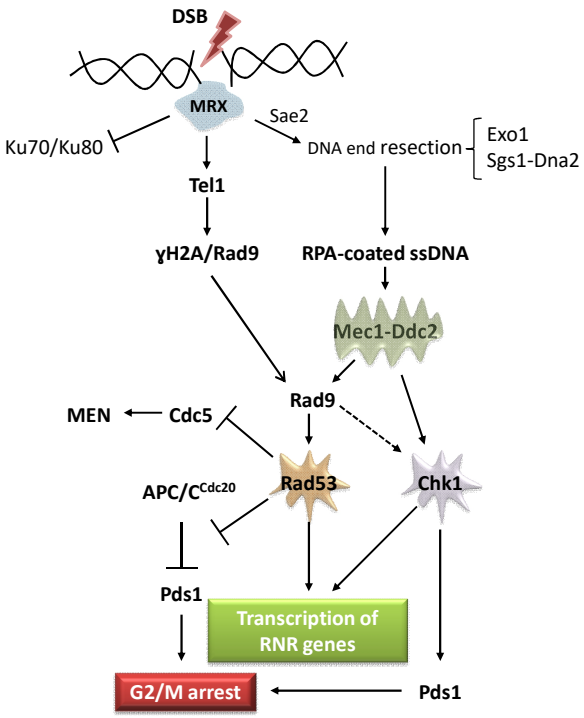


Fig.2. DNA damage checkpoint in *Saccharomyces cerevisiae*. Once a double-strand break (DSB) is generated, the MRX complex is recruited to maintain the broken DNA ends together and to trigger the damage checkpoint response by activating Tel1 and Mec1. After amplification of the signal, the effector kinases Rad53 and Chk1 are activated in order to: 1) promote a cell cycle arrest prior to anaphase (G2/M) by stabilizing the securin Pds1, and 2) induce a transcriptional program that activates genes required for DNA repair, as well as the posttranslational regulation of the ribonucleotide reductase (RNR).

Regarding the effectors involved in the cell cycle arrest, it has been demonstrated that budding yeast securin (Pds1) is required for a normal cell cycle arrest in the presence of DNA damage. This protein is ubiquitinated by the anaphase promoting complex/cyclosome ubiquitin ligase (APC/C) together with Cdc20 (APC/C^{Cdc20}) and consequently degraded as cells enter into mitosis in an unperturbed cell cycle. Importantly, Pds1 destruction is required for sister chromatid separation. In the context of DNA damage, Pds1 is stabilized by the two branches of the DNA damage checkpoint governed by Chk1 and Rad53 (Fig.2) [43-45]. Stabilized securin binds and inhibits the separase Esp1, the protein necessary of the cleavage

of cohesin, thus preventing sister chromatid separation. It has been proposed that Rad53 also blocks the mitotic exit network (MEN) through the inhibition of the polo-like kinase Cdc5 [44, 46] (Fig.2).

In addition to the inhibition of the cell cycle progression, the DNA damage checkpoint triggers a significant transcriptional response. One of the best-characterized outcomes within this response is the induction of high transcription of ribonucleotide reductase (RNR) genes [47]. Importantly, this phenomenon is controlled by the kinase Dun1 [47-49] which is activated by both Mec1 and Rad53 (Fig.2).

2.2. Inactivation of the DNA damage checkpoint.

Once DNA repair has been successfully accomplished, cells must turn off the damage checkpoint to re-enter the cell cycle in a process known as recovery. In yeast, cells that present an unrepairable DSB can also recover from the cell cycle arrest after long checkpoint-mediated delay through a process named adaptation [21, 50]. It is not clear if adaptation and recovery are interrelated mechanisms, but it is clear that the capacity of cells to adapt depends on the extent of DNA damage [21].

Some of the factors that are involved in the process of adaptation are also necessary for the inactivation of the damage checkpoint. One of these proteins is the helicase Srs2 which can remove Rad51 from ssDNA. It has been proposed that, in the absence of Srs2, Rad51 remains in the DNA even when the DNA has been repaired, therefore generating a persistent DNA damage checkpoint signal [51, 52]. Importantly, it seems that removal of DNA lesions is not enough to re-enter the cell cycle. Indeed, it is believed that Srs2 could remove several protein complexes from the DNA to inhibit the checkpoint signaling once the DNA molecule has been repaired.

Another step that is crucial for the maintenance of an active damage checkpoint signal is the DNA resection. Indeed, the longer the ssDNA track persists, the more active the DNA checkpoint is, due to the constant signaling that Mec1 produces when bound to ssDNA. To avoid over-resection, and thus, a hyperactivation of the damage checkpoint, cells have developed several mechanisms to control the extension of the ssDNA generated. It has recently been demonstrated that Rad9 might act as a barrier for the Sgs-Dna2 pathway [53, 54]. Similarly, active Rad53 phosphorylates and inhibits the exonuclease Exo1 [55]. An additional mechanism that inhibits DNA resection has been proposed in mammalian cells. It

consists on the degradation of EXO1 after the generation of DNA damage and involves the ATR-dependent phosphorylation of the exonuclease [56]. This rapid degradation of EXO1 might be crucial to prevent hyper-resection. It is not clear if this mechanism also operates in the budding yeast or other model organisms. In any case, it is reasonable to think the DNA end resection must be inhibited to avoid the generation of long ssDNA tracks that might generate a hyperactivation of the checkpoint. Importantly, uncontrolled DNA resection can lead to chromosome instability. There are two non-mutually exclusive explanations for the contribution of the unbridled resection to genome instability: 1) an extensive resection would shift DSB repair from Rad51-dependent HR to a more mutagenic pathway involving RAD52 [57, 58]; 2) the presence of extensive ssDNA would exhaust the existing pool of RPA leaving ssDNA regions unprotected against cellular nucleases [59, 60]. Considering that EXO1 is phosphorylated by CDK during S/G2 phases and is required for its activity in human cell lines [61] it seems that there is a temporal window for EXO1 activity, delimited by the initial activation by CDK-dependent phosphorylation and the subsequent phosphorylation by ATR to initiate its degradation [56]. Again, whether this mechanism is extended to yeast has to be determined. However, there are not fully Cdk-consensus site in the budding yeast Exo1 and up to date, has not been demonstrated that Exo1 is subjected to degradation in response to DNA insults, suggesting that its regulation might differ from humans.

Since the damage checkpoint is controlled by numerous phosphorylation events it is expected that phosphatases may also play a crucial role in reverting these activating signals to downregulate the response. In this line, it has been well documented that phosphatases are required for cell recovery in response to DNA damage in both mammals and yeast. One clear example is the dephosphorylation of the effector kinase Rad53. Up to date several phosphatases such as protein phosphatase-1 (PP1), protein phosphatase 2C (PP2C) and protein phosphatase 4 (PP4) have been involved in the reversion of Rad53-phosphorylation under different DNA-damaging conditions [62-64]. Interestingly, PP4 is also involved in the dephosphorylation of H2A in yeast and human cell lines to allow an efficient recovery from the checkpoint arrest [65, 66]. If these phosphatases might be also regulating the DNA damage checkpoint inactivation by controlling the resection machinery remains to be investigated.

Altogether, the fine-tune regulation of the resection machinery must be followed to limit the processing of the DNA molecule and prevent a constant signal from the damaged

chromosome. Once DNA repair has been accomplished, the resection machinery must be switched off to allow cell cycle progression and maintain genome stability.

3. Activation of the DNA repair pathway

As previously mentioned, the aim of the DDR is to couple cell cycle arrest with DNA repair. We have revised some important subjects about the checkpoint activation and regulation. Now, we will compile some results that will help us to understand the complexity of the repair pathway. As commented above, DSBs are one of the most hazardous types of DNA damage since defects in the repair of only one DSB can lead to aneuploidy, mutations or cell death. Cells have developed several mechanisms to detect and repair this type of lesion. In this regard, there are two major pathways involved in the restoration of the DNA molecule: non homologous end-joining [67] and homologous recombination [68]. Importantly, these processes highly depend on the nature of the DNA damage and the phase of the cell cycle in which the damage occurs.

3.1. An error-prone pathway essential for cell viability: non homologous end-joining (NHEJ).

Non-homologous end joining consists on the direct ligation of the two broken ends of a DSB. Since NHEJ does not use a template to repair de damaged DNA it is generally considered as an error-prone pathway. Moreover, in some cases the execution of this pathway is associated to some processing of the DNA ends, a situation that can lead to the generation of insertions or deletions. Furthermore, in the presence of multiple DSBs, end joining can result in chromosome translocations or fusions. Still, NHEJ is essential for cell viability during G1 when there is not a sister chromatid available for repair. This mechanism consists generally on three phases: 1) protection, 2) bridging, and 3) ligation of the exposed ends. In *S. cerevisiae,* NHEJ relies on three complexes that interact to each other at the DSB site [69]: MRX, Ku and DNA ligase IV (Dnl4-Lif1-Nej1). Firstly, the Ku heterodimer (Ku70-Ku80) binds and protects DSB ends from degradation, prevents DNA end resection and, in consequence, inhibits HR [23, 70]. In addition, Ku also acts as a scaffold for the recruitment and interaction between NHEJ factors. Importantly, the MRX complex is necessary to maintain DSB ends in close proximity through the zinc-hook of Rad50 [71-73]. Whether the recruitment of the MRX to the break site occurs before or after Ku-binding to the DSB is still unknown. Once Ku and MRX hold together the broken ends, Dnl4, Lif1 and DNA ligase IV are recruited to allow the alignment

and base pairing of the overhangs. Next, the ligation reaction is performed and the NHEJ proteins removed from the break vicinity.

3.2. A conservative DNA repair by homologous recombination (HR).

Homologous recombination is considered a legitimate DNA repair pathway since requires the presence of a template that will be used to copy the information lost during the generation of the DSB [74-76]. HR is considered an error-free pathway and, in consequence, the most accurate repair choice. However, this only applies to recombination between identical sister chromatids. Exchange of genetic information between homologs carries the risk of local loss of heterozygosity (LOH). In addition, a crossover between repeats on different chromosomes can result in chromosome translocations or other types of rearrangements [77].

At the DNA level, HR begins with the nucleolytic processing of the broken DNA ends. As previously mentioned, this process is known as DNA resection and involves the degradation of the 5' to 3' strand in a two-step process governed by the MRX complex and the long-range resection pathways Exo1 and Sgs1-Dna2 (Fig.3). It is worth mentioning that this process is triggered equally at both side of the DSB and two 3'-ended ssDNA are generated. Resected ssDNA is first coated and protected by RPA and then substituted by the strand exchange protein Rad51, a process driven by the recombination mediator Rad52 [78]. ssDNA-bound Rad51 forms the nucleoprotein filament (Fig.3). The nucleofilament is crucial to search for an intact homologous sequence (normally named donor) and to prime DNA synthesis once the strand invasion with the donor has taken place [79]. The simplest way for cells to find a donor sequence is to use the sister chromatid or the homologous chromosome during meiosis. Even if the mechanisms behind the homology search are not well understood, new evidences suggest that both the three dimensional proximity of DSBs/donor sequences into the nucleus and the involvement of several chromatin modifiers are essential for this process [79]. When homologous DNA sequences are found, a heteroduplex between the complementary donor and the 3'-ended nucleofilament is formed in a Rad51-dependent manner. This structure is known as the displacement loop (D-loop). At this point Rad51-dsDNA filaments are formed by accommodating both the donor and the invading strands within the filament. Next, Rad51 dissociates from the dsDNA to expose the 3'-OH required for the DNA synthesis machinery thus allowing the recovery of the information previously lost [80].

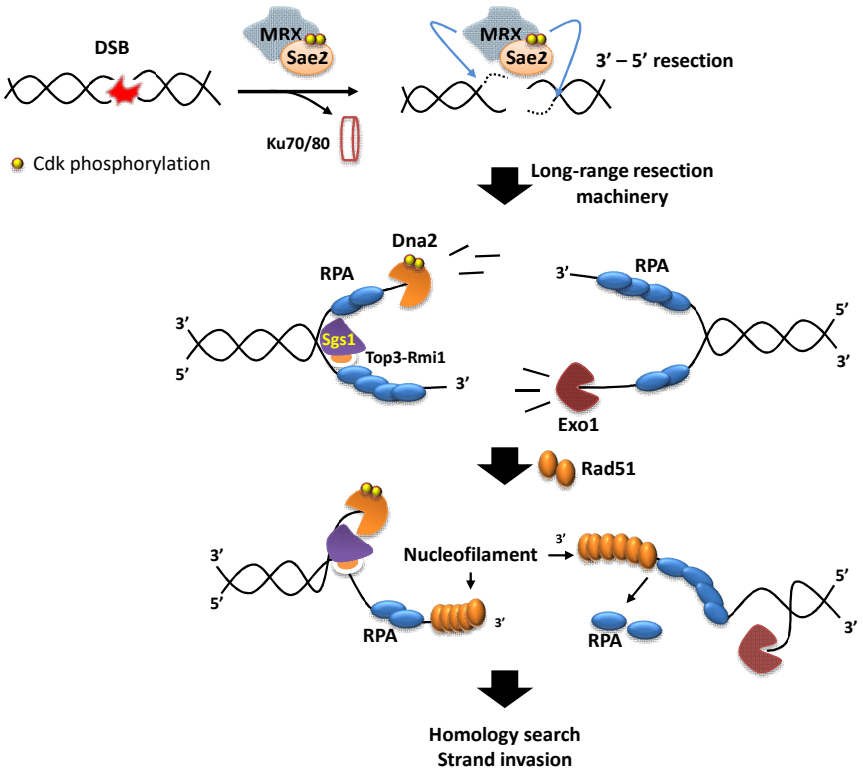


Fig.3. Schematic representation of the initial events require for the homologous recombination. After the initiation of the DNA end resection by the MRX complex and Sae2, Exo1 and Sgs1-Dna2 generate long single-stranted DNA (ssDNA) tracks that are rapidly covered by the replication protein A (RPA). Next, RPA is replaced by Rad51, a crucial component of the homologous recombination (HR) pathway. ssDNA-bound Rad51 forms the nucleofilament, a structure required for the homology search and invasion of the donor sequence that will serve as template to copy the information lost during the generation of the double-strand break (DSB). Importantly, the initiation of the DNA end resection removes Ku heterodimer from the break thus inhibiting non-homologous end joining (NHEJ). Note that either Exo1 or Sgs1-Dna2 can trigger long-range resection at both sides of the break. In this case, one pathway is included at each 5'-ended strand to simplify the scheme.

Once the DNA synthesis has been triggered there are three different routes that can be followed to resolve the D-loop (Fig.4):

1- **Double-strand break repair (DSBR):** this canonical pathway takes place when the second 5'-end of the DSB is engaged to stabilize the D-loop (second end capture) and, as a result, a double Holliday junction (dHJ) is formed [81, 82]. In addition, depending on the resolution of these dHJ intermediates by the action of resolvases/helicases, crossover or non-crossover products can be obtained. Crossovers consist on reciprocal exchanges between chromatids/chromosomes that share homologous sequences. By contrast, non-crossover products generate

unidirectional transfer of genetic information between sequences that share identity (also called gene conversion). Even if it has been demonstrated that crossovers are essential for the generation of genetic diversity through the meiotic program, during mitotic DNA repair cells tend to avoid crossover formation by activating several mechanisms involving helicases and anti-recombinases.

2- **Synthesis-dependent strand annealing (SDSA):** occurs when the invading strand is displaced from the D-loop and anneals with the other broken DNA end of the complementary strand [83]. SDSA is the preferred mechanism used during the repair of a mitotic DSB because this system never forms crossovers between sister chromatids. In this regard it is important to note that while in meiosis, crossovers are generated by the resolution of dHJ via the DSBR, non-crossovers outcomes are mainly produced by SDSA [84, 85].

3- **Break-induced replication (BIR):** takes place when the displacement loop is assembled into a replication fork, a phenomenon that involves the copy of the entire chromosome arm [86]. This mechanism is triggered more often when there is only one DNA end, either due to the loss of the other end or in the process of telomere lengthening in a deficient-telomerase context.

4- **Single strand annealing (SSA):** All the previous routes share their dependency on the recombinant protein Rad51. However, the SSA pathway does not rely on Rad51 for its execution. Instead, the ssDNA generated by the processing of the DNA molecule exposes homologous sequences in the surrounding area f the lesion that anneal and ligate. It is important to remark that SSA entitles the elimination of all information encoded between the two homologous sequences [87] (Fig.4).

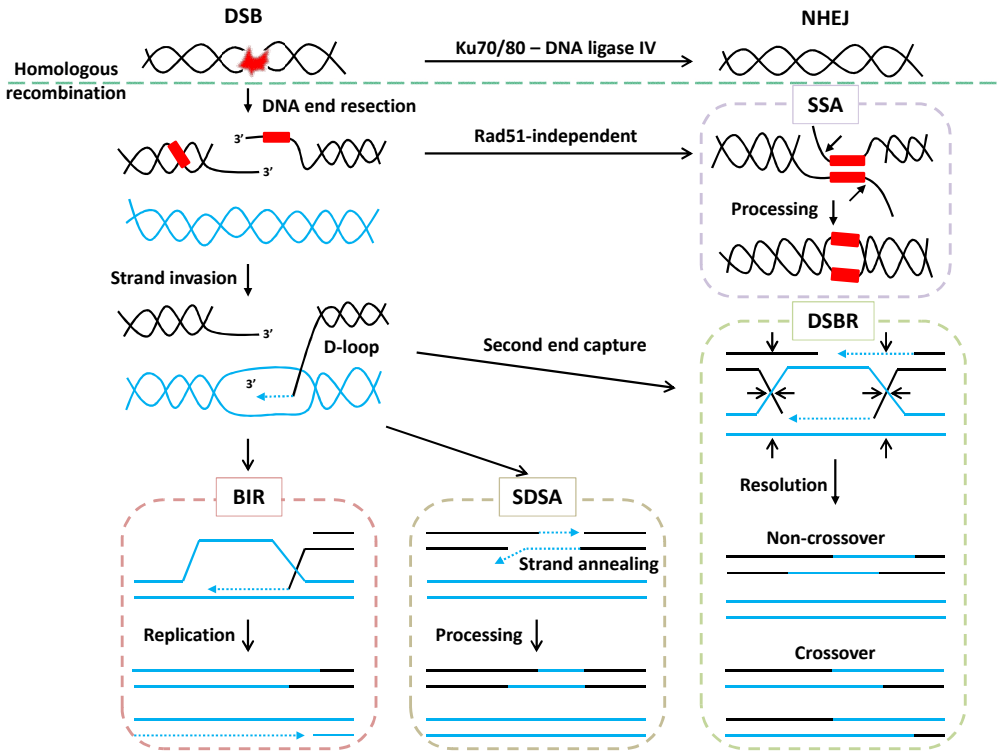


Fig.4. Models for the repair of a double-strand break (DSB). After the generation of DSBs two mutually exclusive pathways can fix it. Non-homologous end joining (NHEJ) joins the broken DNA ends to restore the DNA molecule. If DSB is resected, cells trigger homologous recombination (HR). This pathway involves the generation of a 3'-protuberant end that, together with Rad51, mediate the homology search and strand invasion, generating the displacement loop (D-loop). After priming DNA synthesis, three routes can be invoked. During BIR, which can occur when the other end of the break is absent, D-loop turns into a replication fork capable of both lagging and leading strand synthesis. In SDSA, the nascent strand is first displaced to anneal with the complementary 3'-tail thus promoting DNA synthesis. In addition, nucleolytic processing might be also required during SDSA. In DSBR pathway, the second end is capture and a dHJ intermediate is formed. Resolution of dHJs can occur in either plane (indicated by arrows), generating crossover or non-crossover products. The Rad51-independent pathway SSA is also depicted. During SSA, DNA resection can reveal complementary sequences (red squares) between two repeats, allowing annealing. The 3'-overhangs are then removed and the nick ligated.

3.3. DNA end resection.

As mentioned above, in most eukaryotes DNA end resection consists in a two-step process that initiates with a nucleolytic processing limited to the vicinity of the DSB followed by a long-stretch degradation of the 5'-ended DNA fiber. It is important to note that not all sources of DNA damage generate the same kind of lesion. The presence of secondary structures, chemical modifications or proteins bound to the DNA end supposes a challenge for the resection machinery. In line with this, it has been proposed that the nuclease activity of Mre11 is necessary to process these non-canonical DNA ends [88, 89] but is dispensable

for the resection of “clean” DSBs such as those generated by endonucleases [90]. Consistent with this, it was found that resection initiation at clean DSBs is initiated by an endonucleolytic cleavage of the 5’ strand of the DNA molecule induced by Dna2. When Dna2 is absent, the MRX together with Sae2 can cleave the 5’-ended DNA fiber at more distal sites to initiate resection in an alternative pathway [91].

As explained before, the action of MRX at the DSB allows the recruitment and the access of the nucleases capable of degrading DNA in a 5’ to 3’ direction [29, 92]. These nucleases comprise the exonuclease Exo1 and the endonuclease Dna2, which govern two non-overlapping pathways of long-range resection [32]. Importantly, inactivation of a single pathway results in only a minor defect in resection, due to the redundant function of both routes. However, when both Exo1 and Dna2 are affected simultaneously, significant resection defects are observed [31, 32, 93]. There are some differences between both pathways in terms of regulation and mechanism of action. Next, we will describe some relevant features about these routes.

3.3.1. Exo1 resection pathway.

Exo1 has the ability to release mononucleotide products from dsDNA ends in 5’ to 3’ direction [94]. The enzyme does not need a helicase to unwind DNA and directly produces a long 3’-ended DNA that will mediate the homology search and invasion [94, 95]. Exo1 is involved in several processes such as mismatch repair, DSBR, nucleotide excision repair (NER) and telomere maintenance [96-98]. It has been shown that Exo1 requires some factors to stimulate its processivity [99]. In this line, it has been shown that the MRX together with Sae2 enhance Exo1 function [95, 100]. Moreover, it is believed that other proteins involved in the DNA damage checkpoint and the HR pathway might encourage Exo1 nuclease activity in the context of DNA resection [101].

Regarding its regulation during DNA resection, Exo1 is recruited to the DSB by the MRX complex and Sae2. This recruitment is blocked by the presence of the Ku heterodimer [102-104]. In line with this, Exo1 can function without MRX as long as Ku is absent [88, 95]. The interaction between Exo1 and RPA is more complicated since RPA can both inhibit and stimulate the function of the exonuclease [95, 100]. Supporting this complex interaction, it has been demonstrated that while Exo1 is recruited more efficiently to DSBs in the absence of RPA, the exonuclease is more processive when bound to RPA [95, 105, 106].

3.3.2. Sgs1-Dna2 pathway.

The DNA replication-dependent helicase/nuclease 2 (Dna2) is a bifunctional helicase-nuclease essential for cell viability due to its role in the elimination of long DNA flaps during the Okazaki fragment maturation [107, 108]. In the context of DNA repair, Dna2-dependent resection needs the RecQ helicase Sgs1 (Werner syndrome protein, WRN; or Bloom syndrome protein, BLM; in human cells) [109], to unwind dsDNA in a 3'-5' polarity [32, 110]. Sgs1 functions together with topoisomerase 3 (Top3) and Rmi1 in dissolving dHJ to generate non-crossover products thus preventing sister chromatid exchange and genome instability [110, 111]. Interestingly, it was demonstrated that Top3 and Rmi1 are also involved in the stimulation of end resection by recruiting Sgs1 to DSBs [112, 113]. Once the Sgs1-Top3-Rmi1 (STR) complex is bound to a DSB, it provides the ssDNA substrate required for the Dna2 nuclease to be loaded at the break site [114]. Dna2 endonuleolytically degrades DNA in 5' to 3' direction, generating fragments of two different lengths ranging from 5-100 nucleotides in length [115, 116]. Importantly, it was demonstrated that RPA is critical to establish the correct polarity (5'-3') of the Sgs1-Dna2 pathway. RPA inhibits the degradation of the 3'-ended ssDNA but stimulates the elimination of the 5'-terminated strand [112, 113]. This observation is quite relevant since it was described that Dna2 has both 3'-5' and 5'-3' nuclease activities. In addition, Dna2 is not recruited to DSBs in the absence of RPA thus producing a block in long-range resection [105]. Surprisingly, it was found that the helicase activity of Dna2 unwinds dsDNA only when the nuclease activity is inactivated, revealing that the nuclease masks the unwinding activity of the enzyme [117, 118].

Lately, a further characterization of the Dna2 helicase activity in resection has been made. Levikova *et al.* [116] studied the effect of the helicase-proficient and the helicase-deficient variants of the budding yeast Dna2 in the processing of ssDNA *in vitro*. This work proposed that the helicase activity of Dna2 acts as a motor 5'-3' translocase to allow the tracking of the enzyme through the unwound 5'-terminated DNA strand. Importantly, this movement accelerates the degradation of the ssDNA by its nuclease activity in the presence of ATP and RPA, generating long DNA products that will be further degraded into smaller pieces. Indeed, helicase-deficient variants of Dna2 are not able to generate degradation products longer than 20 nucleotides. It seems that this model fits with the current knowledge about the function and regulation of Dna2 in DNA resection. Importantly, this molecular mechanism might also be conserved in higher eukaryotes since same results were obtained when working with the

human DNA2 [116]. This model also highlights the importance of Sgs1 as leading helicase necessary to unwind dsDNA and to allow Dna2 loading onto ssDNA as previously proposed.

Several studies have revealed that DNA resection by Sgs1-Dna2 is also stimulated by the MRX complex. MRX might recruit Sgs1-Dna2 to the DSB site since Mre11 interacts with Sgs1 and stimulates its helicase activity [92, 112, 113]. Additionally, it was demonstrated that Sgs1 interacts with Rad51 [119]. Even if the meaning of this interaction is still unknown, it would be interesting to see if Sgs1 collaborates with Rad51 in its loading onto ssDNA once generated by the resection machinery [101].

Finally, it is important to remark that Rad9 acts as a barrier for the Sgs1-Dna2 pathway [54, 120, 121]. Indeed, Rad9 also inhibits both Exo1 and Sgs1-Dna2 pathways at uncapped telomeres [122]. Even if the mechanism by which Rad9 inhibits or blocks DNA end resection is still unknown, it seems that depends on chromatin association and oligomerization of Rad9. This suggests a model whereby Rad9 might act as a physical barrier for the resection machinery [54].

3.3.3. Cdk-dependent regulation of the DNA end resection.

As commented before, the Cdk plays an essential role in controlling the DNA resection at multiple levels. Cdk is important for the activation of Sae2 [123, 124] and the MRX complex (Mre11 and Xrs2) in the presence of different sources of DNA damage [125]. This mechanism might be important for the inhibition of NHEJ and promotion of HR. At the resection level, it has been demonstrated that Cdk1 phosphorylates and activates Dna2. Dna2 presents 3 full consensus sequences (S/T-P-x-K/R; Ser17, Ser237 and Thr4) (Fig.5) and 5 minimal S/T-P consensus sites for Cdk1 phosphorylation. However, only the three full consensus sites seem to be phosphorylated by Cdk1, being S17 and S237 the most relevant [120]. In this regard, cells harboring a phospho-deficient version of Dna2 (*dna2*^{S17A,S237A}) showed a significant defect in focus formation when exposed to a DSB. Supporting this observation, Dna2 recruitment to the DSB vicinity is impaired in this mutant [120]. Additionally, long-range resection is highly affected when the three CDK consensus sites are mutated to alanine, confirming the importance of the Cdk1-dependent phosphorylation in the recruitment and activation of Dna2 at the break site. Strikingly, Dna2 nuclear localization depends on Cdk1 phosphorylation since the inhibition of its kinase activity restrained the nuclease at the cytoplasm [126]. Importantly, two of the three full consensus sites (T4 and S17) lie on a nuclear localization signal (NLS) domain (Fig.5), indicating that these two sites might be

crucial in controlling the cytoplasm/nucleus shuttling of Dna2. On the contrary, S237 is near the nuclease domain and surrounded by Mec1/Tel consensus sites (Fig.5). In this regard, it was observed that Dna2 is phosphorylated by Mec1 in response to DNA damage. This phosphorylation depends, at least in part, on previous Cdk1-dependent phosphorylation of Dna2 since *dna2*^{S17A,S237A} double mutant exhibited a decrease in the abundance of phospho-bands [120]. It is not clear if the position of the Cdk sites may interfere with other post-translational modifications but it would be interesting to determine the impact of these modifications on Dna2 function. Surprisingly, human DNA2 lacks the entire N-terminal region of its yeast counterpart including the full CDK consensus sites. This suggests that Cdk-dependent regulation of long-range resection might be different between organisms.

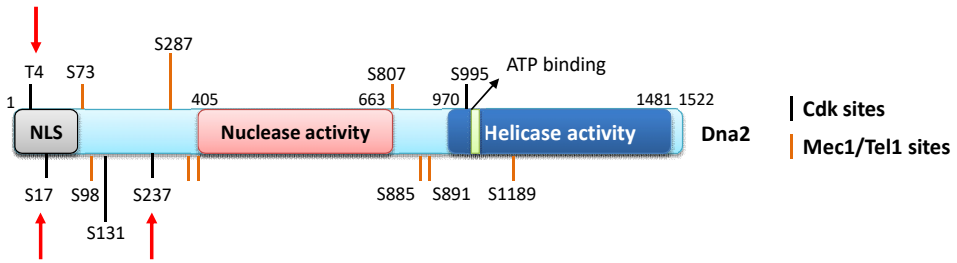


Fig.5. Diagram showing Dna2 sites phosphorylated by different kinases. The nuclear localization signal (NLS), nuclease and helicase domains of Dna2 are depicted. Relevant Cdk and Mec1/Tel1 kinase sites are included. Numbers indicate the position and the total number of amino acids. Note that the NLS is located at the N-terminal region of the protein and that it contains two of the full consensus sites for Cdk1-dependent phosphorylation. Full CDK consensus site are indicated by red arrows.

As mentioned above, Ku is the main responsible for antagonizing resection, thus directing the repair process to NHEJ. To allow DNA repair by HR, Ku must be removed from the break site. In this line, it has been established that in the absence of the heterodimer Ku, Cdk1 activity is dispensable for the initiation of resection by the MRX and Sae2, but is still needed for long-range resection [24]. This indicates that Cdk1 promotes the MRX function by inhibiting the recruitment of Ku at the DSBs. However, it seems that this observation can be explained by an indirect effect of the Cdk1 since removal of most Cdk consensus sites from Ku70 and Ku80 had no effect on the repair of the break in the budding yeast [127].

It is worth mentioning that DNA resection is not only controlled by the subcellular localization and activation of the resection machinery itself. We have to comprehend the DNA processing in a context of chromatin remodeling since it might suppose a natural barrier for the resection processes. In this line, it has been demonstrated that Cdk1 and cyclins (Clb2, Clb5 and Cln2)

are recruited to DSBs to stimulate DNA damage checkpoint activation and DNA end resection [128]. This regulation involves, at least in part, the phosphorylation of the chromatin remodeler Fun30 [128]. Fun30 might produce a DNA conformation suitable for the access of both Exo1 and Sgs1-Dna2 enzymes thus promoting DNA resection both in humans and yeast [121, 129, 130]. Furthermore, it was shown that Fun30's function in resection becomes less important when Rad9 is absence, indicating that this chromatin remodeler is hampering the inhibitory effect imposed by Rad9 [129].

3.4. Repair pathway choice.

When cells are exposed to endogenous or exogenous sources of DNA damage they have to recognize these insults in order to activate an accurate repair mechanism and restore the DNA molecule. To do this, they have to decide between triggering NHEJ or HR, a decision that is collectively known as the repair pathway choice. The first evidence for a direct competition between NHEJ and HR was obtained in mammalian cells where it was shown that elimination of Ku is associated with an increase in the frequency of DNA repair between direct repeats by HR [131]. Nowadays, it has been broadly demonstrated that cell cycle progression governs the repair pathway choice. NHEJ can occur in any phase of the cell cycle, but in *S. cerevisiae*, is limited to the G1 phase when there is no sister chromatids or homologous sequences to serve as donor for HR. Accordingly, NHEJ is reduced in G2/M phases while homologous recombination is encouraged. Importantly, the inhibition of NHEJ in G2 does not depend on the HR pathway but is related with reduced association of Ku and Dnl4/Lif1 proteins at the DSB site due to the action of the Cdk1 [127]. In line with this, Cdk1 controls cell cycle dependent repair pathway choice at multiple steps: 1) activates DNA end resection which is essential for executing of HR but inhibitory to NHEJ [132-134]; 2) Cdk1 is required for later steps of the homologous recombination pathway [132].

As mentioned before the initiation of long-range resection commits cells to repair by HR instead of NHEJ. This is due to the generation of ssDNA which is not a suitable substrate for Ku [135]. It was shown that HR efficiency increases in the absence of Ku, but the reverse is not the case, indicating that cells tend to activate NHEJ before activating DNA end resection [131, 136, 137]. In yeast, removal of Ku from DNA ends is thought to be carried out by MRX and Sae2 clipping activity [88, 138]. Elimination of Sae2 or Mre11 results in a much higher frequency of NHEJ [124]. Similarly, a molecule analog to mirin, which specifically inhibits the MRE11 endonuclease activity and prevents the formation of IR-induced RPA foci, promotes

NHEJ in mammalian cells [139, 140]. On the other hand, elimination of Rad51 or Rad52 in yeast has no effect on NHEJ, consistent with the notion that once resection initiates cells are committed to HR.

4. Phosphatases in the damage response

During the last years, there have been rapid progresses in the characterization of the mechanisms governed by the DNA damage response. As mentioned above, the transmission of the signal along the DNA damage checkpoint and DNA repair pathways is mainly driven by phosphorylation events endorse by specific kinases that phosphorylate DDR components, predominantly at serine and threonine residues of [141-143]. However, less is known about the role of protein dephosphorylation by protein phosphatases and their implication in the restoration of a DNA lesion. Still, it is reasonable to think that the fine-tuning of the response relies on the activity of phosphatases in order to prevent illegitimate activation of the DDR in the absence of damage as well as to allow a rapid cessation of the signal once the DNA lesion has been fixed. Because of this perspective, most of the studies involving protein phosphatases have been focused in their role in counterbalancing DDR-kinases to stimulate cell cycle re-entry upon repair. However, in the last years several studies have revealed that these enzymes are also able to directly modulate the DDR at the repair level, a discovery that has changed the perception of protein dephosphorylation in the response to DNA damage.

To date, several phosphatases have been mainly involved in the DDR such as PP1, PP2A, PP4 and the Cdk-antagonizing phosphatase CDC14. These phosphatases can be classified into two groups on the basis of their sequence, structure and biological activity. PP1, PP4 and PP2A are comprised in the classic Ser/Thr phosphoprotein phosphatases family (PPPs) while Cdc14 forms part of the dual-specificity phosphatase family (DUSP). One peculiarity of these phosphatases is their ability to counteract a great number of kinases. It has been estimated in human cells that there are about 500 protein kinases, while only 150 phosphatases have been described up to date [144, 145]. Due to this difference between the number of kinases and phosphatases, it has always been considered phosphatases as promiscuous enzymes. Today we know that these proteins are indeed selective and tightly regulated enzymes. The discrimination in targets recognition by protein phosphatases is attained by their ability to form specific complexes between a catalytic subunit and multiple regulatory elements that confer specificity to the holoenzyme [146-148].

It is important to note that regulation of protein phosphorylation/dephosphorylation during the DDR is critical to maintain genome integrity and prevent the development of diseases such as cancer. Phosphatases have been involved in the DDR activation after a DNA lesion is generated, and in its inactivation when the damage has been repaired. It is generally accepted that this control might be hijacked by cancer cells to elude the activation of checkpoint pathways during tumorigenesis, allowing tumor cells to grow uncontrolled. Supporting this notion, several types of cancer show an altered regulation of the DDR, a fact that may explain the accumulation of high levels of DNA damage at later stages of the disease. In addition, most oncogenes encode for protein kinases and phosphatases, reflecting the importance of protein phosphorylation in cancer development and progression. Interestingly, protein phosphatases can also operate as tumor suppressors through positive regulation of the DDR [147, 149]. In this regard, these enzymes have been implicated not only in the control of the DNA damage checkpoint, but also in the regulation of the repair mechanisms operating throughout the response.

4.1. The Cdc14 phosphatase.

The cell division cycle 14 (Cdc14) is one of the most studied families within DUSPs. These proteins are characterized by their ability to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues in their substrates. The Cdc14 phosphatases family is highly conserved, and orthologs have been described in several organisms. One special feature of this family is its predisposition to dephosphorylate targets of the Cdk. In particular, the Ser/Thr phosphatase Cdc14 was firstly identified in *S. cerevisiae* as an essential cell cycle protein required for Cdk inactivation and mitotic exit [150].

4.1.1. Cell cycle regulation of Cdc14.

It has been broadly demonstrated that the eukaryotic cell cycle is driven by a series of Cdk-cyclins complexes. In *S. cerevisiae*, there is a single Cdk, Cdc28, that governs all cell cycle transitions by interacting with different cyclins (Clns for G1 and Clbs for S/M cyclins). The simplicity of this model has supposed an advantage for the study of different regulators within the cell cycle. Today we know that S and M phase cyclins must be degraded to allow mitotic exit [151]. In budding yeast, inhibition of the mitotic Cdk-Clbs complexes is triggered by two redundant mechanisms: 1) the degradation of Clbs; and 2) the accumulation of the Cdk inhibitor Sic1 [150, 152]. Degradation of Clbs by proteolysis is a two-step process that depends on the APC/C. As commented before, the ubiquitin protein ligase APC/C^{Cdc20} controls

the metaphase-anaphase transition. It initiates anaphase by promoting the elimination of Pds1 to allow the Esp1-dependent cleavage of cohesin thus allowing chromosome segregation. Besides, APC/C^{Cdc20} also induces the initial degradation of S phase and some M cyclins in a proteasoma-dependent manner. In late anaphase and subsequent G1, APC/C forms a complex with the cofactor Cdh1 (APC/C^{Cdh1}) to participate in the second round of cyclins proteolysis in order to remove all M cyclins, including the major mitotic cyclin Clb2 [153]. In addition to the inactivation of Cdk-Clbs, exit from mitosis needs the reversal of the phosphorylation imposed by these kinases complexes [153]. In this context, the Cdc14 phosphatase plays an essential role both in reversing and inactivating the Cdk-Clbs complexes [150, 154]. Particularly, Cdc14 dephosphorylates and activates Swi5, which is a transcription factor for Sic1, and Cdh1, the cofactor of the APC/C [150, 155].

In budding yeast, the function of Cdc14 is highly regulated by cell cycle-dependent changes in its localization. In G1, S and early M phase, Cdc14 is retained at the nucleolus by interacting with Net1 (also called Cfi1), forming the RENT (Regulator of the Nucleolar silencing and Telophase exit) complex [156-158] (Fig.6). Importantly, nucleolar sequestration of Cdc14 avoids its interaction with its substrates and, therefore, it is considered that the presence of the phosphatase at the nucleolus determines its inactive state. This spatial inhibition is attained by the binding of the N-terminal region of Net1 to the active site of the phosphatase [158]. As cells enter in mitosis, Cdc14 retention at the nucleolus becomes weaker, thus being released in two distinctive waves, first to the nucleus and then to the cytoplasm. These changes in the localization of Cdc14 are promoted by the activation of two interconnected regulatory networks known as FEAR (Fourteen Early Anaphase Release) and MEN, respectively [159] (Fig.6).

FEAR takes place in early anaphase and is mainly driven by the separase Esp1 which, together with Cdc5, the kinetochore protein Slk19 and Spo12 regulate Cdc14 release to the nucleus [159]. On the other side, it has also been established that FEAR-dependent Cdc14 release is endorsed by the effect of the Cdk in Net1 phosphorylation. This Cdk-dependent phosphorylation of Net1 relaxes its interaction with Cdc14, thus liberating the phosphatase to the nucleoplasm [160]. In parallel, Esp1 together with Slk19 inhibit PP2A which dephosphorylates Net1 allowing its phosphorylation by the Cdk [160] (Fig.6). During the last years, the polo kinase Cdc5 has also been identified as an essential player in the regulation of cell cycle-dependent release of Cdc14 during the FEAR pathway (Fig.6). As the Cdk, Cdc5

has also the ability to phosphorylate both Net1 and Cdc14, a phosphorylation that enhances the phosphatase shuttling from the nucleolus to the nucleus [159, 161-163].

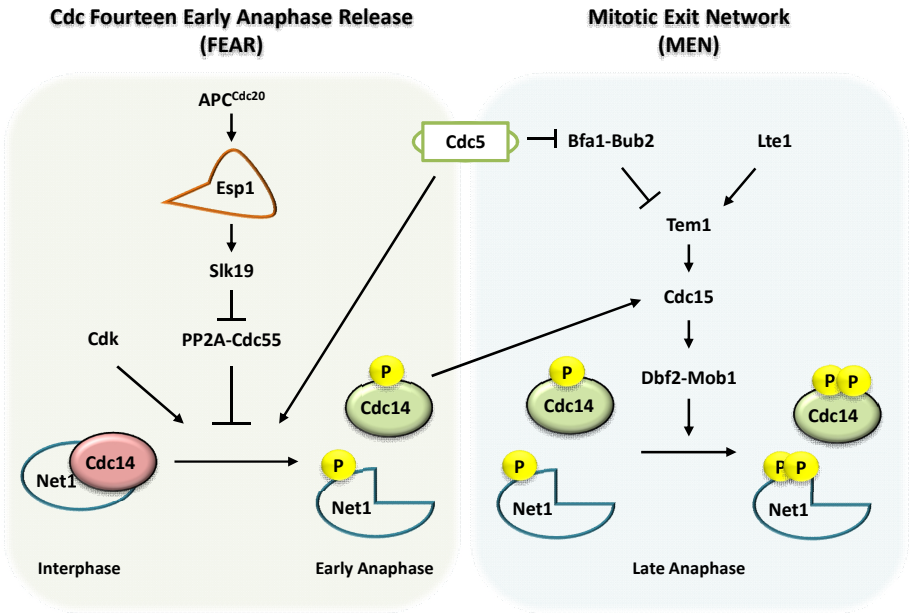


Fig.6. Cdc14 localization is highly controlled during an unperturbed cell cycle in *S. cerevisiae*. Cdc14 is sequestered into the nucleolus by interacting with Net1 during interphase. During early anaphase when the phosphatase spreads first to the nucleus and then to the cytoplasm by the activation of FEAR and MEN pathways. During FEAR, the separase Esp1 together with Slk19 inhibit the function of the phosphatase PP2A thus allowing the Cdk-dependent phosphorylation of Net1. This phosphorylation is required for Cdc14 release to the nucleus. The phosphatase also promotes MEN by acting over the kinase Cdc15 which in turn activates Dbf2-Mob1. Dbf2-Mob1 phosphorylates Cdc14 and promotes its second release. Cdc14 liberated by MEN dephosphorylates several targets that allow mitotic exit. Note that Cdc5 is depicted in between FEAR and MEN representing its dual role in promoting both waves of Cdc14 release.

MEN initiates in late anaphase with the activation of the GTPase Tem1, the upstream member of this pathway [156]. The activity of Tem1 is regulated by the GEF Lte1 and the GAP complex Bfa1-Bub2. Interestingly, Cdc5 has also a role in the regulation of MEN. In this regard, it has been postulated that Cdc5-dependent phosphorylation of Bfa1 and Bub2 blocks its inhibitory effect over Tem1 thus promoting MEN [164-166] (Fig.6). Importantly, Cdk1 controls the activation of Cdc5 first during FEAR and later in MEN [167] confirming the importance of the Cdk in the regulation of the Cdc14 release and providing new evidences about the implication of the Cdc5 in the MEN pathway. Once Tem1 is activated, it transduces the signal downstream to Cdc15 (Fig.6). Interestingly, FEAR-dependent translocation of Cdc14 has also the ability to encourage its own release by dephosphorylating and activating Cdc15, thus contributing to its complete release at late anaphase. However, it is important

to remark that while FEAR-dependent release of Cdc14 potentiates MEN, it is not essential for the correct execution of mitotic exit [168]. Finally, activated Cdc15 stimulates Dbf2-Mob1-dependent phosphorylation of Cdc14 driving its translocation to the cytoplasm [169, 170]. The cytoplasmic localization of the phosphatase allows its interaction with Sic1, Swi5 and Cdh1, a fundamental step to trigger mitotic exit in *S. cerevisiae*.

To better understand the physiological meaning of this two-step release of Cdc14, efforts have been focused on the identification of specific targets for the phosphatase on each pathway. FEAR-mediated release of Cdc14 has been implicated in chromosome segregation [171, 172] and transcription [171, 173, 174]. On the other hand, Cdc14 activated by MEN is essential for mitotic exit [150], cytokinesis [175, 176] and resolution of linked DNA intermediates [177-179].

In contrast to the high dependence of Cdc14 for exiting mitosis in the budding yeast, the *S. pombe* homologue Cdc14-like phosphatase 1 (Clp1; also known as Flp1) is not essential for accomplishing mitosis [180, 181]. This different requirement of Cdc14/Clp1 between budding and fission yeasts has always been attributed to the distinctive roles of both phosphatases in the execution of the mitotic exit program. However, the fact that a severe depletion of Cdc14 in *S. cerevisiae* by using an auxin-inducible degron does not affect mitosis exit has challenged this point of view, and has reunited both organisms under the common idea of multiple phosphatases cooperating in the removal of Cdk phospho-residues throughout mitosis [182]. As in its budding yeast counterpart, *S. pombe* Flp1 localizes at the nucleolus and the spindle pole body (SPB) during G1 and S phases. However, there are some differences between the budding and the fission yeast in terms of release and activation of the phosphatase. Flp1 release from the nucleolus takes place in metaphase and does not depend on the FEAR pathway [183]. Nevertheless, once released relocates first to the mitotic spindle and kinetochores and later at the contractile ring, similarly that in the budding yeast [184]. In vertebrates, three homologous of Cdc14 yeast have been characterized (CDC14A, CDC14B and CDC14C). During interphase, Cdc14A is localized at centrosomes while Cdc14B is mainly nucleolar. As in yeasts, Cdc14B is also liberated from the nucleolus in anaphase to relocate to the sister chromatids [185]. Little is known about the cellular localization and spatial regulation of Cdc14C. However, taking into account the great similarity in protein sequence with Cdc14B, it is thought that both phosphatases might be genetically redundant.

4.1.2. Role of Cdc14 in the DDR.

As mention before, one special feature of the Cdc14 phosphatase family is its predisposition to dephosphorylate targets that have previously been phosphorylated by the Cdk. Since the Cdk controls multiple aspects of the DDR pathway, it is tempting to speculate that the main role of Cdc14 in these processes might be related to its capacity to revert the phosphorylation events imposed by this kinase. In this line, efforts have been focused on understanding the role of this phosphatase family by counteracting Cdk substrates in the DDR. One of the first evidences regarding the role of Cdc14 in DNA damage came from the fission yeast, where Flp1 is translocated from the nucleolus to the nucleus after DNA replication stress induced by HU [186] (Fig.7). Nucleolar release of Flp1 is regulated by the checkpoint kinase Cds1, which is recruited to stalled forks during replication stress. Surprisingly, Flp1 seems to regulate the complete activation of Cds1 through a positive feedback loop that allows a proficient execution of the DNA damage checkpoint in response to replication stress [186] (Fig.7). Corroborating the phosphatase release/activation in response to genotoxic stress, mammalian Cdc14B is also translocated from the nucleolus to the nucleoplasm. Cdc14B liberation promotes the degradation of Plk1 by the APC/C^{Cdh1}, resulting in the stabilization of the DNA damage activator Claspin and the cell cycle inhibitor Wee1, allowing a proficient G2 checkpoint activation [187] (Fig.7). Together with previous observation demonstrating that Cdc14 is required for Cds1 activation in the fission yeast, it seems that the function of the phosphatase is exclusively related to the activation of the DNA damage checkpoint both in yeast and mammalian cells. Unexpectedly, the publication of two independent studies created controversy about the function of the Cdc14 phosphatases family in the DDR. Using both avian and human somatic cell lines it was demonstrated that neither Cdc14A nor Cdc14B are required for DNA damage checkpoint activation. Indeed, Cdc14A/B knockout cells arrested efficiently in G2-phase without affecting the activation of Chk1 and Chk2 in response to irradiation. However, these cells showed defects in repairing endogenous and exogenous DNA damage, accumulated γ-H2AX foci (as marker for double-strand breaks) and developed hypersensitivity to irradiation [188] (Fig.7). Supporting these results, Cdc14b-deficient mouse embryonic fibroblasts (MEFs) exposed to DNA damage also accumulated endogenous DNA damage and triggered senescence. However, no defects in DNA damage checkpoint activation were observed, indicating that the function of the phosphatase is only restricted to promote efficient DNA repair [189]. It is unclear the precise mechanism by which Cdc14b is operating in the repair of a DNA lesion but it seems that Cdc14a is also required for the same process, suggesting that both phosphatases could share at least some substrates and

be redundant in terms of function. Supporting this idea, it has been demonstrated that Cdc14b knockout MEFs have defects in repairing DSBs induced by ionizing radiation (IR) but only at late passages when Cdc14a levels are low [190].

In addition to the role of Cdc14 in DNA damage checkpoint and DNA repair, it has been proposed that the phosphatase is also required for the resolution of intermediates that are generated during the response to DNA lesions. Interestingly, Cdc14 activity is responsible for the accumulation of the active form of the budding yeast Holliday junction resolvase Yen1 [177-179] (Fig.7). The precise activation of this protein is crucial for the coordination of the DNA repair with chromosome segregation in order to maintain genome stability. These evidences suggest that Cdc14 function in the DDR may be not only restricted to control the damage checkpoint and repair processes, but also the precise resolution of recombination intermediates generated during the response to DNA damage (Fig.7). If Yen1 activation by Cdc14 is an active process that accompanies the repair of a DNA lesion during its damage-dependent release or a passive mechanism operating during the FEAR/MEN activation is an interesting question for the future.

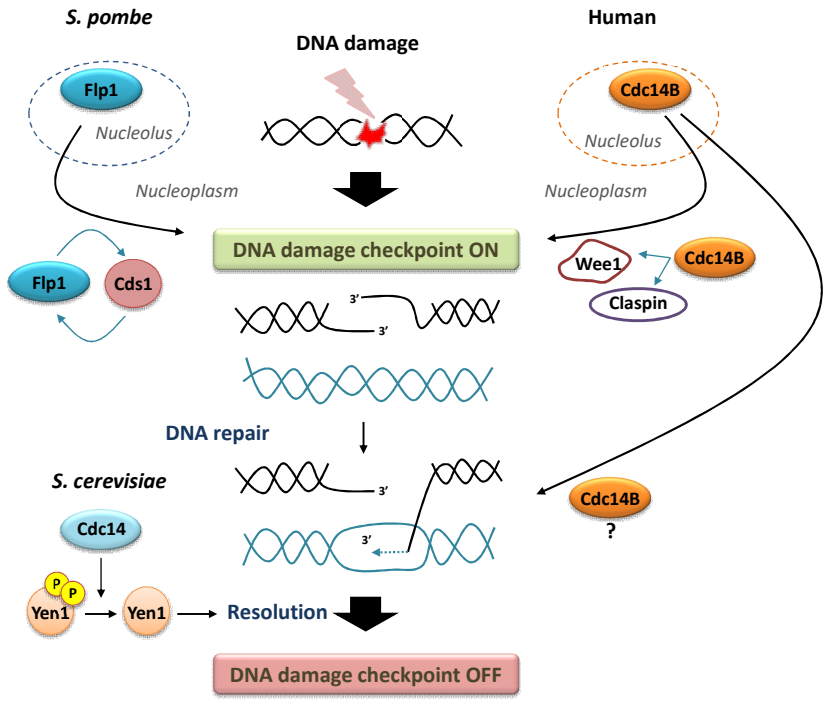


Fig.7. Main roles of Cdc14 in the DNA damage response. Cdc14 has been implicated in the execution of the DNA damage response in different organisms. In *Schizosaccharomyes pombe*, Flp1, the orthologue of *Saccharomyces cerevisiae* Cdc14, is required for the full activation of the kinase Cds1 which in turn promotes the phosphatase release from the nucleolus. Flp1 and Cds1 are both necessary for a proficient DNA damage checkpoint activation. Human Cdc14B is also translocated from the nucleolus to the nucleoplasm in the presence of DNA damage to allow the proficient DNA damage checkpoint activation through the stabilization of Claspin and Wee1. Additionally, it has been proposed that Cdc14B is necessary for DNA repair, but the molecular mechanism behind this function is still unknown. Finally, in *S. cerevisiae* it has been shown that Cdc14 dephosphorylates and activates the resolvase Yen1 involved in the resolution of DNA repair intermediates.

Despite all the information regarding the role of Cdc14 in the DDR, the molecular mechanism by which this phosphatase exerts its function and its regulation during the damage response is still unclear and more effort is required to determine its precise role in the damage response.

Objectives

1. Study the importance of the Cdc14 phosphatase in the execution of DDR and, specifically, in the DNA repair pathway.

2. Identify Cdc14's targets in the DNA damage response.

3. Determine the physiological significance of Cdc14 dephosphorylation of DDR targets in response to a DNA lesion.

1. Regulation of Cdc14 localization during the DNA damage response

1.1. Cdc14 function is essential to maintain cell viability when cells are exposed to different genotoxic stresses.

Several studies have demonstrated that protein kinases play a key role in the DDR by modulating the activation of several components at different stages during the response [141-143]. In particular, the role of Cdk has been well documented in the DDR and DSB repair [124, 132, 133, 191, 192]. This observation together with the notion that the Cdc14 phosphatase has predilection to dephosphorylate Cdk targets, prompted us to characterize its implication in the DDR. To this end, we first tested the effect of the DNA-damaging agent methyl methanesulphonate (MMS) on the growth of serial diluted cells carrying the temperature-sensitive allele *cdc14-1* (Fig.8A). Since Cdc14 is an essential gene in the budding yeast, this assay was performed at a set of different temperatures ranging from 25°C to 33°C. Both the *cdc14-1* mutant and the isogenic wild-type strain grew at 25°C with and without MMS (YPD plates). Surprisingly, cells lacking Cdc14 function showed a severe defect when growing at 30°C in the presence of the genotoxic compound (Fig.8A). However, no differences between the wild-type and the *cdc14-1* strain were detected in the absence of MMS at this temperature. This indicates that we can separate the requirement for both cell cycle and DNA repair functions of Cdc14 by just simply growing cells at the semipermissive temperature of 30°C. Since we could not detect any defect in cell cycle progression but in response to DNA damage, we considered 30°C as the semipermissive temperature to specifically address Cdc14´s roles in the DDR. At 33°C, both treated and untreated cells were not able to survive due to the essential role of Cdc14 in mitotic exit [150].

To further characterize the role of Cdc14 in the presence of different genotoxic compounds, we plated serial dilutions of wild-type and *cdc14-1* mutants in the presence of the UV-mimic 4-nitroquinoline-1-oxide (4NQO), the ribonucleotide reductase inhibitor hydroxyurea (HU), the radiomimetic drug phleomycin which produces DSBs, and the microtubule-destabilizing agent benomyl at semipermissive temperature (Fig.8B). A dimethyl sulfoxide (DMSO) plate was used as control. Strikingly, *cdc14-1* cells showed a great sensitivity in all media tested thus extending the importance of the phosphatase in response to a great variety of DNA

lesions (Fig.8B). It is important to mention that the sensitivity of *cdc14-1* mutants differed between the various genotoxic compounds tested, suggesting that the phosphatase may have different functions depending on the type of DNA insult infringed. In all, we conclude that Cdc14 function is essential for cell viability when exposed to different sources of DNA damage.

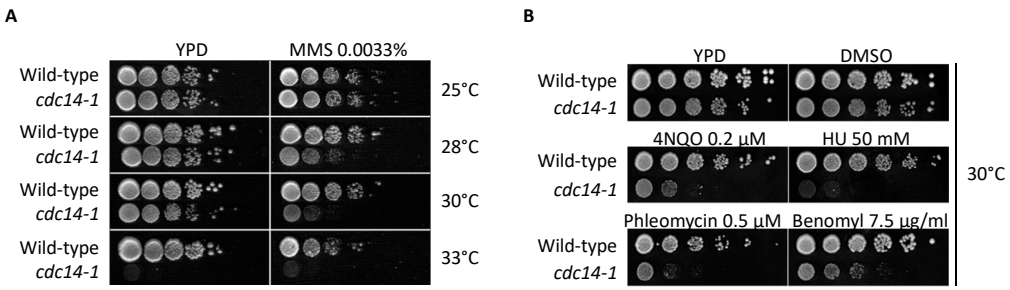


Fig.8. Cdc14 function is required for cell viability under DNA damage. Tenfold serial dilutions of wild-type and *cdc14-1* cells dropped and grown in solid rich media (YPD) or plates containing methyl methanesulphonate (MMS) **(A)**, dimethyl sulfoxide (DMSO), 4-nitroquinoline-1-oxide (4NQO), hydroxyurea (HU), phleomycin or benomyl **(B)** at indicated temperatures.

1.2. Cdc14 is transiently released in the presence of DNA damage.

We have previously seen that Cdc14 activity is essential to maintain cell viability in response to genotoxic stress. This suggests that the phosphatase must be activated during the damage response to execute its function. Considering that the activation of Cdc14 is highly regulated during an unperturbed cell cycle by its translocation from the nucleolus to the nucleoplasm, it is plausible that Cdc14 activation in response to DNA damage might also be subjected to changes in its localization pattern. To test for this hypothesis we tagged the endogenous *CDC14* and *CNM67* (as spindle pole body marker) genes with yellow fluorescent protein (YFP) and red fluorescent protein (RFP), respectively, and determined their cellular localization at different time intervals after adding phleomycin to the culture. While in pre-treated cells all Cdc14 signal was comprised at the nucleolus, five hours after the addition of the drug a small fraction of the protein was released to the nucleoplasm (Fig.9A). Surprisingly, Cdc14 release was transient, since the signal from nucleoplasm decayed around 10 hours after adding the drug, rendering Cdc14 exclusively to the nucleolar area again. Importantly, the partial release of Cdc14 in response to DNA damage was not sufficient to promote mitotic entry as most cells remained arrested in metaphase with short spindles as denoted by the presence of two Cnm67-RFP foci located at the mother cell and aligned to the axial plane of the cell (Fig.9A and 9B). Moreover, the effector

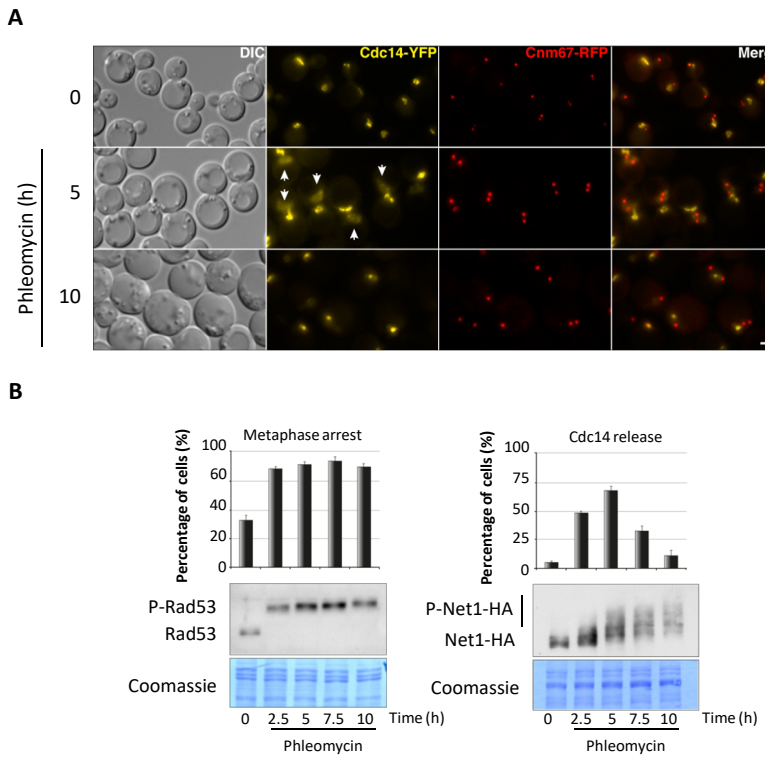


Fig.9. Cdc14 is transiently released from the nucleolus in response to DNA damage. Cells were grown overnight and treated with phleomycin at a final concentration of 1 µM. Samples were taken at different intervals to determine the localization of Cdc14 and the phosphorylation levels of Rad53 and Net1. **A)** Live-cell imaging of Cdc14-YFP and Cnm67-RFP. Cnm67 was used as a SPB reporter to determine the spindle length. Arrows indicate cells with Cdc14 released to the nucleoplasm. Scale bar: 3 µm. **B)** Graphs represent the average percentage ± SD from three independent experiments of cells arrested in metaphase (left graph) and with Cdc14-YFP signal at the nucleoplasm (right graph). Western blots to analyze Rad53 and Net1-6HA phosphorylation during the entire experiment are included. For Net1, 10 µM Phos-Tag gels were used. Coomassie staining is shown as a loading control.

checkpoint kinase Rad53 was phosphorylated throughout the entire experiment, confirming a proficient DNA damage checkpoint activation (Fig.9B). Concomitantly with the DNA-damage dependent nucleolar release of Cdc14, Net1 became phosphorylated (Fig.9B). It is already known that Net1 phosphorylation is a prerequisite to facilitate the phosphatase shuttling and activation in an unperturbed cell cycle [160]. This observation might also be extended to the Cdc14 release observed during the response to DNA damage. Indeed, the phosphorylation pattern of Net1 detected in the presence of DNA lesions by Western blot resembled those observed during anaphase progression in an unperturbed cell cycle (Fig.9B vs Fig.10, 75 min from alpha-factor release). However, there are two characteristics that make damage-dependent Cdc14 release different from an undamaged situation: 1) Net1 hyperphosphorylation in mitosis endorses a full nucleolar release of Cdc14 by the MEN

pathway, while Cdc14 damage release resembles the partial exclusion observed during FEAR activation (Fig.10, 60 min picture); 2) while Cdc14 disappears from the nucleoplasm around 7.5 hours after adding the drug, Net1 still accumulates higher phosphoforms (Fig.9B). Thus, we conclude that Net1 phosphorylation by itself does not account for the nucleolar/nucleoplasm shuttling of Cdc14 along the DDR and, therefore, additional mechanisms might be involved in the regulation of Cdc14 re-localization during the damage response.

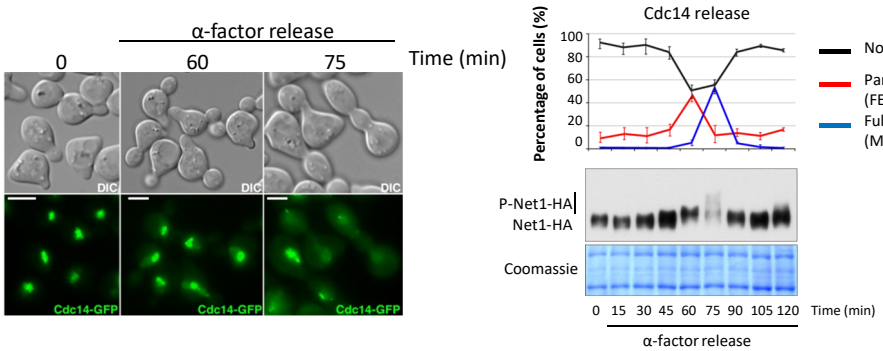


Fig.10. Localization of Cdc14 and Net1 phosphorylation during an undamaged cell cycle. Cells carrying Cdc14-GFP and Net1-6HA were synchronized in G1 by adding the pheromone α-factor and released into fresh media to allow cell cycle progression. Samples were taken at different intervals to follow Cdc14 release (left panel) and Net1 phosphorylation (right panel). A representative image of non-released (0), partially released (60 min) and fully released (75 min) Cdc14 from the nucleolus is shown. Scale bar: 3 µm. Graph represent the percentage ± SD of three independent experiments of cells with no released, partially released and fully released Cdc14 in an unperturbed cell cycle. Western blot analysis showing Net1 phosphorylation along the experiment is included. The separation of the phospho-bands was achieved as described in Fig.9B. Coomassie staining is included as a loading control.

To rule out an indirect cell cycle effect in the Cdc14 nucleolus/nucleoplasm shuttling profile, we induced DNA damage by adding phleomycin to previously G2/M nocodazole arrested cells and followed Cdc14 localization at different time points. As before, Cdc14-YFP was transiently released to the nucleoplasm (Fig.11). However, under these conditions the maximum levels of Cdc14 at the nucleus were reached by 90 minutes after the treatment with the drug (Fig.11). Cdc14's disappearance from the nucleoplasm was attained by 180 minutes after phleomycin addition. Again, Cdc14 exclusion in response to the treatment was not enough to endorse mitotic entry as most cells remained blocked in metaphase with short spindles (Cnm67-RFP as SPB marker) during the entire experiment (Fig.11).

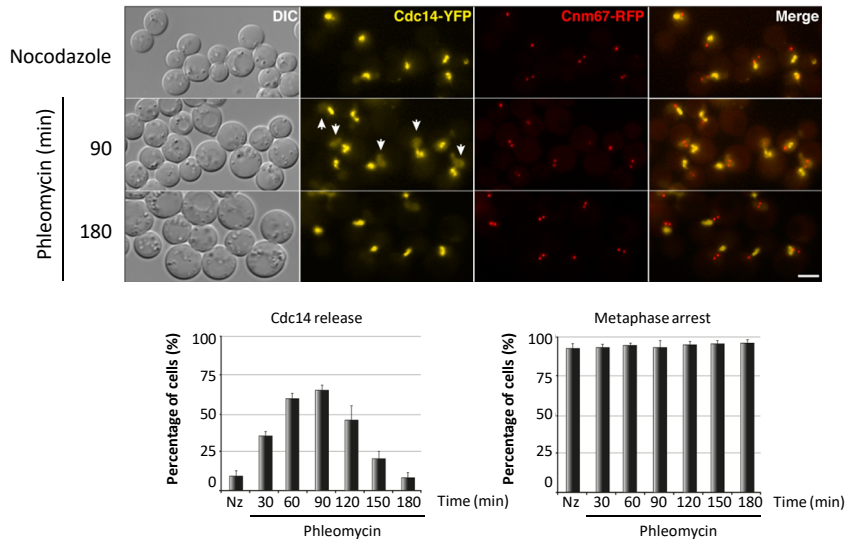


Fig.11. Cdc14 re-localization during the DNA damage response does not depend on cell cycle progression. Cells expressing Cdc14-YFP and Cnm67-RFP were grown overnight and synchronized in G2/M with nocodazole (Nz) prior treatment with phleomycin (1 µM). Samples were taken every 30 minutes to follow Cdc14 re-localization. Arrows indicate cells with Cdc14 signal at the nucleoplasm. The efficiency of the blockage was performed as described in Fig.9. Scale bar: 3 µm. Graphs represent the percentage ± SD of cells with Cdc14 nucleolar exclusion (left graph) and cells arrested in metaphase (right graph) from three independent experiments.

Our previous results showed that Cdc14 is released from the nucleolus to the nucleoplasm in the presence of multiple DSBs generated by phleomycin. However, we wondered if this observation was subjected to this particular context of DNA damage. In addition, we were also interested in a deeper characterization of the phosphatase's role in the DDR, therefore we decided to study the localization pattern of Cdc14 in response to a single DSB. For this purpose, we took advantage of the budding yeast JKM139 background which harbors the homothallic switching endonuclease (HO) gene under the control of the inducible GAL1 promoter at chromosome IV [132] (Fig.12). This system allows the timely regulation of the HO expression by adding galactose/glucose to the media. Once the HO endonuclease is expressed, it will generate a site-specific DNA break in a unique target sequence located at chromosome III in the mating-type (*MAT*) locus. Importantly, this strain lacks the transcriptionally inactive loci *HMR*a and *HML*α, normally present at both arms of the same chromosome to avoid the repair of the DSB, thus generating a persistence DNA break (Fig.12). To determine if Cdc14 was able to shuttle between the nucleolus and the nucleoplasm under these conditions, overnight asynchronous cell cultures were supplemented with galactose and pictures were taken at different intervals. In this case, endogenous *CDC14* gene was tagged with the green fluorescent protein (GFP) and the protein Ddc2 (used as DSB reporter)

with the cyan fluorescent protein (CFP). In addition, Cnm67-RFP was employed as SPB marker.

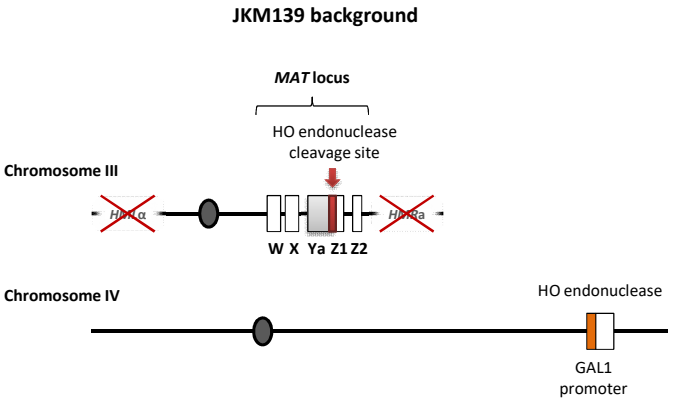


Fig.12. Schematic representation of the JKM139 background. *MAT* locus and the site-specific region recognized by the HO endonuclease are located at chromosome III. In this background, *HML* and *HMR* loci were deleted to avoid the repair of the double-strand break (DSB) generated by the expression of the endonuclease. Note that the gene coding for the HO enzyme is under the inducible GAL1 promoter at chromosome IV.

As expected, Cdc14 was transiently released from the nucleolus in response to a single unrepairable DSB, peaking at 5 hours from the induction (Fig.13). Remarkably, once released from the nucleolus, Cdc14 was observed accumulated at a distinctive focus in some cells. This Cdc14 focus overlapped with the Ddc2-CFP signal (DSB reporter) and the Cnm67-RFP (SPB reporter) in 50% of the cells, indicating that Cdc14 could have a role in the regulation of the SPBs and/or the DSBs. These results confirm that a single DNA break generated by the HO endonuclease is sufficient to promote Cdc14 nucleolar/nucleoplasm re-localization. Taking together, we conclude that the Cdc14 phosphatase is partially and temporary released from the nucleolus in response to different sources of DNA damage.

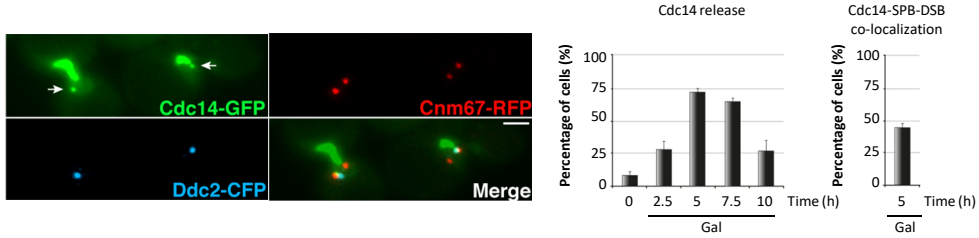


Fig.13. Cdc14 translocates from the nucleolus to the nucleoplasm in the presence of a single double-strand break. Live-cell imaging of Cdc14 localization in the presence of a non-repairable double-strand break (DSB) at the *MAT* locus. Cells carrying Cdc14-GFP, Cnm67-RFP and Ddc2-CFP were grown overnight in YP + raffinose. A DSB was produced by the HO endonuclease at chromosome III after galactose addition. Images were taken before and after the induction of the endonuclease. Ddc2 and Cnm67 were used as DSB and SPB reporters, respectively. Micrographs display the maximum projection of nine planes. Arrows indicate Cdc14 foci and co-localization of Cdc14 signal with the DSB and the SPB. Scale bar: 3 µm. Graphs represent the percentage ± SD from three independent experiments of cells with Cdc14 at the nucleoplasm (left graph) and the percentage of Cdc14-SPB-DSB co-localization after 5 hours in the presence of the DSB (right graph).

2. Characterization of the Cdc14 function during the repair of a DSB

2.1. Cdc14 activity is not required to execute a proficient DNA damage checkpoint.

There are two non-mutually exclusive interpretations that could explain the essential function of Cdc14 in response to DNA damage: 1) an involvement in the DNA damage checkpoint activation; or 2) a direct role in the DNA repair process. It is not clear in the bibliography the involvement of Cdc14 on each. In one hand, some evidences indicate that the phosphatase is required for the activation of the DNA damage checkpoint both in the fission yeast and mammalian cells. On the contrary, other studies have revealed that Cdc14 is dispensable for a proficient DNA damage checkpoint activation but is necessary for the efficient execution of the DNA repair pathway [188, 189].

To test for a putative function of the phosphatase in the DNA damage checkpoint of *S. cerevisiae* we employed a budding yeast strain which contains an intact *MAT* locus and the HO endonuclease under the control of the GAL1 promoter (Fig.14A, yXW2). In this system, upon the induction of the endonuclease, the break generated at the target sequence of the *MAT* can be repaired by gene conversion using the *HMR* or the *HML* loci available at both arms of chromosome III (Fig.14A). To allow the repair of the lesion, glucose must be added to repress the expression of the endonuclease. HO enzyme's half life is around 10 minutes, and it has been shown that it is rapidly degraded by the proteasome once glucose is added to the media [193]. On the contrary, a constant DSB will be formed when cells are incubated in the constant presence of galactose. We induced a constant and unique break by adding

galactose to asynchronous wild-type and *cdc14-1* cell cultures and measured the efficiency of cells to block in G2/M by the activation of the DNA damage checkpoint. We can also analyze the evolution of the lesion by using a DNA probe that hybridizes near the *MAT* locus in Southern blot assays (Fig.14A, diagram). No differences in the generation and the stability of the DSB were observed between both strains along the duration the experiment as denoted in the Southern blot (Fig.14A).

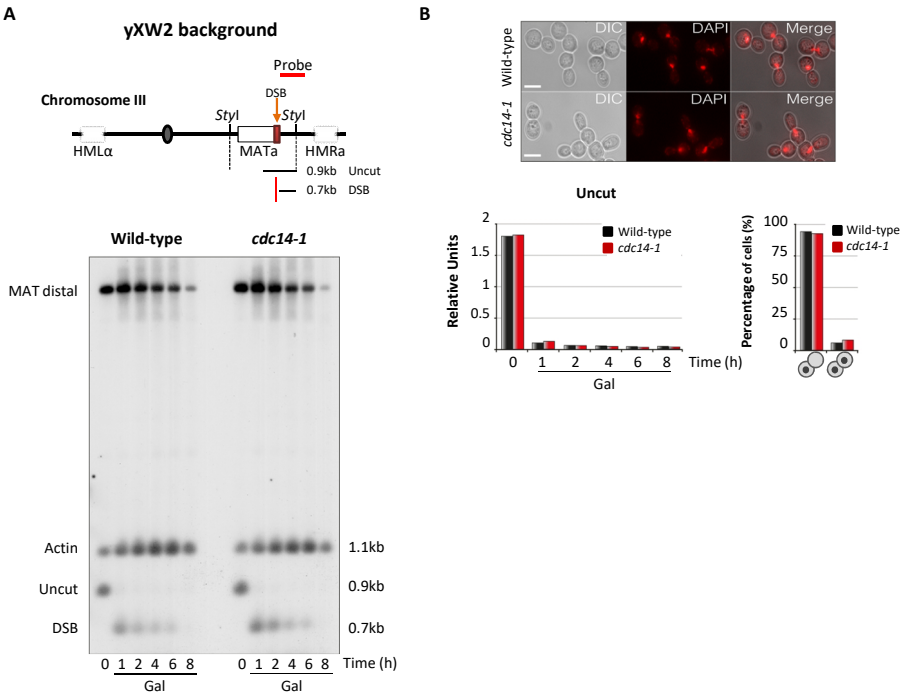


Fig.14. Cdc14 is not required for the activation of a proficient DNA damage checkpoint. Asynchronous cell cultures grown at 25°C in raffinose-containing media were incubated at the restrictive temperature (33°C) in the presence of galactose to induce a constant double-strand break (DSB). Samples were collected at the indicated time points to visualize cells at the microscope and to perform DNA extraction for Southern blotting. **A)** Diagram depicts the yXW2 background employed in this experiment. Restriction sites, DSB position and the *MAT*a distal probe used to visualize the break are included. DNA was digested with *Sty*I, subjected to electrophoresis and blotted. Blot shows the production and the stability of the break of wild-type and *cdc14-1* cells during the experiment. An additional probe for the actin gene was included as loading control. **B)** Micrographs show wild-type and *cdc14-1* mono-nucleated cells blocked in G2/M 8 hours after the generation of the DSB. Scale bar: 5µm. Graphs show the quantification of the Uncut fragment normalized with actin (left) and the percentage of cells blocked in G2/M at time point 8 hours (right).

Importantly, after 8 hours in the presence of the DSB, both wild-type and *cdc14-1* strains showed more than 90% of mono-nucleated G2/M arrested cells (Fig.14B, micrographs). This result indicates that Cdc14 function is not required to activate a proficient DNA damage checkpoint, confirming previous results obtained by other groups [188, 189]. In addition,

this finding suggests that the sensitivity of cells lacking Cdc14 function to different genotoxic compounds could be explain by a possible function of the phosphatase in DNA repair.

2.2. DNA repair is impaired in the absence of Cdc14 activity.

As mention in the introduction, the main role of the DDR is to couple DNA repair with cell cycle progression. Once the damage response is triggered, cells execute different mechanisms to encourage the repair of the broken DNA. In the budding yeast, there are two major pathways involved in the repair of a DSB: NHEJ and HR. It is well documented that the Cdk plays a key role in the repair pathway choice and HR [127, 192]. Following the same reasoning as before, we wondered if Cdc14 could be involved in reverting the effects imposed by the Cdk in this context. To test for this hypothesis, we employed strains harboring different variants of the homing endonuclease HO system and determined the efficiency of DNA repair and the decision between NHEJ and HR in the presence/absence of the phosphatase. These approaches can be combined with standard genomic assays to study several aspects of the repair pathway, including DNA resection, homology search, invasion (D-loop formation) and recombinational events. To start dissecting the role of Cdc14 in DNA repair, we focused on three distinct scenarios to examine the regeneration of the DNA molecule: 1) intra-chromosomal DNA repair, 2) inter-chromosomal gene conversion and 3) repair pathway choice. It is important to remark that during DNA repair by HR, once the D-loop is formed and the information has been copied from the donor sequence, there are two ways to resolve this structure: 1) synthesis-dependent strand annealing (SDSA), and 2) formation of a double Holliday junction (dHJ) through double-strand break repair (DSBR). By using the strains mentioned above, we can determine the efficiency/preference in the execution of these pathways.

2.2.1. Analysis of intra-chromosomal DNA repair in cells lacking Cdc14 activity.

It has been proposed that intra-chromosomal DNA repair at the *MAT* locus occurs mainly by SDSA [194] as the donor sequences are located in the same chromosome (Fig.15). In this background, once the DSB is generated at the *MAT* locus (by expressing the HO endonuclease) it can be repaired by homologous recombination using either the *HML*α or the *HMR*a loci as template (Fig.15). The restoration of the DNA molecule is associated with the incorporation of distinct restriction enzyme sites present at the donor, a feature that facilitates the discrimination between the DNA repair products generated. These fragments

can be analyzed by Southern blot using a *MAT*a-specific probe that hybridizes at the DSB proximity (Fig.15).

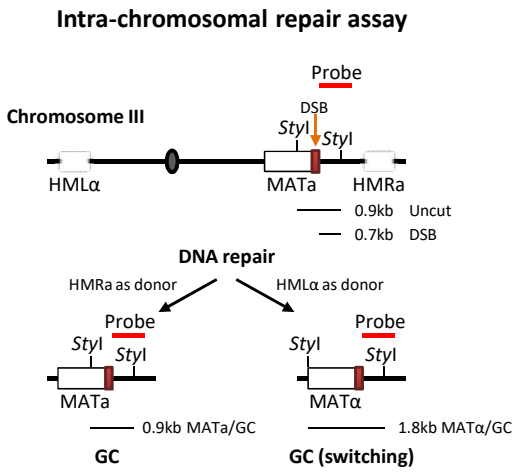


Fig.15. Diagram depicting the intra-chromosomal DNA repair system. Schematic representation of the background used in the intra-chromosomal DNA repair assay. The location of the *MAT*a-specific probe, the restriction sites and the enzyme employed in Southern blot experiments are indicated. The repair products that can be generated during gene conversion (GC) are also represented. Arrow indicates the position of the double-strand break (DSB).

To test if Cdc14 is involved in the intra-chromosomal DNA repair pathway, wild-type and *cdc14-1* cells carrying this system were incubated for 2 hours in the presence of galactose. HO was subsequently repressed by glucose addition to allow the repair of the DSB generated at the *MAT* locus. The kinetics of the repair process was determined by measuring the distribution and intensity of the bands obtained in the Southern blot experiment. While both strains generated the DSB with the same kinetics, cells lacking Cdc14 activity repaired the broken DNA slower and less efficiently than wild-type cells when occurred by restoration of the original *MAT*a or switched to *MAT*α allele (Fig.16, MATa/GC and MATα/GC bands). Note that both the kinetics of the appearance and the intensity of the repair products are diminished in cells lacking Cdc14 activity. In addition, we monitored the phosphorylation levels of the damage checkpoint kinase Rad53 throughout the experiment. The isogenic wild-type strain did not show changes in the mobility of Rad53 (Fig.16), consistent with previous results indicating that DNA damage checkpoint kinases are not active during intra-chromosomal DNA repair. By contrast, Rad53 was significantly hyperphosphorylated in the *cdc14-1* background, mirroring the failure in the execution of the SDSA observed in the absence of the phosphatase. Still, we cannot exclude an indirect effect on Rad53 phosphorylation when inactivating Cdc14 under these experimental conditions.

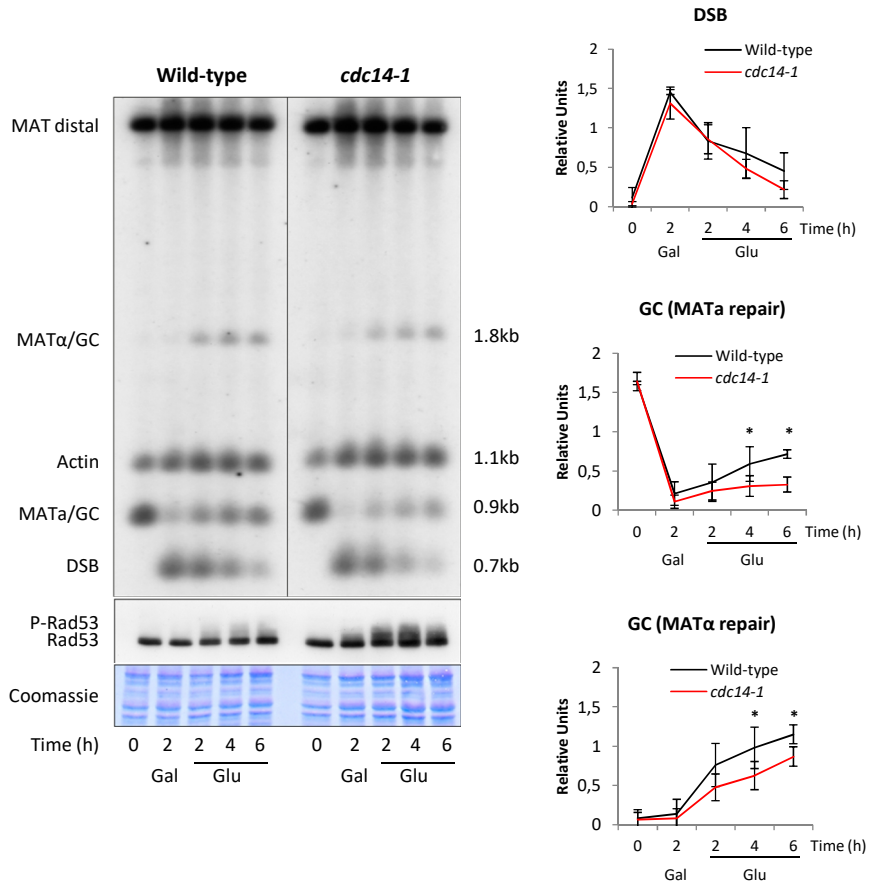


Fig.16. Cdc14 function is required to execute an accurately DNA repair by SDSA. Wild-type and *cdc14-1* cells were grown overnight in YP raffinose (25°C) and galactose was added to express the HO endonuclease during 2 hours. Next, cell cultures were shifted to 33°C to inactivate Cdc14 activity and supplemented with glucose to repress the expression of the endonuclease. Samples were taken at different intervals and the repair of the break with the *HM* donor sequences was attained by Southern blot. Genomic DNA was digested with *Sty*I prior separation on agarose gels and blotted. Blots were hybridized with a probe corresponding to the *MAT*a-distal sequence. A second probe against actin gene was included as loading control. DNA fragments size represented in figure 15 are included to visualize the repair of the double-strand break (DSB). Immunoblot showing Rad53 phosphorylation during the mating-type switching experiment is shown. Coomassie blue staining is depicted as loading control for the Western blot. Graphs show quantification of DSB and gene conversion (GC) which leads to restoration of the *MAT*a or switching to *MAT*α, all normalized against actin. Mean ±SD from three independent experiments is represented. Asterisks depict statistically significant differences determined by a two-tailed unpaired Student's *t*-test, p-value<0.05.

2.2.2. Inter-chromosomal DNA repair is defective in *cdc14-1* mutants.

To determine if Cdc14 is also participating in gene conversion events when the donor sequence is located in a different chromosome we used an inter-chromosomal DNA repair assay. In this system, a *MAT*a locus containing the HO target sequence is located at chromosome V, while the endogenous *MAT*a sequence at chromosome III has been

substituted for a *MAT*a-inc version [195] (Fig.17). This *MAT*a-inc sequence carries a point mutation that avoids the recognition by the HO endonuclease. It is important to remark that the *MAT*a-inc sequence is the only template available for DNA repair since this strain lacks both *HMR* and *HML* loci. Once the *MAT*a-inc sequence at chromosome III is copied to the *MAT*a sequence on chromosome V during the repair process, cells become resistant to further HO cleavages, allowing the use of galactose throughout the entire experiment.

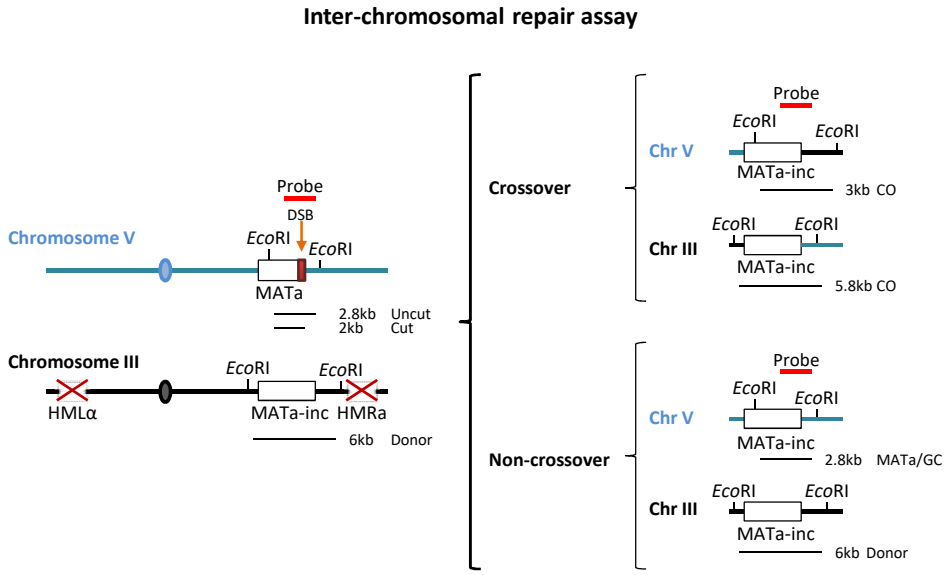


Fig.17. Schematic representation of the inter-chromosomal DNA repair assay. The location of the *MAT*-specific probe, the restriction sites and the enzyme employed in Southern blot experiments are indicated. The crossover and non-crossover products can be distinguish by Southern blot because of their differences in the product size. *MAT*a-inc contains a point mutation at the HO specific-sequence which renders it insensible to endonuclease cleavage. Arrow indicates the position of the double-strand break (DSB).

It has been postulated that during inter-chromosomal DNA repair a dHJ is formed. When this DNA structure is resolved, two different outcomes can be produced: a crossover and a non-crossover product [196]. A change in the ratio between crossover and non-crossover is associated with defects in the resolution of recombinant intermediates. The formation of a crossover is detected by the appearance of a different band size in Southern blot experiments due to the incorporation of distinctive restriction sites present at the donor sequence (Fig.17).

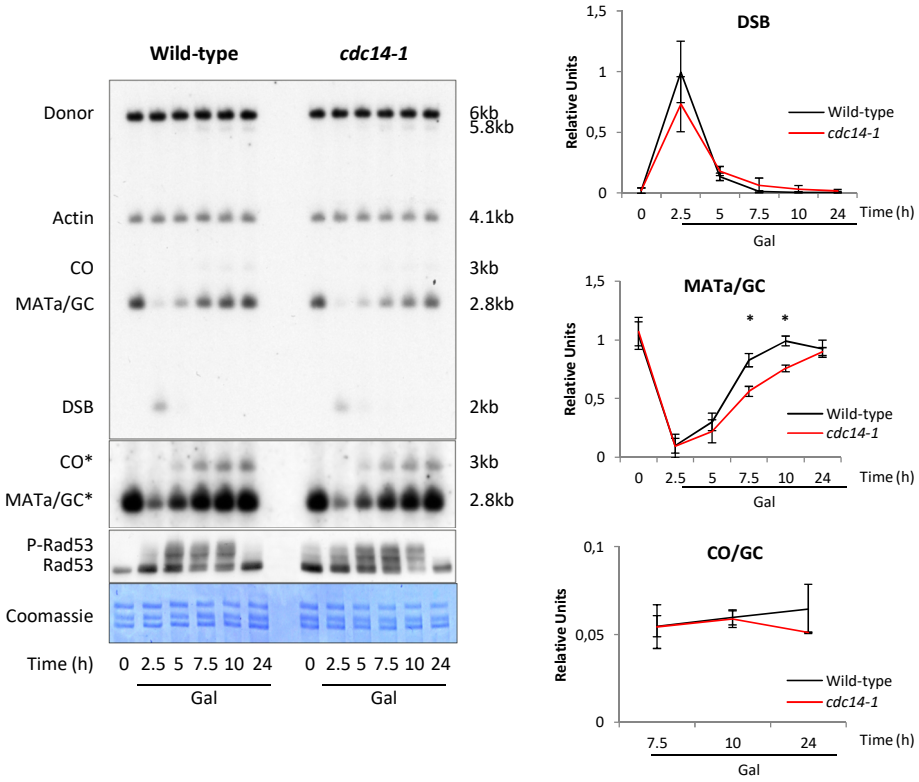


Fig.18. Inter-chromosomal DNA repair is impaired in the absence of Cdc14 activity. Exponentially growing cells (YP raffinose, 25°C) were incubated with galactose to express the homing endonuclease which generates the double-strand break (DSB) at chromosome V. Cell cultures were shift to the restrictive temperature to inactivate Cdc14's function. Samples were taken at different time points up to 24 hours in the presence of galactose. DNA was extracted, digested with *Eco*RI and blotted. Blots were hybridized with a *MAT*-specific sequence and an actin probe as loading control. The DNA repair fragments size represented in figure 17 are shown to visualize gene conversion and crossover formation. Asterisk denotes an overexposed film to detect crossovers (CO). Immunoblot of Rad53 was performed as previously described. Coomassie blue staining is included as loading control. Graphs show the quantification of the DSB, gene conversion (GC) which leads to the appearance of the *MAT*a-inc at chromosome V, and the ratio between crossover and non-crossover. Mean ±SD from three independent experiments is represented. Asterisks indicate statistically significant differences assessed by a two-tailed unpaired Student's *t*-test, p-value<0.05.

Cells growing exponentially were subjected to HO endonuclease expression by adding galactose to the media. Samples were taken a different time points and Southern blots were performed to follow both the dynamics of the repair product formation and the crossover vs non-crossover ratio. As previously observed, both wild-type and *cdc14-1* mutants showed the same kinetics in the generation of the DNA break upon HO induction (Fig.18, DSB). However, wild-type cells repaired the lesion faster and more efficiently than *cdc14-1* mutants (Fig.18, MATa/GC product). It is important to remark that gene conversion with no associated crossover was the predominant form of repair in both strains. In addition, no differences

were detected in the proportion of crossover formation between the wild-type and the *cdc14-1* mutant among cells that repaired the DNA break (Fig.18, CO in overexposed film and ratio CO/GC). Importantly, no defects in DNA damage checkpoint activation were observed in the presence/absence of Cdc14 function since Rad53 showed the same phosphorylation pattern in both strains (Fig.18). This observation confirms that Cdc14 function is not required for the correct execution of the damage checkpoint. Altogether, these experiments demonstrate that Cdc14 is necessary to fulfill DNA repair by ectopic recombination but is dispensable for triggering the DNA damage checkpoint response.

2.2.3. The repair pathway choice is not affected in the absence of Cdc14.

One of the first events that take place during the repair of a DNA break is the decision to activate HR or NHEJ, a phenomenon known as the repair pathway choice. This complex program stimulates one DNA repair mechanism or the other depending on two different features: 1) the phase of the cell cycle when the damage occurs, and 2) the availability of a donor sequence to be used as template. Importantly, it has been well documented that the Cdk plays an important role in this process [127, 132, 133, 197]. Cdk activation targets several components of the repair machinery to enhance recombinational DNA repair. As Cdc14 is the only known phosphatase that counteracts Cdk-dependent phosphorylation, it is tempting to speculate that Cdc14 could also be implicated in the decision to repair by HR or NHEJ. To determine the putative influence of Cdc14 in the repair pathway choice we used a background strain that allows us to distinguish between DNA repair by gene conversion or NHEJ. This background contains a *MAT*a locus at chromosome III and only the *HMLα* sequence to be used as donor for the repair of the HO-induced break [23, 198]. Therefore, DNA repair by HR is obligatorily associated with mating-type switching from *MAT*a to *MAT*α. In contrast, if cells execute NHEJ they will maintain the mating-type as *MAT*a (Fig.19). Importantly, the incorporation of the *HML*α allele into the *MAT* locus introduce a new restriction site, facilitating its detection by using a specific probe that hybridizes in the *MAT* sequence (Fig.19). Asynchronous cell cultures of wild-type and *cdc14-1* mutants were incubated during 1.5 hours in the presence of galactose to express the homing endonuclease. HO expression was then repressed by adding glucose to the media and samples were taken at several time points to determine the regeneration of the DNA molecule. As seen before, *cdc14-1* cells showed defects in gene conversion as denoted by the lower intensity of the switched product when compared to the isogenic wild-type (Fig.20A, MATα/GC).

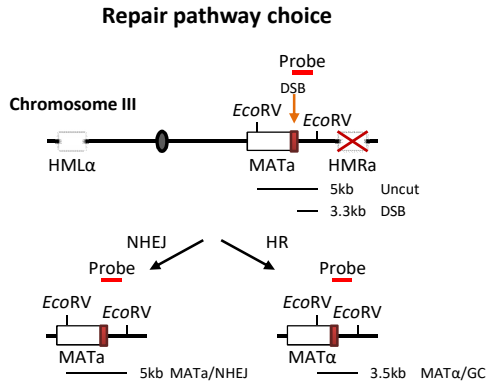


Fig.19. Schematic representation of the repair pathway choice assay. Diagram showing the genomic structure of the strain employed to analyze the repair pathway choice. The position of the *MAT*-specific probe, the restriction sites and the enzyme employed during Southern blot experiments are represented. The used of NHEJ and HR can be distinguish because the repair products generated contain different sites for the restriction enzyme. Arrow indicates the location of the double-strand break (DSB).

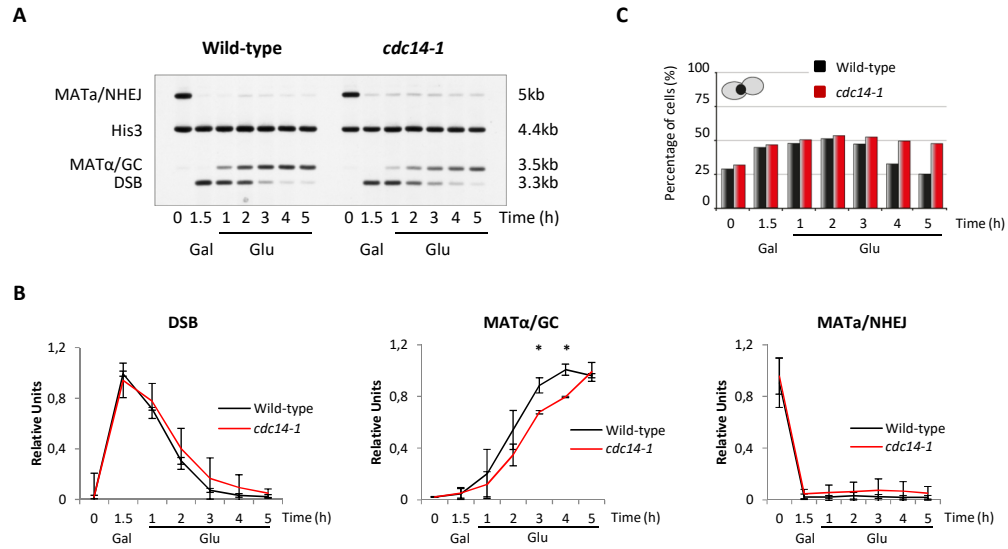


Fig.20. Cdc14 is not involved in the repair pathway choice. Asynchronous cell cultures of wild-type and *cdc14-1* cells (YP raffinose, 25°C) were incubated during 1.5 hours in the presence of galactose at the restrictive temperature (33°C). Glucose was added to repress HO expression thus allowing the repair of the break. **A)** Physical analysis of wild-type and *cdc14-1* cells harboring the repair pathway choice assay. Samples were taken at indicated time points; DNA was extracted, digested with *Eco*RV and separated on agarose gels prior blotting. Blots were hybridized with a probe corresponding to the *MAT*a-specific sequence. A second probe to detect His3 gene was included as a control for the amount of DNA loaded in each line. Fragments size are included to visualize the repair products depicted in figure 19. **B)** Graphs represent quantification of the double-strand break (DSB) formation, the mating-type switching by gene conversion (MATα/GC), and restoration of the MATa by NHEJ (MATa/NHEJ), all normalized with His3. Graphs show the mean ± SD from three independent experiments. Asterisks depict statistically significant differences determined by a two-tailed unpaired Student's *t*-test, p-value<0.05. **C)** Bar graph represents the percentage of mono-nucleated wild-type or *cdc14-1* cells arrested in G2/M at each time point of the experiment.

Note that while most wild-type cells repaired the DSB by 3 hours after glucose addition, *cdc14-1* spent around 5 hours to exhibit a comparable repair product (Fig.20A and 20B, MATα/GC). Supporting the delay in DNA repair observed in the absence of the phosphatase, *cdc14-1* cultures presented an extended G2/M arrest with an increased number of mono-

nucleated cells (Fig.20C, graph). Importantly, the accumulation of the NHEJ repair product showed no significant differences between the wild-type and *cdc14-1* mutant (Fig.20A and 20B, Mata/NHEJ), indicating that Cdc14 does not contribute to DSB repair pathway choice.

Conjointly, all these findings demonstrate that Cdc14 is necessary for an accurate DNA repair by homologous recombination, both by SDSA and dHJ. Moreover, taking into account that the repair pathway choice is activated only during the initial steps of the repair process, it seems that the role of the phosphatase in controlling HR takes place later in the repair response.

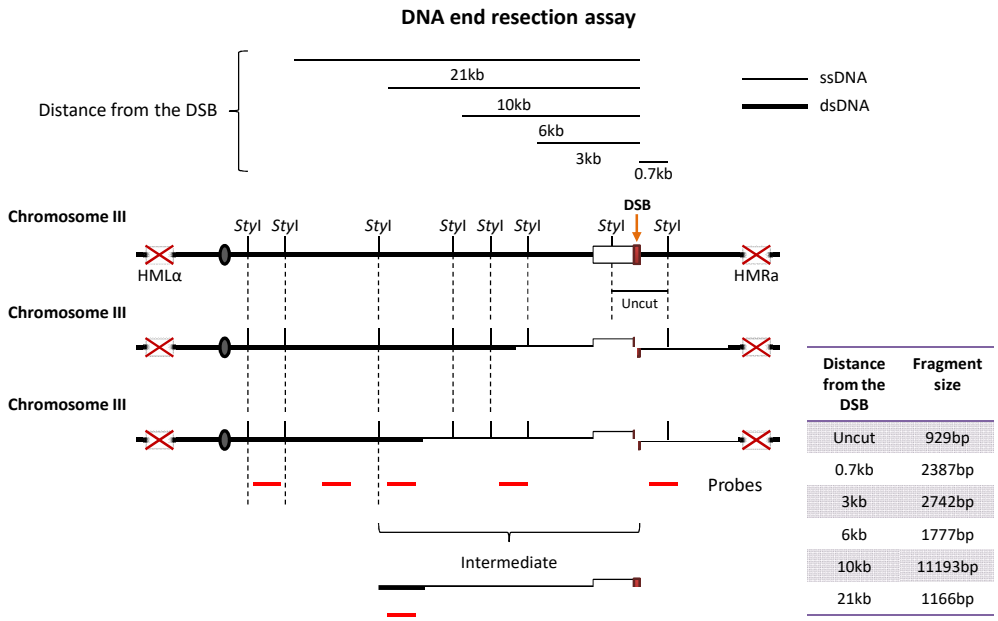
3. Role of Cdc14 in DNA end resection

3.1. The activation of the DNA end resection is not affected in the absence of Cdc14 function.

The processing of the DNA molecule and, in consequence, the generation of a 3'-ended ssDNA is one of the initial steps of the repair process. We have previously mentioned that this step is regulated by Cdk-dependent phosphorylation events. Indeed, both the initial processing of the DNA molecule by the MRX complex together with Sae2 and the further activation of the long-range nucleases highly depend on the Cdk [120, 124, 125, 192]. For this reason, we first decided to study the possible implication of the Cdc14 phosphatase in the DNA end resection process. If the phosphatase is dephosphorylating any component of the resection machinery we should detect some differences in the processing of the DNA molecule when Cdc14 function is impaired. To this end, we employed the JKM139 background which contains a non-repairable DSB due to the elimination of both *HML/HMR* loci (Fig.12). This genetic system supposes a useful tool when studying DNA resection because it prevents the execution of following steps of the repair pathway that could mask any phenotype associated with the processing of the DNA molecule. Therefore, JKM139, allows us to specifically focus on the resection activity. By using this approach we can analyze in detail the velocity and track length of the DNA resection and the influence of each DNA damage-dependent factor in the process.

Physical experiments have been extensively used to study different aspects of the DNA resection. They involve the design of DNA probes that hybridize at increasing distances from the DSB. As the 5'-ended DNA fiber is eliminated when the resection machinery moves forward, restriction enzymes are unable to recognize and cleave ssDNA sites and, therefore,

the intensity of the bands corresponding to a specific DNA fragment diminishes. In addition, we can detect and quantify the intermediates that are generated during resection. These intermediates consist on DNA molecules with both dsDNA/ssDNA that are formed when one of the two restriction sites flanking the sequence that recognizes the probe is processed by the resection machinery (Fig.21). In terms of detection, we can easily observe the intermediates because they migrate lower and are less intense than those DNA fragments composed exclusively by dsDNA (which maintain intact restriction sites). Importantly, they provide valuable information about the DNA resection velocity and efficiency since the accumulation of these bands associate with a decrease in the velocity of the DNA molecule processing.



Distance from the DSB	Fragment size
Uncut	929bp
0.7kb	2387bp
3kb	2742bp
6kb	1777bp
10kb	11193bp
21kb	1166bp

Fig.21. Schematic representation of the DNA resection assay. The genetic background used for this approach is the JKM139 (Fig.12). Diagram includes the distances from the HO endonuclease cut site analyzed by Southern blot. Restriction sites and probes employed during this approach are depicted. Note that once DNA resection moves forward ssDNA is generated and restriction enzymes are not able to recognize the specific sites. Orange arrow indicates the location of the double-strand break (DSB). An intermediate of DNA end resection is shown as example. Table includes the *Sty*I-*Sty*I DNA fragments length for each probe used in the resection assay.

We designed several probes to recognize specific DNA regions located at 0.7, 3, 6, 10 and 21kb from the HO-specific site (Fig.21) to study the kinetics of short- and long-range resection. Wild-type and *cdc14-1* cells were synchronized in G2/M by nocodazole treatment. After blocked, the HO endonuclease was expressed by adding galactose and cell cultures

were transferred to the restrictive temperature (33°C) to inactivate Cdc14 function. Samples were taken at different time points and Southern blots were performed to visualize DNA resection velocity and total track length. Both the wild-type and the *cdc14-1* backgrounds generated the DSB with the same efficiency (Fig.22). As shown in figure 22, while DNA resection machinery moves away from the DNA lesion, the signal coming from the 0.7kb disappears (see 3, 4, and 5 hours from galactose addition). No differences between wild-type and *cdc14-1* cells were observed in terms of DNA resection at short distances from the DSB (Fig.22, 0.7kb and intermediates R1/R2).

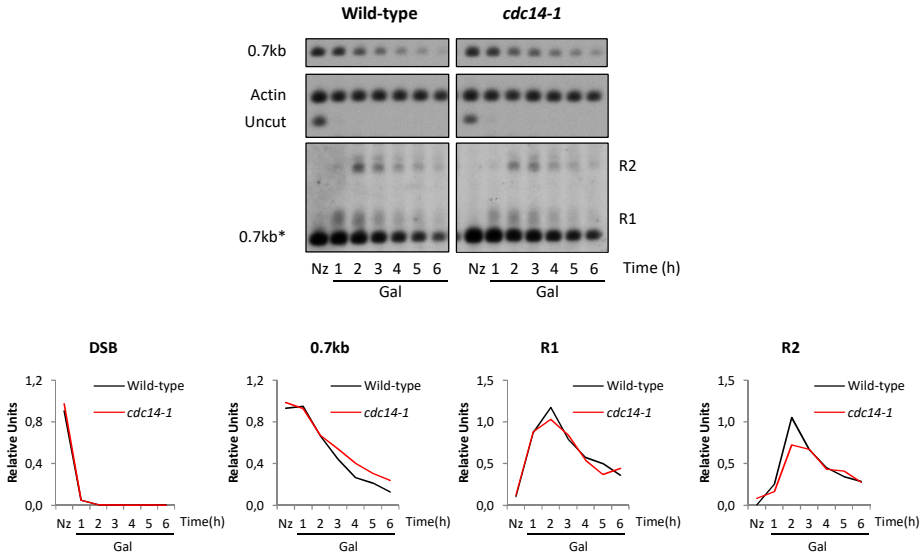


Fig.22. Cdc14 is not required for short-range resection. Wild-type and *cdc14-1* cells were synchronized in G2/M with nocodazole (Nz, YP raffinose, 25°C) and then shifted to the restrictive temperature to inactivate the function of the phosphatase (33°C). HO endonuclease was expressed by adding galactose to the media. Samples were taken at different time points, DNA extracted and digested with *Sty*I. DNA fragments were separated on agarose gels and blotted. Fragment sizes are mentioned in figure 21. Blot shows the band corresponding to the 0.7kb probe located near to the double-strand break (DSB). A second probe for the actin is included as loading control. Asterisk denotes an overexposed film to visualize the resection intermediates (R1 and R2). Graphs represent the quantification of the DSB, the 0.7kb probe and intermediates R1/R2, all normalized against actin.

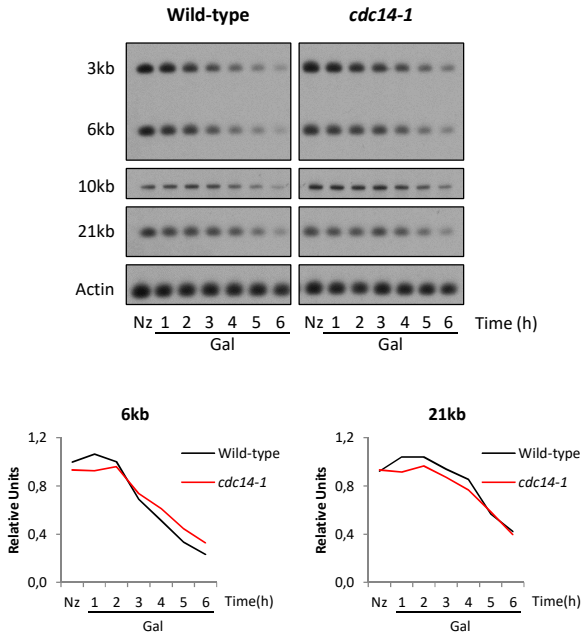


Fig.23. Cdc14 function is dispensable for long-range resection. Wild-type and *cdc14-1* samples from the experiment depicted in figure 22 were digested with *Sty*I and blotted. Blot shows the bands corresponding to several probes located at increasing distances from the break (3, 6, 10 and 21kb). An additional probe against actin is included as loading control. Graphs represent the quantification of the 6kb and 21kb probes normalized to actin.

At longer distances from the break, the velocity of resection was also identical between both strains since the decay of signals coming from probes that hybridize at increasing distances from the break occurred with the same kinetic (Fig.23; 3, 6, 10 and 21kb bands). Graphs containing the quantification for 6kb and 21kb probes are included in figure 23 as examples. These results indicate that Cdc14 is not required for the activation of DNA end resection.

3.2.The absence of Cdc14 function produces a delay in DNA long-range resection in cells lacking Sgs1.

As mention in the introduction, long-range resection is triggered by two independent pathways involving the exonuclease Exo1 and the Dna2/Sgs1-Top3-Rmi1 complex. Importantly, the helicase activity of Sgs1 is required for the nuclease Dna2 to degrade the unwound ssDNA while Exo1 is able to execute its function by its own [32, 77, 110, 113]. It has been demonstrated that the inactivation of a single pathway has only a minor effect in DNA resection, because the other mechanism can efficiently compensate. Indeed, major resection defects were detected when both pathways are disrupted simultaneously [31, 32, 93]. Taking into account that both pathways are redundant, we cannot discard that either Exo1 or Dna2 might be masking the function that Cdc14 exerts over the resection machinery. To address this issue, we depleted *EXO1* and *SGS1* in both wild-type and *cdc14-1* backgrounds and determined their influence in resection. Deletion of Sgs1 in commonly used to abrogate Dna2's function since the nuclease cannot process dsDNA by itself. It is important to note

that we did not eliminate Dna2 because it is essential for cell viability due to its role in the Okazaki fragment maturation [107, 199].

If Cdc14 is modulating Sgs1-Dna2 activity, we should observe differences in the kinetics of DNA resection between the *exo1Δ* and *cdc14-1 exo1Δ* mutants since the Sgs1-Dna2 pathway is the only DNA resection pathway available in these backgrounds. Additionally, we would expect the same phenotype between *cdc14-1* and *cdc14-1 sgs1Δ* double mutant since both Sgs1 and Cdc14 might be acting over the same pathway. We induced a single DSB in a JKM139 background following the same experimental conditions previously mentioned for the resection assays, and analyzed the efficiency to resect the DNA molecule by Southern blot. A single mutant *exo1Δ* showed the same kinetics than a double *cdc14-1 exo1Δ* in the generation of the DSB. No differences were observed between both strains in short-range resection efficiency (Fig.24, 0.7kb fragment).

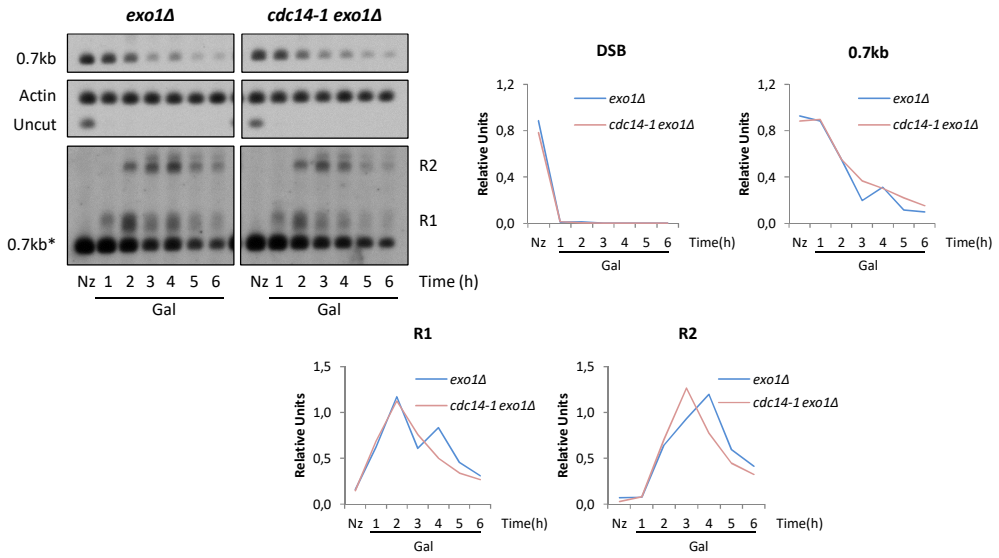


Fig.24. The absence of Cdc14 has no effect over the *exo1Δ* mutant. Cell cultures were synchronized in G2/M with nocodazole (Nz, YP raffinose, 25°C) and then shifted to the restrictive temperature to inactivate the function of the phosphatase (33°C). HO endonuclease expression was attained by adding galactose to the media. Samples were taken at different time points, DNA extracted and digested with *Sty*I. DNA fragments were separated on agarose gels and blotted. Blot shows the 0.7kb band recognized by the probe located near to the double-strand break (DSB). A second probe for the actin is included as loading control. Asterisk denotes an overexposed film to detect the resection intermediates (R1 and R2). Graphs represent the quantification of the break, the 0.7kb probe and intermediates R1/R2, all normalized with actin.

The analysis of the resection intermediates showed that *exo1Δ* cells presented a delay in the accumulation of R2. While the peak of accumulation for this intermediate was around 2 hours

after galactose addition in wild-type cells (Fig.22), an *exo1Δ* mutant showed a maximum intensity by 4 hours (Fig.24). On the other hand, a *cdc14-1 exo1Δ* double mutant exhibited a mild phenotype in the kinetics of the R2 intermediate, being accumulated 3 hours after the generation of the DNA break (Fig.24). In addition, a similar slight delay in long-range resection at later time points was detected in both *exo1Δ* and *cdc14-1 exo1* when compared to the wild-type (Fig.23, wild-type and Fig.25; see 21kb band). However, the absence of the phosphatase did not exacerbate or rescue the phenotype of the *exo1Δ* single mutant (Fig.24 and 25), indicating that Cdc14 is not modulating the Sgs1-Dna2 pathway.

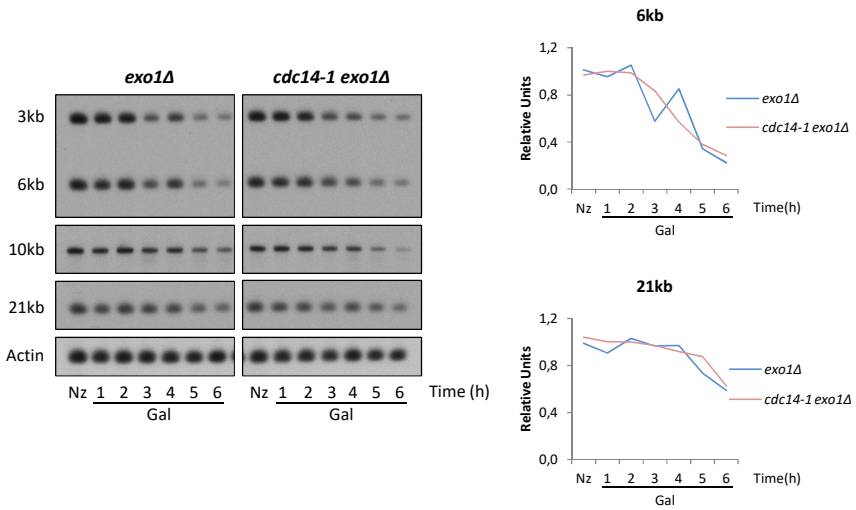


Fig.25. Cdc14 function is dispensable for long-range resection even in the absence of Exo1. Samples from experiment shown in figure 24 were subjected to Southern blotting using probes at 3, 6, 10 and 21kb from the break site. An additional probe for the actin is included to control the amount of DNA loaded in each line. Graphs represent the quantification of the 6kb and 21kb probes normalized with actin.

On the other hand, if Cdc14 is acting over the Exo1 pathway, this effect should be more evident in the absence of *SGS1* when the Exo1 pathway is the unique source of long DNA resection. When measuring the contribution of Sgs1 deletion in resection efficiency, a minor delay was detected in short- and long-range resection between the wild-type and *sgs1Δ* strains (Fig.22, 23, 26 and 27). Additionally, the R2 intermediate accumulated at later time points and remained longer in cells lacking Sgs1, manifesting the overall contribution of Sgs1 in DNA resection efficiency at short distances from the break (Fig.26). However, a *cdc14-1 sgs1Δ* double mutant showed the same kinetics of accumulation for the R2 intermediate even if it was less abundant in comparison to the *sgs1Δ* (Fig.26). In addition, cells lacking both Cdc14 and Sgs1 presented a delay in long-range resection when compared to *sgs1Δ* single

mutant denoted by the persistence of signals coming from probes located far away from the DSB site (Fig.27, 6kb and 21kb probes). These results suggest that Cdc14 might be participating in the correct activation of the resection process by enhancing Exo1 activity.

Altogether, it seems that Cdc14 is not involved in the activation of the DNA resection machinery at short distances from the break. However, our experiments demonstrate that Cdc14 might participate in controlling the resection process at long distances from the break by modulating Exo1 activity. However, we believe this phenotype do not explain the defects in DNA repair previously described in *cdc14-1* cells because:

1) The effect of Cdc14 in Exo1 regulation is only visible at far distances from the DNA break. Taking into account that short resection (2kb) is enough to execute proficient SDSA/BIR, it is quite unlikely that this phenotype could explain the lack of HR observed in the absence of the phosphatase.

2) Deletion of Exo1 has a minor effect in the overall execution of the HR pathway [31, 32], suggesting that the slight reduction of Exo1 activity in the absence of Cdc14 function cannot account for the global defect in DNA recombination observed in *cdc14-1* cells.

3) Exo1 does not contain any Cdk consensus site on its sequence, indicating that the possible interaction between Cdc14 and Exo1 is probably reflecting an indirect effect.

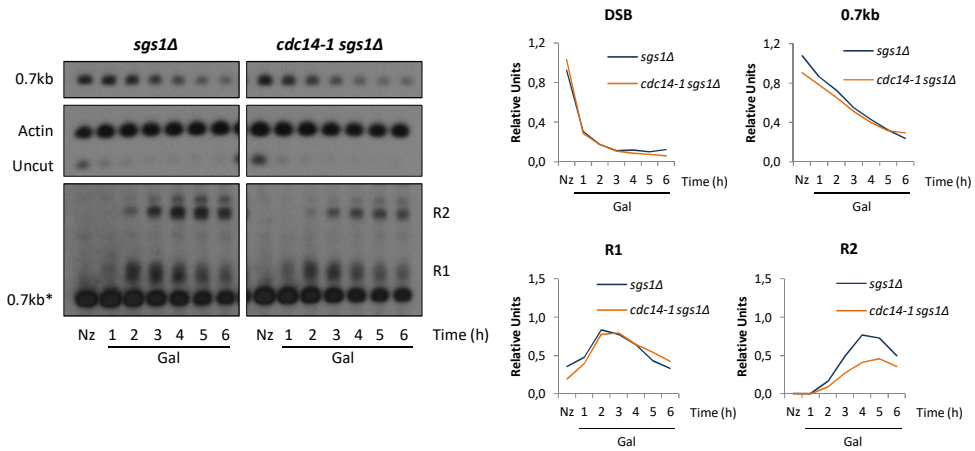


Fig.26. The *sgs1Δ* and *cdc14-1 sgs1Δ* double mutant showed the same kinetics in short-range resection. Same experimental conditions as those detailed in figure 24 were followed. Samples were taken at different time points, DNA extracted and digested with *Sty*I. DNA fragments were separated by gel electrophoresis and blotted. Blot shows the band corresponding to the 0.7kb probe. An additional probe against the actin gene is included as loading control. Asterisk indicates the overexposed film to visualize the resection intermediates R1 and R2. Graphs represent the quantification of the double-strand break (DSB), the 0.7kb probe and intermediates R1/R2, all normalized against actin.

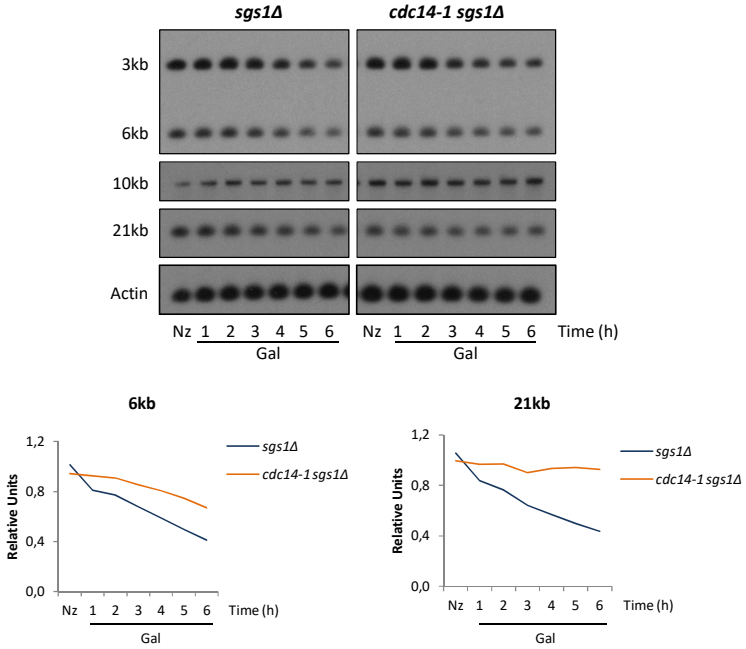


Fig.27. Cells lacking both Cdc14 activity and the helicase Sgs1 showed defects in long-range resection. Samples from the experiment described in figure 26 were digested with *Sty*I, separated on agarose gels and blotted. Blot shows the bands corresponding to the 3, 6, 10 and 21kb probes. An additional probe against the actin gene is included as loading control. Graphs represent the quantification of the 6kb and 21kb probes, both normalized to actin.

4. Importance of Cdc14 activity in the inhibition of the DNA end resection

4.1. Lack of Cdc14 activity is associated with a hyper-resection phenotype.

Once we confirmed that *cdc14-1* mutants were able to search for an homology sequence and form the displacement loop we wondered if Cdc14 might be implicated in switching DNA resection off. To study this step we employed the resection approach used in the previous sections of this work (Fig.21). The combination of several probes that recognize DNA sequences at increasing distances from the DSB in the AC1595 allows us to examine not only the disappearance of DNA fragments once the resection moves forward but also the timing and the efficiency of the DNA synthesis represented by the recovery of the DNA molecule. The same experimental approach that the one described in figures 38 and 39 were followed. Samples were collected at different intervals; DNA was extracted, separated on agarose gels and blotted. Wild-type and *cdc14-1* mutant cells generated the DSB with the same efficiency (Fig.41). In addition, no significant differences in the disappearance of the 0.7kb, uncut or DSB fragments were detected between both strains. These results correlate with our previous observations demonstrating that Cdc14 is not required for the activation/maintenance of the resection process at short distances from the DSB (shown in Fig.22 and 23).

Interestingly, cells lacking the function of the phosphatase showed a dramatic deficiency in the restoration of the 0.7kb and uncut bands when compared to wild-type (Fig.41), corroborating our previous findings that *cdc14-1* cells are defective in DNA repair. This difference in the restoration of the signal between the wild-type and the *cdc14-1* mutant was further confirmed by probes located at increasing distances from the HO-break (Fig.42, 6Kb and 21Kb). Astonishingly, while the resection machinery only reached the distance of 21Kb from the HO break at 4 hours in the wild-type strain, cells lacking Cdc14 activity rendered a continuous resection at 21Kb (Fig.42).

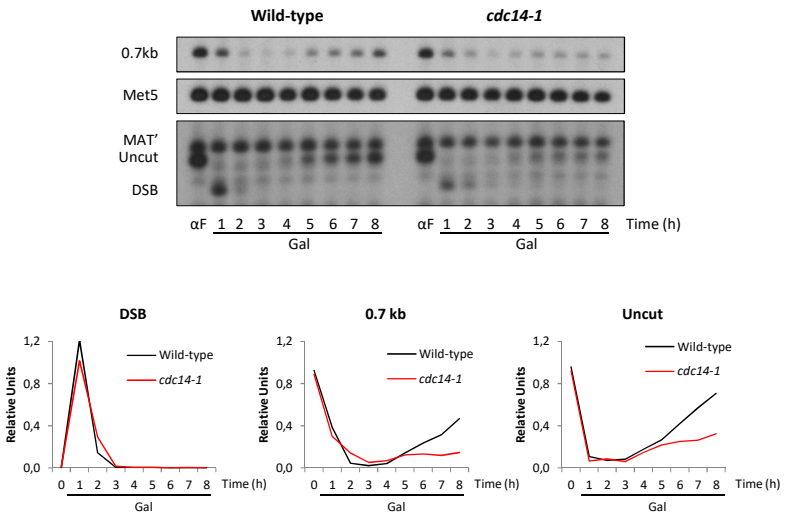


Fig.41. Cells lacking Cdc14 activity are unable to restore the DNA molecule once DNA resection has occurred. Wild-type and *cdc14-1* cells were subjected to one repairable double-strand break (DSB) following the experimental conditions described in figure 38. DNA was digested with *Sty*I, separated on agarose gels and subjected to Southern blotting. The approach followed to design the probes is detailed in figure 21. Blot shows DNA fragments detected by the Mat-specific probe (0.7kb, MAT', Uncut and DSB). An additional probe against met5 gene is included to control de amount of DNA loaded in each line. Graphs show the quantification of the indicated products relative to met5.

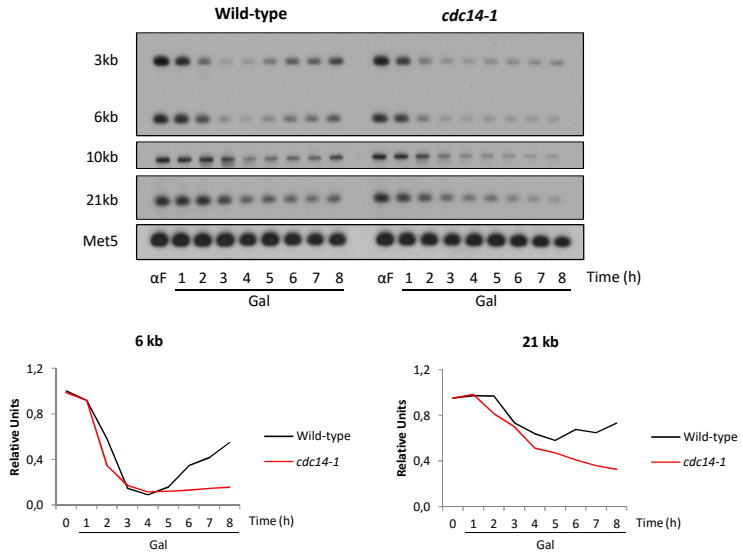


Fig.42. DNA end resection is hyperactive in *cdc14-1* cells. Samples from the experiment described in figure 41 were digested with *Sty*I. DNA fragments were separated on agarose gels and blotted. Southern blot was performed as previously mentioned (Fig.21 using probes at 3, 6, 10 and 21kb from the break). Blot shows the kinetics of disappearance/recovery of signal coming from these probes. An additional probe to detect met5 gene is included as loading control. Graphs show the quantification of DNA fragments analyzed at 6kb and 21kb from the DSB, both normalized against met5.

The hyper-resection phenotype observed in the absence of Cdc14 activity suggests that the phosphatase might be required for the downregulation of the resection machinery once the donor sequence has been found. Indeed, it is reasonable to think that this process must be highly regulated to avoid the formation of long tracks of ssDNA that can generate genome instability as commented in the introduction. Thus, we conclude that Cdc14 is required for the restoration of the DNA molecule by inhibiting the DNA resection machinery.

4.7. Cdc14 dephosphorylates and inactivates Dna2 to allow the restoration of the DNA molecule.

4.7.1. The hyperactivation phenotype *observed in* cdc14-1 *cells does not depend on Exo1.*

The generation of long ssDNA tracks once a DSB is produced depends on two non-overlapping mechanisms: Exo1 and Sgs1-Dna2 complex. In order to pinpoint which is the pathway operating behind the hyper-resection phenotype observed in *cdc14-1* mutants we deleted Exo1 and Sgs1 in the AC1595 background and measured the contribution of these two nucleases in the process. We speculated that if one of these two pathways is hyperactive in the absence of Cdc14 activity, the elimination of the responsible nuclease function would revert the extended resection observed in *cdc14-1* cells. To this end, asynchronous cell cultures of single and double mutants were grown overnight in raffinose-containing media. Next, galactose was added to induce the HO-break at the restrictive temperature and samples were taken at different time intervals. It is important to remark that henceforth, all experiments were extended up to 24 hours from the induction of the DSB to have a complete view of the DNA resection and repair process.

The analysis of the short-range resection in *cdc14-1* cells showed that the hyper-resection phenotype was present even at 24 hours from the induction of the DSB, even though we observed a slight and transient recovery of the 0.7kb band by 9 hours from the HO expression (Fig.46, 0.7Kb band). Importantly, this DNA synthesis was detected in the whole genome sequencing experiment (Fig.43). This temporary re-synthesis of the DNA molecule disappeared at later time points, supporting the hypothesis of an unbridled DNA resection in the absence of Cdc14 function as previously commented. Indeed, a defect in the recovery of the repair product was detected (Fig.46, Uncut), denoting that hyper-resection is compromising the recovery of the DNA molecule. Interestingly, elimination of Exo1 in *cdc14-1* cells did not present any significant change in the resection dynamics close to the DSB when compared to a single *cdc14-1* mutant (Fig.46, 0.7kb and Uncut bands). In addition, lack of

Exo1 in *cdc14-1* cells did not improve the repair of the HO-induced break (Fig.46), confirming that Exo1 and Cdc14 work in different pathways within the resection process.

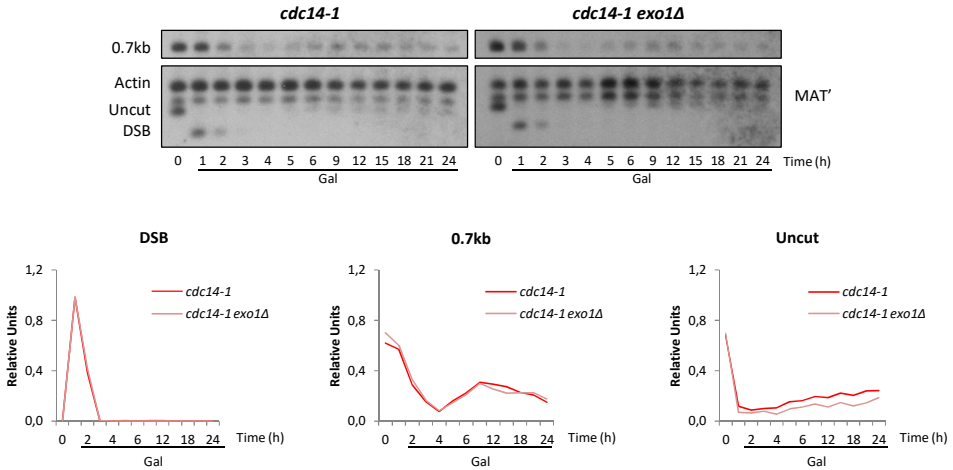


Fig.46. Lack of Exo1 does not revert the hyper-resection phenotype of *cdc14-1* mutants. Asynchronous cultures of cells lacking Cdc14 and both Cdc14 and Exo1 (YP raffinose, 25°C) were incubated in the presence of galactose to induce the HO endonuclease and shifted to the restrictive temperature (33°C). Samples were taken at different time points (until 24 hours) and subjected to Southern blotting as previously described (Fig.41). Probes and DNA fragments are described in figure 21. Blot includes the bands corresponding to the 0.7kb probe (0.7kb; Uncut; and double-strand break, DSB). Note that this probe also recognizes the MAT' donor sequence. An additional probe against actin is included to control the amount of DNA loaded in each line. Graphs show the quantification of the indicated products relative to actin.

Next, we extended this experimental approach to long-range resection. As in previous experiments, we observed a mild and transient recovery of the DNA products in *cdc14-1* single mutant by 9 hours after the generation of the DSB. Curiously, this effect was observed when measuring the 6kb fragment but not the 27kb band (Fig.47), suggesting that DNA re-synthesis in the absence of Cdc14 activity is a very limited event. Importantly, we did not find any difference in the long-range resection profile between a single *cdc14-1* mutant and a double *cdc14-1 exo1Δ* mutant, again confirming that Exo1 is not the exonuclease controlled by Cdc14 during the repair of a DSB (Fig.47).

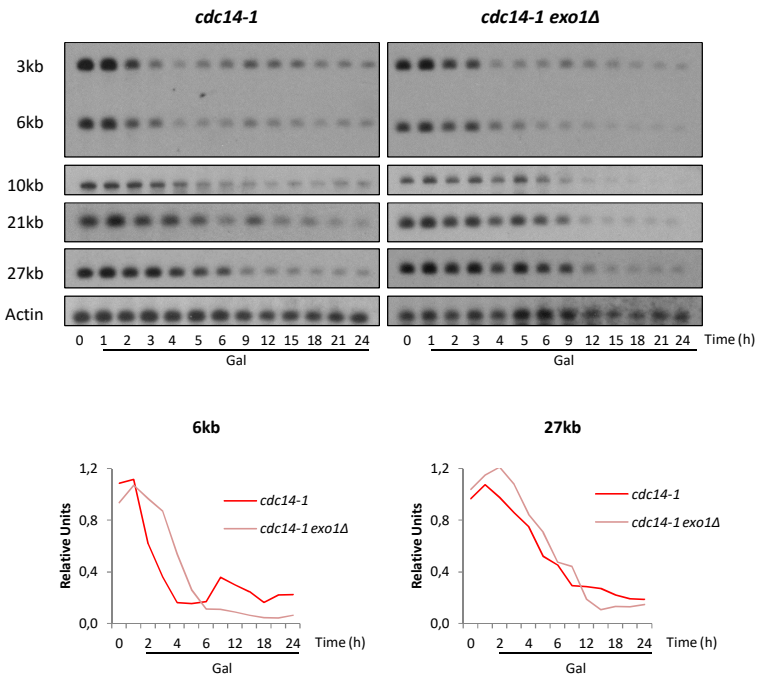


Fig.47. Lack of Exo1 does not rescue the phenotype of hyper-resection observed in *cdc14-1* cells. Samples obtained from the experiment mentioned in figure 46 were digested with *Sty*I, separated on agarose gels and blotted. Blot shows DNA fragments detected by 3, 6, 10 and 21kb probes and actin as loading control. Restriction sites, probes and DNA fragments size are included in figure 21. An additional probe that hybridizes at 27kb from the break is included. Graphs show the quantification of the indicated products relative to actin.

Overall, these experiments confirm that Cdc14 is required for shutting off resection once the donor sequence has been found in order to allow the re-synthesis of the DNA molecule. Cdc14 function in the control of resection does not involve Exo1, suggesting that the phosphatase might be acting over a different pathway to restrain resection.

4.7.2. Cdc14 acts in the same pathway than Sgs1-Dna2.

We have previously seen that the hyper-resection phenotype observed in the absence of Cdc14 activity is Exo1-independent. It has been demonstrated that DNA end resection in response to a DSB depends not only of Exo1, but also on the Sgs1-Dna2 pathway. Interestingly, Dna2 activation in response to DNA damage directly depends on the Cdk activity [120]. Indeed, it has been reported that phosphorylation at serines 17 and 237 by the Cdk is responsible for the activation of the nuclease in response to different sources of DNA damage [120]. Taking into account that Cdc14 has predilection to remove Cdk-dependent phosphorylation, it is tempting to speculate that Cdk/Cdc14 could be forming part of a

molecular switch that controls the timely execution of the resection process by acting over the nuclease Dna2. To attain this question, we decided to investigate whether elimination of Dna2 function in the absence of Cdc14 activity could rescue the hyper-resection phenotype observed in *cdc14-1* cells. Since *DNA2* is an essential gene, we decided to work with a *sgs1Δ* mutant, as it has been reported that Dna2's role in DNA resection depends on the helicase activity of Sgs1 [32, 112, 113]. Thus, we constructed a double *cdc14-1 sgs1Δ* mutant and determined the kinetic of short/long-range resection and its involvement in the repair of a DSB.

The absence of Sgs1 had no effect in short-range resection and in the restoration of the DNA molecule in cells lacking Cdc14 function (Fig.48). Both strains triggered DNA end resection with the same efficiency as previously observed in the non-repair assays (Fig.26). Interestingly, an improvement in the recovery of the 0.7Kb and the uncut band signals was detected at later points in the double *cdc14-1 sgs1Δ* mutant when compared to a single *cdc14-1* background (Fig.48, 21h and 24h). Surprisingly, the absence of Sgs1 in *cdc14-1* cells significantly alleviated the hyper-resection phenotype associated to the lack of the phosphatase when measuring long-range resection (Fig.49). While elimination of Cdc14 renders cells to an unrestrained resection during the entire experiment, *cdc14-1 sgs1Δ* double mutant was able to stop resection activity by 9 hours from the induction of the DSB. In consequence, 6kb and 27kb fragments were restored to levels comparable to the wild-type (even when timing between both experiments is different) (Fig.49 vs Fig.32). It is important to remark that the reversion of the hyper-resection phenotype of the *cdc14-1* mutant at long distances from the break did not correlate with the recovery of the uncut band, meaning that DNA repair is still impaired in the absence of the phosphatase. This observation suggests that in addition to inhibit DNA resection, Cdc14 must have other roles to ensure the correct regeneration of a DNA lesion, and sustains the idea that the phosphatase might be acting downstream the resection machinery to encourage a proficient DNA repair. In this line, our group has demonstrated that the phosphatase is required for the stabilization of the metaphase spindle and the recruitment of DSBs to the SPB proximity to allow the legitimate repair of these lesions [202]. Altogether, these findings demonstrate that Cdc14 acts as an inhibitor of the Sgs1-Dna2 pathway, a feature that is especially relevant to avoid an excessive resection.

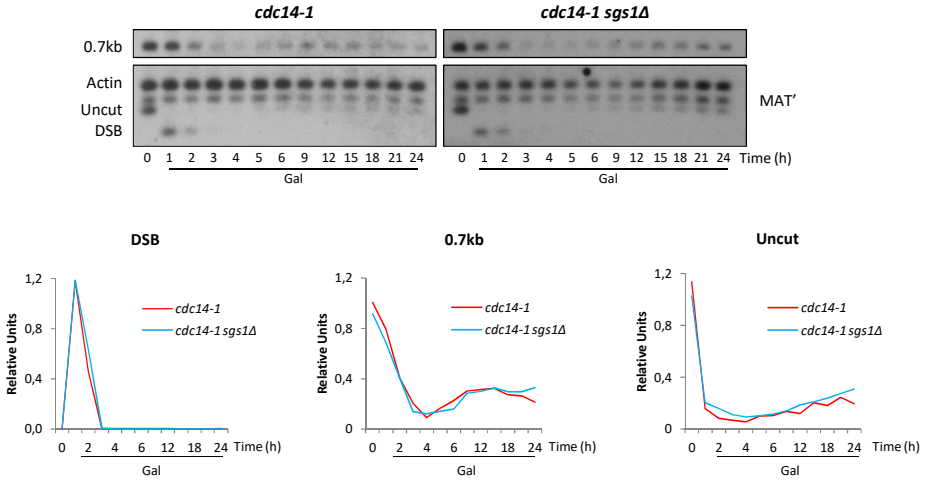


Fig.48. The absence of Sgs1 does not revert the phenotype of *cdc14-1* cells at short distances from the break. Growing conditions and probes employed in this experiment are described in figure 46 and 21, respectively. Blot shows DNA fragments detected by 0.7kb and actin probes. Note that 0.7kb probe also binds to the MAT' donor sequence. Graphs show the quantification of the indicated products normalized to actin.

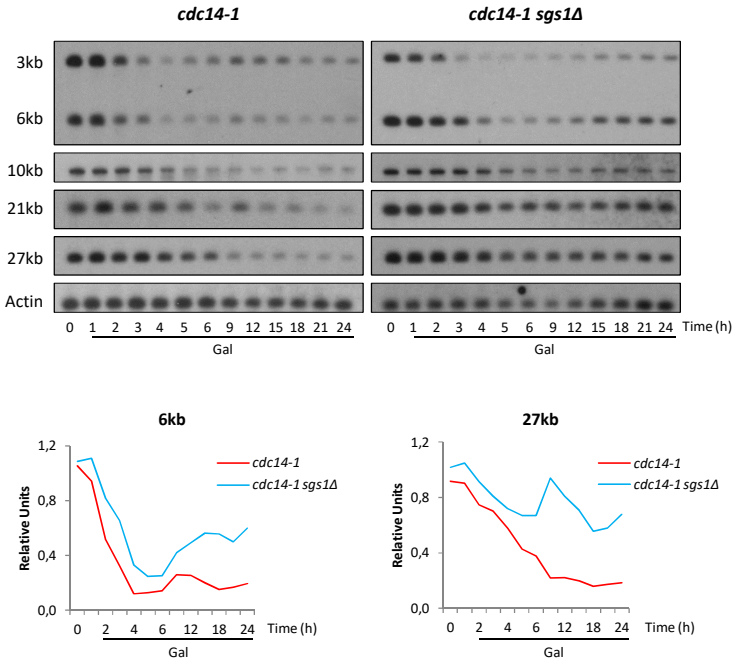


Fig.49. Lack of Sgs1 rescues the hyper-resection phenotype of *cdc14-1* cells. Experimental conditions and probes are described in figure 46 and 21, respectively. Samples from the experiment depicted in figure 48 were digested with *Sty*I. DNA fragments were separated by gel electrophoresis and blotted. Blot shows DNA fragments detected by 3, 6, 10 and 21kb and actin probes as loading control. An additional probe that hybridizes at 27kb from the break is included. Graphs show the quantification of the indicated products (6kb and 27kb) relative to actin.

4.7.3. Dna2 is a potential target of Cdc14 during the DNA damage response.

Our previous results indicate that Cdc14 is activated and released in the presence of DNA damage to inhibit resection thus limiting the amount of ssDNA at the time when DNA synthesis must occur. In particular, we know that Cdc14 acts in the same pathway than Sgs1-Dna2 and that the inhibition of this pathway by the phosphatase might be necessary to allow the proficient recovery of the DNA molecule.

While we were dissecting the implication of Cdc14 in the DNA processing we performed a quantitative phospho-proteomics approach to identify possible targets of the phosphatase in response to a DSB. This type of experimentation would provide us biochemical evidences regarding the Cdc14's function in the DDR. We decided to combine mass spectrometry with titanium dioxide phospho-peptide enrichment because it has been well documented that this methodology is one of the most reliable technique to identify protein phosphorylation. We developed this experiment in collaboration with the Cell Cycle Group and the Mass Spectrometry and Proteomics Facility at the Clinical Science Centre in London. By using this approach we screened for proteins containing quantitatively low levels of phosphorylated residues after the induction of the HO endonuclease that only occur when Cdc14 is active (putative targets of the phosphatase). Wild-type and *cdc14-1* cells of JKM139 background (Fig.12) were synchronized in G2/M to avoid cell cycle-dependent changes in the global phosphorylation status, and galactose was added to the media to express the endonuclease at the restrictive temperature (Fig.50). Cell cultures growing in YP-glucose were also carried out in parallel as undamaged negative control. The identified targets were grouped into different GO categories depending on the protein's molecular function. A large number of new potential Cdc14 targets previously described for their role in the DDR were identified (Fig.50). Within this category, quantitative comparison between wild-type and *cdc14-1* mutants before and after the DSB induction was applied to classify the discovered targets according to their relative phosphorylation status across the experimental conditions. To this end, we established a ratio which the higher it is, the more phosphorylation of a particular protein was found in *cdc14-1* mutant when compared to the isogenic wild-type strain, specifically during the DDR. Among the identified targets we found a series of proteins involved in the recognition and processing of the DSB and several SPB-associated proteins (Fig.50).

From all the targets isolated we focused our attention on the nuclease Dna2 since it could explain, at the biochemical level, our previous results pointing out a link between Cdc14 and DNA end resection. Dna2 has been described as one of the most processive enzymes operating during resection. As mentioned in the introduction it has been described that Dna2 has both nuclease and helicase activities but requires the action of the Sgs1 helicase. Sgs1 unwinds dsDNA and generates the ssDNA substrate for Dna2 to be loaded and degrade DNA endonucleolytically. At this point, we had several evidences indicating that Dna2 could be one of the Cdc14 targets in the DDR: 1) Dna2 is activated by Cdk in response to different sources of DNA damage [120], thus it is tempting to speculate for a role of a phosphatase in dephosphorylating this protein in the context of DNA damage; 2) Cdc14 acts over the Sgs1-Dna2 pathway to inhibit DNA resection and to allow DNA repair; 3) mass spectrometry revealed that Dna2 is hyperphosphorylated in the absence of Cdc14 activity. Thus, we decided to focus on the possible role of Cdc14 in the negatively regulation of the resection process by acting over Dna2.

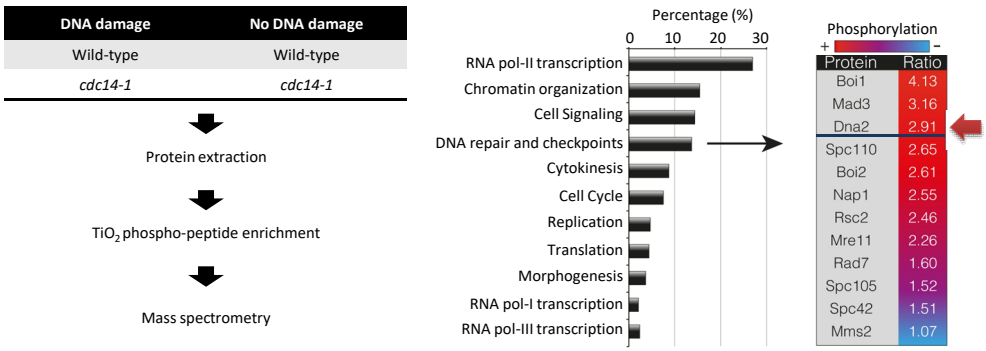


Fig.50. Identification of Cdc14 targets during the DNA damage response by mass spectrometry. Cell cultures were synchronized in G2/M by adding nocodazole (YP raffinose, 25°C) and then shifted to the restrictive temperature to inactivate Cdc14 function. Next, galactose was added to express the HO endonuclease for 4 hours. Workflow for the mass spectrometry experiment is included. Differential phosphorylation of phospho-peptides detected between wild-type and *cdc14-1* were grouped into broad categories depending on the molecular function of the proteins (graph). The table (right panel) represents the DNA damage and checkpoint-related proteins with Cdc14-dependent hyperphosphorylated status and the relative ratio between the wild-type and the *cdc14-1* backgrounds during the DNA damage response. Arrow points out Dna2, one of the putative targets of Cdc14 during the DDR with highest ratio. Red and blue indicate relative amount of the residue phosphorylation between both strains (red, high; blue, low).

4.7.4. Dna2 is hyperphosphorylated in cells lacking Cdc14 activity.

To further characterize the molecular mechanism that governs Cdc14-dependent regulation of the DNA resection we wondered if Cdc14 was acting directly on the regulation of Dna2

phosphorylation. As mentioned above, the activation and localization of Dna2 in the presence of DNA damage is highly controlled by Cdk-dependent phosphorylation events. Thus, we looked for changes in the phosphorylation status of the nuclease that might reflect the modulation of its activity during the response to a DNA lesion and its reliance on the Cdc14 phosphatase. To attain this hypothesis, we examined the phosphorylation profile of Dna2 during the response to a repairable DSB in the AC1595 background. In order to avoid cell cycle-dependent variations in the levels of Dna2 phosphorylation, we synchronized cells in G1 by alpha-factor and released them in the presence of galactose to induce the expression of the HO endonuclease. Both wild-type and *cdc14-1* strains were arrested in G2/M between 1.5 and 3 hours after the generation of the DSB (Fig.51A). However, while a wild-type strain re-entered the cell cycle about 6-7 hours from the DSB induction, cells lacking the Cdc14 function remained blocked the entire experiment probably due to the defects in DNA repair commented before (Fig.51A). Interestingly, while both strains presented a similar Dna2 phosphorylation profile by 1.5-3 hours from the DSB induction, *cdc14-1* cells showed higher levels of Dna2 phosphorylation within time points 3-4.5 hours when compared to the wild-type isogenic strain (Fig.51B, upper blot). It is important to remark that we did not expect to detect higher differences in terms of phosphorylation as it has been proposed that Dna2 is phosphorylated by both Cdk and Mec1, making difficult the assessment of the Cdc14's contribution in the steady-state phosphorylation of the nuclease. Mirroring this statement, Dna2 presented multiple phospho-forms in response to DNA damage when analyzed under our experimental conditions.

It is important to remark, that Dna2 hyperphosphorylation in the absence of Cdc14 activity occurred by 3-4.5 hours, perfectly correlating with the G2/M arrest (Fig.51A). This indicates that Dna2 dephosphorylation takes place at some stage of the DNA damage response. Remarkably, this time point fits with the timing of Cdc14 release from the nucleolus in response to DNA damage. Corroborating this result, Rad53 was active during the indicated interval of time in both strains, demonstrating that Cdc14 might in principle be the responsible for Dna2 dephosphorylation during the DNA damage checkpoint arrest (Fig.51B, bottom panel).

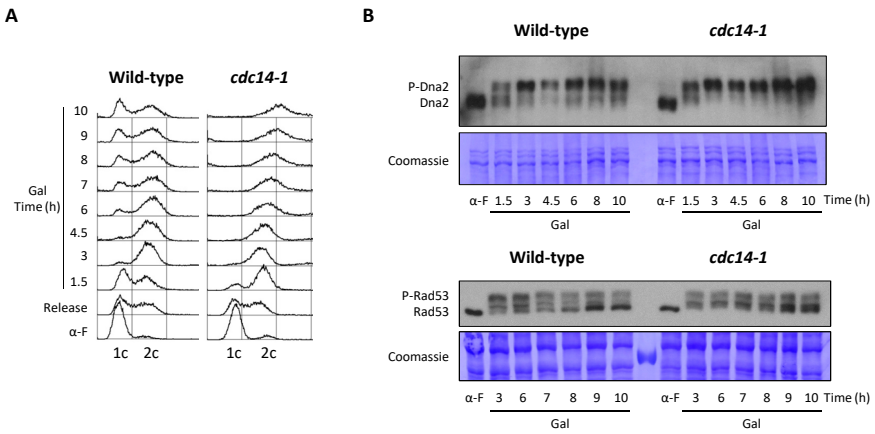


Fig.51. Dna2 is hyperphosphorylated in the absence of functional Cdc14. Wild-type and *cdc14-1* cells were synchronized in G1 by adding alpha-factor (YP raffinose, 25°C) and released into fresh media containing galactose (YP raffinose and galactose 2%). Cell cultures were incubated at the restrictive temperature (33°C) to inactivate Cdc14. Samples were taken at several points and processed for FACS and Southern blotting analyses. **A)** FACS showing DNA content of wild-type and *cdc14-1* at different intervals. **B)** Immunoblots including Dna2 (upper blot) and Rad53 (bottom) phosphorylation are included. For Dna2, gels containing 10μM of Phos-Tag were employed. Coomassie blue staining is included as loading control.

4.7.5. Dna2 foci formation and Cdc14 nucleolar release occurs simultaneously.

If Cdc14 is targeting Dna2 during the DDR it is reasonable to think that both proteins might interact at some point within the damage response. Taking together that Cdk-dependent phosphorylation of Dna2 controls its nuclear localization and considering that Cdc14 might be inhibiting the function of the nuclease, it is tempting to speculate for changes in the localization pattern of Dna2 along the damage response. To test for this hypothesis we analyzed Cdc14-GFP and Dna2-RFP signals following the same experimental conditions than in the previous experiment. Pictures were taken every hour from the generation of the DSB to a total of 6 hours. Supporting our previous results, we found that Cdc14 is transiently released from the nucleolus to the nucleoplasm at 4-5 hours from the induction of the break both in wild-type and *cdc14-1* cells (Fig.52). In addition, we found that Dna2 formed discrete foci in the presence of DNA damage (Fig.52) as has previously been shown [120].

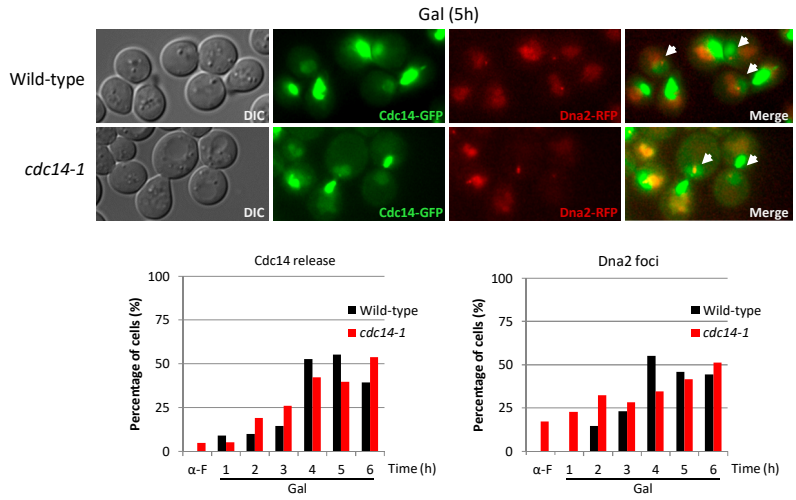


Fig.52. Cdc14 release overlaps with the appearance of Dna2 foci in the context of DNA damage. Live-cell imaging of Cdc14 and Dna2 localization in the presence of a repairable double-strand break (DSB) at the *MAT* locus. Wild-type and *cdc14-1* cells carrying Cdc14-GFP and Dna2-RFP were grown overnight in YP + raffinose and synchronized in G1 by the alpha-factor pheromone (25°C). Next, cells were released and incubated in the presence galactose to induce the HO endonuclease. Cell cultures were shifted to the restrictive temperature (33°C) to inactivate Cdc14 activity. Images were taken before and after the generation of the DSB. Micrographs display the maximum projection of nine planes. Arrows indicate cells with nuclear Cdc14 signal overlapping with Dna2 focus at 5 hours from the HO-induced break. Graphs represent the percentage of cells with Cdc14 at the nucleoplasm (left graph) and the percentage of cells presenting Dna2 focus (right graph) at different time points.

In a wild-type strain, Dna2 foci are formed within 2-4 hours from the induction of the lesion and begun to disappear at later time points, coinciding with the formation of the DNA repair products (Fig.38 and 43). Importantly, the Cdc14 transient release perfectly overlapped with the timing of Dna2 foci disappearance (Fig.52), suggesting that Cdc14 might be targeting Dna2 to promote its exclusion from the DNA. Supporting this hypothesis, a *cdc14-1* strain displayed a higher percentage of cells containing Dna2 foci since the beginning of the DSB generation. Importantly, while no differences in the number of Dna2 foci were detected at later points when compared to the wild-type strain, in the absence of Cdc14 activity, there was a tendency for these foci to remain for longer (Fig.52).

Taking together, it seems that Cdc14 has the ability to dephosphorylate Dna2 in response to a DSB. Interestingly, as Cdk-dependent phosphorylation of Dna2 has been correlated with the activation of the nuclease, we can conclude that the activity of Cdc14 over Dna2 is a prerequisite to inhibit its nuclease activity, a mechanism that might facilitate the inhibition of the DNA resection process and in consequence the repair of the DSB.

4.7.6. The hyper-resection observed in cdc14-1 mutants depends on Dna2.

Even though Sgs1 is considered the helicase required for Dna2 to exert its function as 5'-3' nuclease during the DNA resection, its activity has also been linked to different DNA repair processes. In this line, it was demonstrated that Sgs1 works together with Top3 and Rmi1 in dissolving double Holliday junctions to prevent the formation of crossovers and, in consequence, sister chromatid exchanges and genome instability [110, 111]. In addition, taking into account that Dna2 is hyperphosphorylated in the absence of Cdc14, we wondered if the elimination of Dna2 activity might bypass the over-resection phenotype observed in *cdc14-1* cells. As *DNA2* is an essential gene, we decided to construct an auxin-inducible degron (AID) for the nuclease. This system obtained from plants is an efficient tool for the rapid degradation of essential proteins and can be adapted to most eukaryotes. Auxins are included in a family of plant hormones that control gene expression of many aspects involved in growth and development [203]. One example of these hormones is the indole-3-acetic acid (IAA), a natural auxin that binds to the F-box transport inhibitor response 1 (TIR1) protein [204, 205]. Auxin binding to TIR promotes the interaction between TIR and the aid degron of the target protein. SCF-TIR1 acts as an E3 ubiquitin ligase and recruits the E2 ligase which polyubiquitylates the aid degron resulting in rapid degradation of the latter by the proteasome [206, 207]. All eukaryotes have multiple forms of SCF in which an F-box protein determines substrate specificity, but orthologs of TIR1 and IAAs are only found in plants. By transforming yeast cells with the corresponding TIR1 is possible to recreate the SCF-TIR1 complex and target a specific protein fused to the aid degron to be degraded by the proteasome in an auxin-dependent manner.

To this end, Dna2 was tagged with 9Myc-AID to induce its degradation by the ubiquitin-proteasome pathway in a *cdc14-1* background. Asynchronous cells were incubated in the presence of galactose at the restrictive temperature and after 6 hours of HO induction, cells were divided into two different cultures. One culture was treated with IAA and the control was incubated in the presence of mock ethanol (ETOH). The time point 6 hours was chosen because is when most of the DNA resection has taken place and DNA synthesis occurs (Fig.38 and 43). Cells were harvested at different time points and processed for Western and Southern blotting. As shown in figure 53A the addition of a unique dose of IAA (final concentration 1 µM) is sufficient to induce the degradation of the nuclease Dna2 which remained absent for the next 18 hours. Both cell cultures (ETOH and IAA) showed the same kinetics in terms of generation of the DSB and no significant differences were observed when

measuring the resection at short distances from the break or the recovery of the DNA molecule (Fig.53B, 0.7kb and Uncut). However, both treatments showed an improvement in the recovery of the DNA molecule when compared to *cdc14-1* and *cdc14-1 sgs1Δ* denoted by the restoration of the uncut band (compare figure 48 with figure 53B). This finding reveals that: 1) cells incubated with mock behaved like those lacking Dna2, and 2) deletion of Dna2 restored the DNA repair defects observed in the absence of Cdc14 phosphatase. Importantly, cells treated with IAA rescued the phenotype of hyper-resection of *cdc14-1* single mutants at long distances from the DSB (Fig.54, 3kb, 6kb, 21kb and 27kb bands). Note that even in the absence of IAA (mock), cells harboring Dna2-AID-9Myc showed a mild inhibition of resection and a mild improvement in the restoration of the DNA molecule as commented above (Fig.54, ETOH vs IAA vs *cdc14-1*). Graphs included in figure 54 also shows *cdc14-1* data from the experiment represented in figure 47 to facilitate the comparison between the AID experiment and the single mutant lacking the phosphatase's function. The fact that cells incubated with mock exhibited a moderate reversion of the *cdc14-1* phenotype can be explained by a partially loss of function of the nuclease during the damage response, even when the tagging of the protein had no effects on cells growing in unperturbed conditions (data not shown). Independently of this effect, the inhibition of resection was strengthened in the presence of IAA than in mock samples, corroborating that this nuclease is the responsible for the hyperactivation of the DNA resection in the absence of Cdc14. Importantly, the recovery of the DNA molecule was stable until 24 hours (Fig.54, 6kb and 27kb products), providing further evidence that DNA resection was inhibited under these conditions.

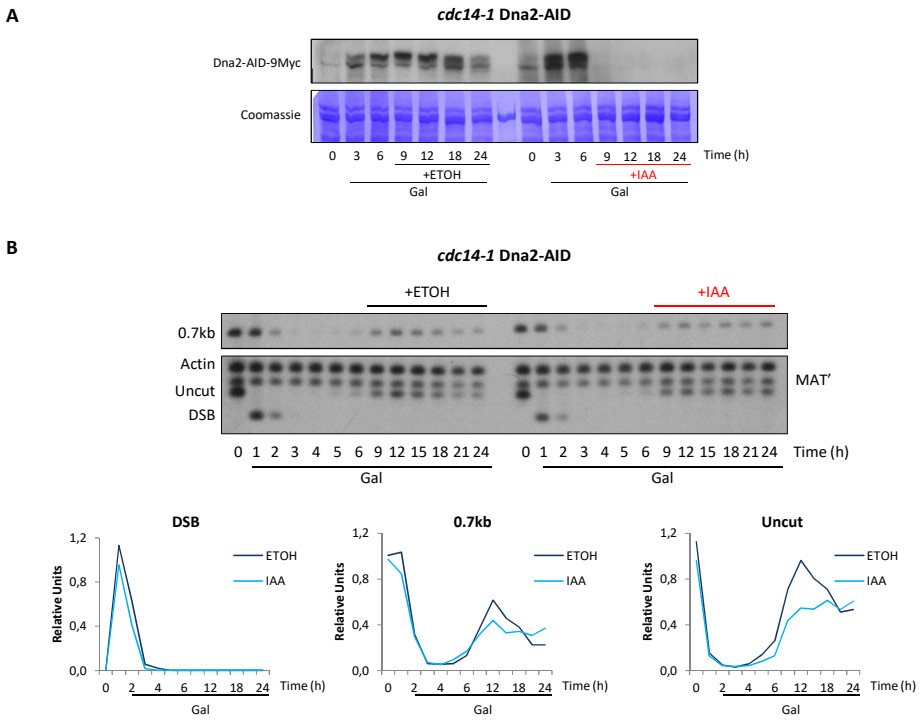


Fig.53. Lack of Dna2 has no apparent effect on *cdc14-1* cells when studying the short-range resection. Asynchronous *cdc14-1* cells containing the auxin-inducible degron (AID) system were grown in YP raffinose at 25°C and then incubated in the presence of galactose at the restrictive temperature (33°C) to induce the expression of the HO endonuclease and to inactivate Cdc14. In addition, ETOH or indole-3-acetic acid (IAA) at final concentration of 1µM were added 6 hours after the generation of the double-strand break (DSB). IAA was used to promote the degradation of Dna2-9Myc-AID. Samples were taken at several time points and processed for Western and Southern blotting. **A)** Immunoblot showing the presence/absence of Dna2-9Myc before/after IAA addition. Coomassie blue staining is included as loading control. **B)** Blots show the fragments detected with the 0.7kb and actin probes. Note that 0.7kb probe also recognizes the MAT' donor. Probes were designed as shown in figure 21. Graphs represent the quantification of the indicated products normalized to actin.

Overall, our data demonstrate that Cdc14 release during the DDR is required to dephosphorylate and inhibit Dna2 once the homology search and the D-loop formation have occurred. The inhibition of the DNA resection together with other functions of the phosphatase, are necessary for the accomplishment of a proficient DNA repair.

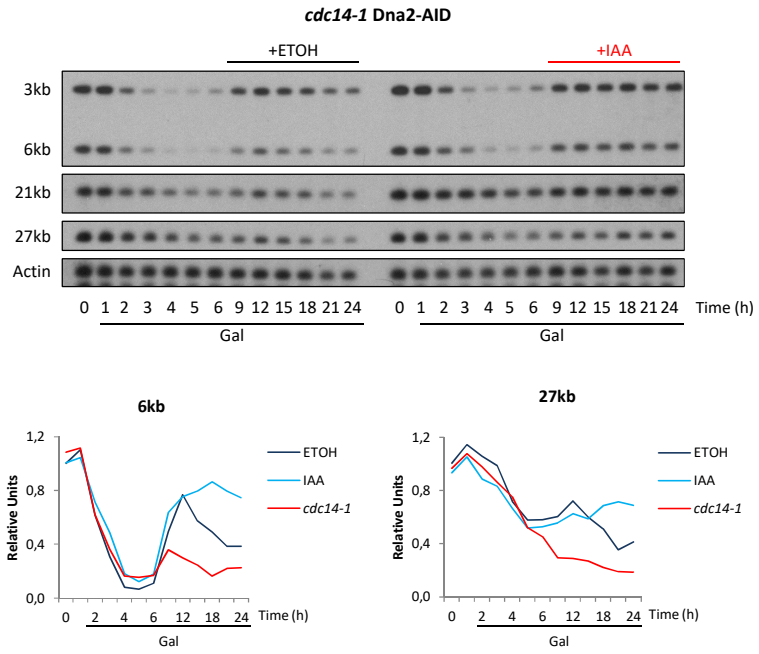


Fig.54. The hyper-resection observed in *cdc14-1* cells depends on Dna2. Samples from the experiment described in figure 53 were digested with *Sty*I. Next, DNA fragments were separated on agarose gels and blotted. Blot were hybridized with 3, 6, 21 and 27kb probes previously described (Fig.21). Graphs represent the quantification of the indicated fragments normalized against actin. Note that *cdc14-1* data from figure 47 is included in the graph to facilitate the comparison with this experiment.

Discussion

1. Cdc14 is required for the inhibition of the DNA end resection

Phosphorylation events that occur in the presence of DNA damage have been thoroughly studied for the last years, focusing mainly in the activation of several signaling pathways and kinases involved in the response to a DNA break. Even if it is generally accepted that reversion of phosphorylation is determinant for restoring the effect imposed by protein kinases, there have only been sporadic studies about protein dephosphorylation during the execution of the DDR. On the other hand, it has been broadly demonstrated that Cdk plays a key role in the modulation of the DDR [4, 61, 127, 128, 143]. In particular, DNA resection is probably one of the earliest events modulated by the protein kinase in response to DNA damage [26, 27, 61, 77, 123, 127, 143, 208]. This tight control exerted over resection is necessary for the accurate activation of the subsequent events of the repair cycle, including the DNA damage checkpoint and the recombinational pathway. Taking into account that the phosphatase Cdc14 has a special predilection for Cdk substrates and that it has been implicated in the DDR in both fission yeast and mammals [186, 188, 189], we hypothesized that the Cdc14 might be responsible for reverting the effect imposed by the Cdk during the damage response in *S. cerevisiae*. In this line, we have focused our attention in elucidating the importance of the protein phosphatase Cdc14 along the repair of a DNA break in the budding yeast.

Undoubtedly, one of the most relevant results presented in this work is the phenotype of hyper-resection observed in the absence of the Cdc14 function. Surprisingly, even if the phosphatase is not required for the initiation of the DNA end resection, it is necessary to switch it off, thus allowing cells to efficiently repair the DNA break. By combining physical and whole genome sequencing approaches we have provided robust evidences that prove the implication of Cdc14 in resection inhibition. Remarkably, Cdc14 function is not required for DNA damage checkpoint activation under these experimental conditions. These results reinforce previous studies indicating that the involvement of Cdc14 in the DDR is directly linked to its ability to enhance DNA repair rather than checkpoint activation, as proposed in avian, human and mice models [188-190].

Importantly, our data demonstrate that Cdc14's role in resection inhibition is limited to the Sgs1-Dna2 pathway, particularly to the modulation of Dna2 phosphorylation. It has been previously demonstrated that the nuclease Dna2 is translocated to the nucleus by Cdk-

dependent phosphorylation events [126]. In addition, Dna2 forms foci in response to DNA damage, and two Cdk consensus sites (S17 and S237) are enough for its function during the DDR [120]. Consistent with this, we found in our mass spectrometry assay that Dna2 is phosphorylated during the response to a DSB. Surprisingly, this nuclease was hyper-phosphorylated in response to a single DSB in the absence of Cdc14 function. Indeed, we have validated that Cdc14 is the phosphatase implicated in the dephosphorylation of Dna2 upon DNA repair. How might Cdc14 negatively regulate Dna2 activity? As commented before Dna2 phosphorylation by Cdk enhances its transport into the nucleus during the damage response. Thus, it is tempting to speculate that the Cdc14-dependent dephosphorylation of the nuclease might exclude the protein from the nucleus, therefore inhibiting its function at the DNA break. Supporting this idea, we found that in a wild-type strain Dna2 foci started to disappear when DNA repair products were formed (time points 5-6 hours), while in the *cdc14-1* mutants Dna2 foci had a tendency to accumulate (Fig.52). We believe the balance Cdk-Cdc14 is determinant for the localization and activation of the nuclease. In this regard, it has been recently proposed that Sgs1 binds to the ssDNA and stays immobile until Dna2 is recruited to initiate DNA resection in the budding yeast [209]. It is important to note that even Dna2 has both helicase and endonuclease activities, the most accepted model suggests that operates as a 5'-3' translocase that slides and cleaves ssDNA only when Sgs1 and RPA are present [116]. Altogether, it seems that Dna2 recruitment to ssDNA is essential for the correct function of the Sgs1-Dna2 machinery, so the timely regulation of Dna2 may be crucial for the execution of the DNA resection. Whether Cdk phosphorylation of Dna2 enhances its nuclease activity and/or binding to ssDNA, or by contrast, is a process that specifically acts through its cytoplasm/nucleoplasm translocation is still unknown.

2. Importance of the fine-tune regulation of the DNA end resection

Our data show that Cdc14 is essential for cell viability when exposed to different genotoxic stresses. As shown in figure 8, not all genotoxic compounds have the same effect over cells lacking Cdc14 function, suggesting that the phosphatase might have different functions in the DDR. Thus, depending on the type of DNA damage infringed cells lacking Cdc14 activity might exhibit differential sensitivity to different genotoxic agents. This is in line with previous studies indicating that phosphatases that share common targets may behave differently according to the context in which the DNA damage occurs [146, 210]. Importantly, this observation could explain the controversial results obtained between yeast and mammalian

cell regarding the implication of Cdc14 in DNA damage checkpoint activation and/or DNA repair.

In this work we have shown that *cdc14-1* mutants exhibited a hyper-resection phenotype. What is the consequence of an unbridled DNA resection? It has been demonstrated that hyper-resection is deleterious for cell survival because of many reasons: 1) long ssDNA tracks are unstable over the time and clustered mutations can be generated [211, 212]; 2) the generation of long 3'-overhangs may exhaust the pool of RPA thus generating free ssDNA regions that are susceptible to be degraded by cellular nucleases [59, 60]; 3) extensive ssDNA molecules would trigger more mutagenic pathways of HR due to the presence of repetitive sequences that may promote recombination events [57, 58]. On the other hand, DNA end resection is essential for the activation of a proficient DNA damage checkpoint and DNA repair by homologous recombination [4, 26, 27, 29, 32, 44, 55, 75, 101, 127, 132, 133, 143, 197, 208]. Therefore, cells have to activate DNA resection to allow the repair of the DNA break but they have to strictly control the extent of the ssDNA generated to allow the recovery of the DNA molecule and re-enter into the next cell cycle round. Supporting this idea, we have also found that in addition to the hyper-resection phenotype observed in *cdc14-1* mutants, these cells are not able to repair the broken DNA, probably due to their inability to switch DNA resection off. Accordingly, elimination of Dna2 activity in *cdc14-1* cells bypasses the DNA repair defects observed in the absence of the phosphatase indicating that Cdc14 is acting exclusively over Dna2 (Fig.53B, Uncut band). Surprisingly, deletion of Sgs1 rescued the hyper-resection phenotype of *cdc14-1* cells but did not show an improvement in the repair of the uncut fragment (Fig.48 and 49). This finding can be explained by the importance of Sgs1 in DNA repair by HR [110, 119, 195, 213-215]. So, even if we block DNA resection by removing this helicase cells will not be able to fulfill DNA repair because Sgs1 is required for subsequent steps of the repair pathway. Supporting this hypothesis, Sgs1 interacts with Rad51 [119, 214] to facilitate its recruitment to ssDNA while it is unwinding the dsDNA [216].

We have to take into account that the DNA repair failure observed in cells lacking Cdc14 could be explain by an inefficient DNA synthesis due to the uncontrolled resection observed in these mutants. Interestingly, we have shown that *cdc14-1* cells are competent to timely form a D-loop, indicating that homology search and invasion of the donor sequence occur normally. These data suggest that DNA hyper-resection does not interfere with the formation of a stable D-loop. Surprisingly, *cdc14-1* cells showed a transient recovery of the

DNA molecule at short distances from the break (Fig.43 and 46, time points 6 and 9 hours), indicating that activation of DNA synthesis is competent in the absence of the phosphatase. The further disappearance of this signal was probably due to the steady activity of the Sgs1-Dna2 pathway over the new synthesized strand in *cdc14-1* cells. Supporting this idea, elimination of Dna2 resulted in the complete recovery of the DNA molecule (Fig.53 and 54). These findings fit into a model whereby Cdc14 dephosphorylates Dna2 in response to DNA damage to turn Sgs1-Dna2-dependent DNA resection off, thus promoting an efficient recovery of the DNA molecule by DNA polymerases and ligases (Fig.55).

However, this is probably not the only mechanism of regulation operating at this point of the repair pathway, since it has been proposed that the DNA synthesis machinery has an important role in inhibiting Exo1 pathway to allow the restoration of the DNA molecule [217]. This suggests that must be a crosstalk between the DNA synthesis and resection machineries. In this scenario, Cdc14 might collaborate with the DNA synthesis to downregulate long-range resection activity (Fig.55). As mentioned before, the fine-tune regulation of these processes is essential for HR, therefore it is reasonable to think that more than one mechanism may control Exo1 and Sgs1-Dna2 pathways. Indeed, it has been demonstrated in budding yeast that Exo1 is phosphorylated by Rad53 to restrain its exonuclease activity and avoid excessive resection. This result has been reproduced in mammalian cells, where EXO1 is phosphorylated and degraded rapidly in the presence of DNA damage to avoid over-resection [56].

3. Cdc14 localization during the DDR

In the budding yeast, Cdc14 is maintaining inactive during interphase by its retention into the nucleolus by interacting with Net1. This interaction is relaxed in mitosis to promote Cdc14 release, a feature that encourages mitotic exit. In this work we have determined that Cdc14 nucleolar release is not restricted to mitosis, as cell exposed to DNA damage are also able to promote Cdc14 release and activation. Our model proposes that Cdc14 activation in response to a DNA lesion is crucial to inactivate the DNA resection by dephosphorylating Dna2 (Fig.55). Therefore, we should be able to detect changes in the subcellular localization of the phosphatase specifically at this stage of the repair pathway. Indeed, we found that Cdc14 is transiently released from the nucleolus to the nucleoplasm in response to one or multiple DSBs about 5 hours from the induction, a time point that correlates with the inactivation of resection, the appearance of DNA repair products and the disappearance of Dna2 foci (Fig.29,

35, 38, 41 and 52). This finding is in line with previous results coming from *S.pombe* and mammals showing that Cdc14 orthologues Flp1 or Cdc14B are translocated to the nucleus in response to DNA damage [186, 187]. Thus, it is tempting to speculate that the mechanism of Cdc14 activation in response to DNA breaks might be conserved.

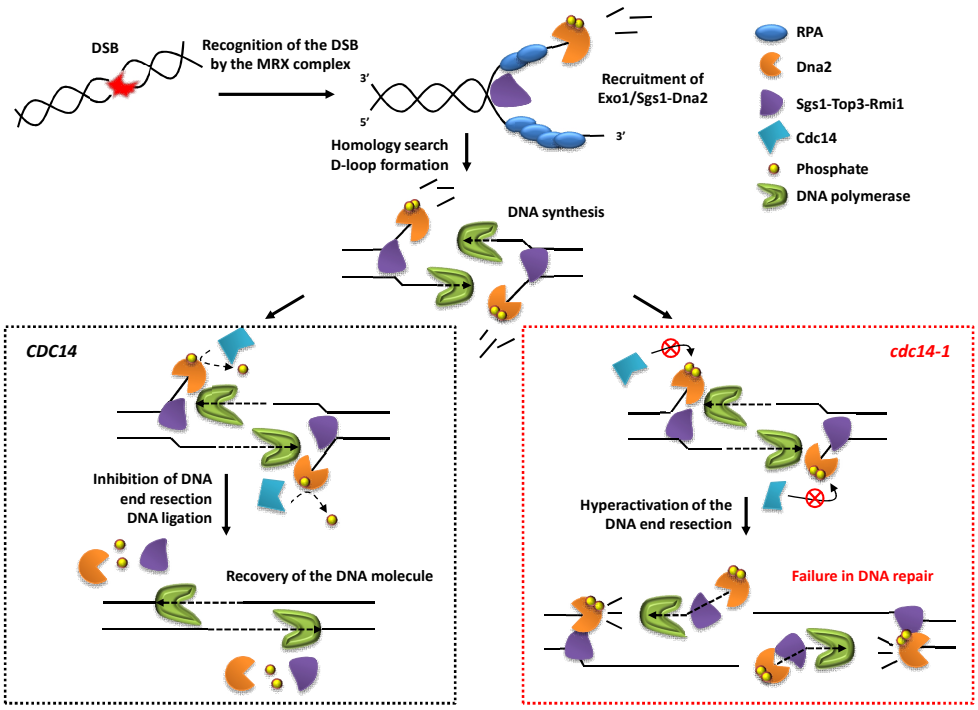


Fig.55. Model of Cdc14-dependent inhibition of the DNA end resection. After the recognition of the double-strand break (DSB) by the MRX complex, the nucleases Exo1 and Sgs1/Top3/Rmi1-Dna2 are recruited to the lesion to process the 5'-ended strand. These two pathways generate the 3'-overhang of single-strand DNA (ssDNA) required for the homology search and invasion. Once cells have copied the information from the donor sequence, the DNA resection machinery must be switched off. In the presence of functional Cdc14 (CDC14, left bubble), Dna2 is dephosphorylated and removed from the DNA molecule thus inhibiting the action of the Sgs1-Dna2 pathway. This allows the machinery involved in the DNA synthesis to restore the DNA molecule and finish with the repair process. In the absence of Cdc14 function (*cdc14-1* background, right bubble), Dna2 remains phosphorylated and hyperactive, thus generating long ssDNA tracks. Under this condition, the DNA synthesis may start, but the presence of active Sgs1-Dna2 machinery would eliminate the new synthesized molecule. In consequence, DNA synthesis and repair are not accomplished. Even if not included in the scheme, Exo1 pathway might be functional under these conditions.

Moreover, the re-localization of the phosphatase coincides with Net1 phosphorylation. Taking into account that Net1 phosphorylation is associated with the release of Cdc14 in an unperturbed cell cycle it is intuitive to think that a similar mechanism might be operating in response to DNA damage to exclude the phosphatase from the nucleolar region. Interestingly, Cdc14 nucleolar liberation in response to DNA damage resembles the partial and transient exclusion that the phosphatase experience during FEAR activation (Fig.9 and

52 vs Fig.10). These observations suggest that DNA damage-dependent Cdc14 liberation might be linked to a FEAR-related activation. As FEAR-dependent Cdc14 release does not induce mitotic exit, cells ensures a controlled activation of the phosphatase to execute its function in DNA repair but avoiding cell cycle progression. Even-though, all data point to a FEAR activation in response to DNA damage, we still detected Cdc14 release under DNA-damaging conditions in the absence of the separase Esp1 (data not shown). This observation together with the persistence of Net1 phosphorylation even when Cdc14 localized at the nucleolus at late points during the damage response (Fig.9B), suggest that additional mechanisms might be controlling the nucleolar/nucleoplasm shuttling of the phosphatase during the DDR. It has been proposed that in addition of the Net1 phosphorylation, Cdc5-dependent phosphorylation of Cdc14 is required for the full release of the phosphatase in an unperturbed cell cycle. While Net1 phosphorylation seems to occur during MEN and requires Cdk activity, Cdc14 phosphorylation is limited to the FEAR pathway [163, 167]. Additionally, it seems that Cdk governs Cdc5 function both in FEAR and MEN indicating it might be crucial in the regulation of Cdc14 release. In this line, we have detected that Cdc14 is phosphorylated under DNA damage (data not shown). However this phosphorylation seems to be restricted to MEN since takes place during checkpoint deactivation and cell cycle reentry. Surprisingly, we have found that Cdc14 is posttransductional modified by SUMO (small ubiquitin-like modifier) during its exclusion from the nucleolus in the presence of a DSB (data not shown). By using a SUMO-prediction software, we have found only one bona fide SUMOylation site located at the NoLS (nucleolar localization sequence) of Cdc14. Since SUMO modification has been associated with the regulation of the subcellular localization of several proteins, we can hypothesize that Cdc14 SUMOylation at the NoLS is required for the nuclear localization of the phosphatase in the context of damage. Supporting this idea, some preliminary results coming from our laboratory suggest that Cdc14 SUMOylation is necessary for the correct function of the phosphatase in the repair of a DNA lesion. However, we still do not know if this SUMO modification has a role in the spatial regulation of Cdc14 in the context of DNA damage..

It is important to remark that Cdc14 liberation in response to DNA damage temporary occurs between 2.5 and 7 hours from the generation of a DSB. Even Cdc14 liberation might be well explained by its SUMO-dependent modification, we still missing how the phosphatase is removed from the nucleus at later time points. As mention above, Cdc14 is also subjected to phosphorylation about 7 hours from the DSB induction. In principle this result might suggest that Cdc14 phosphorylation could be the signal to remove the phosphatase from the

nucleoplasm. It is well known that Dbf2-Mob1-dependent phosphorylation of Cdc14 during MEN occurs next to the NLS to translocate the phosphatase into the cytoplasm [218]. Curiously we detected high levels Cdc14 phosphorylation at these residues during cell cycle reentry in cells subjected to DNA damage (mass spectrometry data). This finding suggests that Cdc14 disappearance from the nucleoplasm might be restricted to the effect of MEN once cells scape from the DNA damage checkpoint and reenter the cell cycle. However, the fact that cells containing a non-reparable HO system execute a similar transient Cdc14 release (Fig.13) indicates that nucleoplasmic removal of Cdc14 takes place even when the damage checkpoint is still active. This result clearly indicates that Cdc14 phosphorylation enhances its nucleoplasm elimination in a MEN-independent manner and that further mechanism might control the localization of Cdc14 in response to a DNA damage.

In summary, we propose that Cdc14 release in response to a DNA break inhibits DNA resection during the DDR (Fig.55). Importantly, the inhibition of the resection machinery by Cdc14 might be highly regulated to avoid too low/high levels of resection. This temporary control of resection is at least in part controlled by the activity of Cdc14 along the repair cycle. Cdc14 temporary exclusion from the nucleolar compartment ensures a precise mechanism to turn resection off only when the donor sequence has been found. We believe that fine-tuning modulation of the phosphatase plays an essential role in this mechanism, ensuring a legitimate DNA repair to maintain cell viability in response to DNA damage.

Conclusions

1. The activity of the Cdc14 phosphatase is essential for cell viability when cells are exposed to genotoxic stresses in *Saccharomyces cerevisiae*.
2. Cdc14's function is dispensable for a proficient DNA damage checkpoint activation.
3. DNA repair analyzed by SDSA and DSBR systems is impaired in cells lacking Cdc14 activity. On the contrary, repair pathway choice occurs normally in the absence of the phosphatase.
4. Cdc14 is transiently released from the nucleolus to the nucleoplasm in response to one or multiple DSBs.
5. Cdc14 is not required for the activation and execution of DNA end resection at short or long distances from the break.
6. DNA repair system of AC1595 background supposes a powerful tool to study DNA resection and repair by SDSA through the combination of physical and sequencing approaches.
7. Genome-wide sequencing of AC1595 provides detailed information about generation of DSBs, resection, directionality of DNA synthesis, fidelity of copy, and length of DNA copied.
8. Cdc14 activity is required to inhibit DNA resection through Sgs1-Dna2 pathway once homology search and strand invasion have occurred.
9. Cdc14 dephosphorylates and inactivates the nuclease Dna2 during the DNA damage response to switch DNA resection off and prevent the generation of long ssDNA tracks that would impede the recovery of the DNA molecule.
10. Cdc14-dependent dephosphorylation of Dna2 is necessary to allow cells the accomplishment of DNA repair pathway.

YEAST STRAINS, GROWING CONDITIONS AND MEDIA

Saccharomyces cerevisiae strains

The confirmation of yeast strains generated during this work was done by polymerase chain reaction (PCR) and sequencing. PCR was performed with cell extracts obtained by heat lysis from isolated colonies. These methods are extensively described in the next section. All yeast strains used in this work are listed in Table 1.

Table1. Genotype and name of each *S. cerevisiae* strain used in this work.

Strain	Genotype	Reference
AC40	*MAT*a *leu2 ura3 his3 trp1 ade2 cdc14-1*-GFP::KanMX4	**L. Aragón**
AC224	*MAT*a-inc HOΔ *hml*::ADE1 *hmr*::ADE1 *ade1-100 leu2-3,112 lys5 trp1*::hisG *ura3-52 ade3*::GAL::HO *arg5,6*::MATa::HPH	**J. Haber**
AC243	*MAT*α HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO	**J. Haber**
AC244	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp*::hisG *HML*α *hmr*::ADE1 *ade3*::GAL-HO	**J. Haber**
AC245	*MAT*α HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *cdc14-1*-GFP::KanMX4	**This Study**
AC248	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp*::hisG *HML*α *hmr*::ADE1 *ade3*::GAL-HO *cdc14-1*-GFP::KanMX4	**This Study**
AC263	*MAT*a HOΔ *HML*α *HMR*a *leu2-3,112 his3-11,15 ade2-1 can1-100 ura3-1 trp1-1 bar1*::hisG *ade3*::GAL::HO	**J. Haber**
AC265	*MAT*a HOΔ *HML*α *HMR*a *leu2-3,112 his3-11,15 ade2-1 can1-100 ura3-1 trp1-1 bar1*::hisG *ade3*::GAL::HO *cdc14-1*-GFP::KanMX4	**This Study**
AC277	*MAT*a-inc HOΔ *hml*::ADE1 *hmr*::ADE1 *ade1-100 leu2-3,112 lys5 trp1*::hisG *ura3-52 ade3*::GAL::HO *arg5,6*::*MAT*a::HPH *cdc14-1*-GFP::KanMX4	**This Study**
AC406	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO	**J. Haber**
AC417	*MAT*α HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO DDC2-RFP::Nat CDC14-YFP::HPH CNM67-CFP::KanMX4	**This Study**
AC427	*MAT*α HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *cdc14-1*-YFP::HPH DDC2-RFP::Nat CNM67-CFP::KanMX4	**This Study**
AC527	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO SPC110-RFP::Nat CDC14-GFP::KanMX4	**This Study**

AC582	*MAT*α HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO DDC2-GFP::KanMX4 CDC14-RFP::Nat NET1-6HA::HPH	**This Study**
AC995	*MAT*α HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO CNM67-RFP::Nat CDC14-GFP::KanMX4 DDC2-CFP::TRP	**This Study**
AC1312	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO CDC14-9Myc::HPH	**This Study**
AC1314	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *cdc14-1*-9Myc::HPH	**This Study**
AC1391	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *exo1Δ*::TRP *cdc14-1*-9Myc::HPH	**This Study**
AC1415	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *exo1Δ*::TRP CDC14-9Myc::HPH	**This Study**
AC1588	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO CDC14-9Myc::HPH *sgs1Δ*::Nat	**This Study**
AC1595	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2'	**This Study**
AC1608	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *cdc14-1*-9Myc::HPH *sgs1Δ*::Nat	**This Study**
AC1640	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2' CDC14-9Myc::HPH	**This Study**
AC1642	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2' *cdc14-1*-9Myc::HPH	**This Study**
AC1915	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2' *cdc14-1*-9Myc::HPH *sgs1Δ*::Nat	**This Study**
AC1919	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2' *cdc14-1*-9Myc::HPH *exo1Δ*::URA3	**This Study**
AC1968	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya-HO-inc-Z1-Z2	**This Study**
AC1982	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2' *cdc14-1*-9Myc::HPH Dna2-AID-9Myc::Nat PADH1-TIR::URA3	**This Study**
AC2039	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2' CDC14-GFP::KanMX4 Dna2-RFP::Nat	**This Study**
AC2042	*MAT*a HOΔ *ade1-100 leu2,3-112 lys5 ura3-52 trp1*::hisG *hml*::ADE1 *hmr*::ADE1 *ade3*::GAL-HO *arg5,6*::Ya'-HO-inc-Z1-Z2' *cdc14-1*-GFP::KanMX4 Dna2-RFP::Nat	**This Study**

Construction of temperature sensitive mutants of S. cerevisiae

The temperature-sensitive mutant carrying the *cdc14-1* allele was created by transforming yeast strains with the plasmids pAC5 or pAC58 previously linearized with *Kpn*I. pAC5 contains *cdc14-1*-GFP-KanMX4 cassette which was created by PCR amplification of the *cdc14-1*-GFP-KanMX4 locus from strain AC40 and cloned into a TOPO® vector (Life Technologies). pAC58 was created by substitution of the GFP-KanMX4 by 9-Myc-HPH. Yeasts were seeded on plates containing the corresponding antibiotic (G418 or hygromycin for pAC5 and pAC58, respectively) and confirmed by PCR and sensitivity to restrictive temperature.

Construction of strain used in genome-wide sequencing experiments

The AC1595 strain used for the repair assays by genome-wide sequencing was created using the *delitto perfetto* mutagenesis system. Briefly, AC406 strain (JKM139) was first transformed with a counterselectable reporter (CORE) cassette containing two markers (KanMX4 and URA3). This cassette was integrated in the *ARG5,6* locus at chromosome V. Next, the entire cassette was replaced by the *MAT*' sequence coming from pAC99 previously digested with *Sal*I and *Kpn*I. pAC99 was created by cloning the *MAT*' sequence into the pUC57 plasmid. *MAT*' includes Ya-Z1-Z2 regions containing polymorphisms every 45-60 bp and an *HO*-inc sequence. *MAT*' was created by gene synthesis (GeneCust). The substitution of the CORE cassette by the *MAT*´ locus was attained by selecting cells on 5-Fluoro Orotic Acid (5-FOA) plates. Colonies able to grow on media containing 5-FOA were confirmed by their inability to grow on -URA3 and G418 plates. All colonies isolated were subjected to subsequent PCR analysis and sequencing to verify the presence of the *MAT*´ sequence at the *ARG5,6* locus.

Construction of Dna2-AID strain

Endogenous *DNA2* gene of AC1642 strain was tagged at C-terminal region with the aid degron-9myc::Nat cassette [219]. Oligonucleotides used for the generation of the cassette are included in Table 2. After transformation, cells were grown on YPD (yeast extract-peptone-dextrose) plates supplemented with nourseothricin sulfate (cloNAT). Colonies were confirmed by PCR (see Polymerase chain reaction and Analysis of yeast colonies by PCR sections) and then transformed with YMK728 plasmid previously digested with *Stu*I. YMK728 contains F-box transport inhibitor response 1 (TIR1) from rice *Oryza sativa* (Os-TIR1) under the ADH1 promoter [206, 207]. Cells containing Os-TIR1 were selected on synthetic minimal medium (SD) plates lacking uracil and supplemented with adenine, histidine, leucine and

tryptophan. Individual colonies were tested by lethality when exposed to 250-500 µM of indole-3-acetic acid (IAA) since Dna2 protein is essential for cell viability. Those colonies that grew in YPD but were sensitive to IAA were selected. To confirm the degradation of Dna2, cell cultures grown in YPD were incubated in the presence of 1 µM of IAA. Samples were taken at different intervals before and after IAA addition, protein extracted and subjected to Western blotting.

Tagging and deletion of endogenous genes in S. cerevisiae

Epitope tagging of endogenous genes was performed by gene targeting using PCR products as described in [220]. Deletion of endogenous genes was attained by PCR-based amplification of the resistance gene to hygromycin B (HPH) or cloNAT using pAGs plasmids (Euroscarf)[221]. URA3 and TRP1 genes ware also used to generate deletions in *ura3-52* [222] or *trp1::hisG* strains, respectively. The cassette was flanked by homologous sequences to the upstream and downstream region of the open reading frame (ORF) of the target gene. The oligonucleotides used for targeting/deletion of endogenous genes are listed in Table 2.

Table2. Name and sequence of oligonucleotides employed for gene targeting/deletion.

Name	Sequence (5' → 3')	Description
DDC2-S2	ACAAGGTTTCTATAAAGCGTTGACATTTTCCCCTTTT GATTGTTGCCTTAATCGATGAATTCGAGCTCG	To tag *DDC2* at C-terminal
DDC2-S3	ATCTAACCACACTAGAGGAGGCCGATTCATTATATA TCTCAATGGGACTGCGTACGCTGCAGGTCGAC	To tag *DDC2* at C-terminal
Cnm67-S2	TTTAGTTACAATTACAACAATTTATCTATTGACTCCG TTAATAAAAATTAATCGATGAATTCGAGCTCG	To tag *CNM67* at C-terminal
Cnm67-S3	AGAGGTGTCTGGACCATCTGTATGATCATATCCTGG AGAAGATGGTGAAGCGTACGCTGCAGGTCGAC	To tag *CNM67* at C-terminal
Cdc14-S2	TAAGTTTTTTTATTATATGATATATATATATATAAAA ATGAAATAAATTAATCGATGAATTCGAGCTCG	To tag *CDC14* at C-terminal
Cdc14-S3	CTACAAGCGCCGCCGGTGGTATAAGAAAAATAAGT GGCTCCATCAAGAAACGTACGCTGCAGGTCGAC	To tag *CDC14* at C-terminal
Spc110-S2	TAGGAGTCGATGTACATACGAGAAATATGATGATA GAGTAAGCGATACTAATCGATGAATTCGAGCTCG	To tag *SPC110* at C-terminal
Spc110-S3	TGCGAATACTAAGAGATAGAATTGAGAGTAGCAGC GGGCGTATATCTTGGCGTACGCTGCAGGTCGAC	To tag *SPC110* at C-terminal
Net1-S2	TTTTTACTAGCTTTCTGTGACGTGTATTCTACTGAGA CTTTCTGGTATCAATCGATGAATTCGAGCTCG	To tag *NET1* at C-terminal
Net1-S3	AGAAGCCAAGTGGTGGATTTGCATCATTAATAAAA GATTTCAAGAAAAAACGTACGCTGCAGGTCGAC	To tag *NET1* at C-terminal
Dna2-S2	GCTGTGATAGCTTTCCTGTTATGGAGAAGCTCTTCT TATTCCCCCTGTCAATCGATGAATTCGAGCTCG	To tag *DNA2* at C-terminal
Dna2-S3	TCGTAAGCGATAAACCTATCATAAAGGAAATTCTAC AAGAGTATGAAAGTCGTACGCTGCAGGTCGAC	To tag *DNA2* at C-terminal
Exo1 KO fwd	ACCACATTAAAATAAAAGGAGCTCGAAAAAACTGA AAGGCGTAGAAAGGACAGCTGAAGCTTCGTACGC	To delete endogenous *EXO1* gene

Exo1 KO rev	TTTTCATTTGAAAAATATACCTCCGATATGAAACGT GCAGTACTTAACTTGCATAGGCCACTAGTGGATCTG	To delete endogenous *EXO1* gene
Sgs1 KO fwd	ATTATTGTTGTATATATTTAAAAAATCATACACGTAC ACACAAGGCGGTACAGCTGAAGCTTCGTACGC	To delete endogenous *SGS1* gene
Sgs1 KO rev	TTGGCGAATGGTGTCGTAGTTATAAGTAACACTATT TATTTTTCTACTCTGCATAGGCCACTAGTGGATCTG	To delete endogenous *SGS1* gene

The generation of cassettes both for tagging and deletion of endogenous genes was attained by using the Expand Long Template PCR System (Roche). The mixture was prepared according to the manufacturer's recommendations in a final volume of 50 µl. The PCR protocol included two rounds of amplification: an initial 10 cycles program (94°C 20 sec, 58°C 30 sec, 68°C 2.5 min) followed by a 25 cycles of the amplification program (94°C 25 sec, 58°C 30 sec, 68°C 2.5 min and increasing 20 sec every cycle). A final extension of 7 min at 68°C was added.

The PCR product was integrated in the genome of the budding yeast by transformation (see *S. cerevisiae* transformation for details). Before plating transformants onto selective media, cells were grown for 4-5 hours in YPD at room temperature on a rotator to allow the expression of the transformed drug resistance markers. Cells were seeded and selected on YPD plates containing HPH (300 µg/ml), G418 (200 µg/ml) or cloNAT (100 µg/ml), or SD plates lacking uracil or tryptophan. To confirm the tagging/deletion of the target gene a standard PCR colony test was performed (see Polymerase chain reaction section). To further validate gene deletions, a second PCR was performed from genomic DNA of those positive clones coming from the colony test. Genomic DNA was obtained as describes below in the Southern blot protocol. For this amplification, 200 ng of genomic DNA were used as template in a final volume of 25 µl. The upstream/downstream primers synthetized to confirm gene tagging/deletion were designed to amplify outside the recombination sequences previously used to direct the integration of the cassette. These primers are listed in Table 3.

Table3. Name and sequence of primers used to verify the integration of targeting/deletion cassettes.

Name	Sequence (5' → 3')	Description
KanB	CTGCAGCGAGGAGCCGTAAT	Reverse primer to check cassette integration.
DDC2-C3	GCCTCTTATTACGAAGATGCC	Forward primer to check *DDC2* tagging
Dna2 -244	AAGTACAATCGGCTCTGTTCC	Forward primer to check *DNA2* tagging
Cnm67 #1	CGAAAGATTCCGTTGCTGGC	Forward primer to check *CNM67* tagging
Cdc14 -215	CATTATCCCAAAGTTTCGCGC	Forward primer to check *CDC14* tagging
Spc110 -263	CACTTCCAGACGATGATGAAC	Forward primer to check *SPC110* tagging

Net1 -224	GTGATGATGAATCTAGCTCTG	Forward primer to check *NET1* tagging
Exo1 -113	GTCCTTGCTCCTTCAGGTATA	Forward primer to check *EXO1* deletion from genomic DNA
Exo1 +109	CTGTCCTACTTTACTGGGCAT	Reverse primer to check *EXO1* deletion from genomic DNA
Sgs1 -154	CTGGGTGATCATTGGTGATAC	Forward primer to check *SGS1* deletion from genomic DNA
Sgs1 +177	TGCACACCACAATATGTCGTG	Reverse primer to check *SGS1* deletion from genomic DNA

Culture media preparation and growing conditions

For most experiments cells were grown in YEP (1% yeast extract and 2% peptone) containing 2% of raffinose as carbon source to avoid the expression/repression of the GAL-inducible promoter. When working with plasmids and auxotrophy-based selection, SD containing yeast nitrogen base (YNB) without aminoacids and complete supplement (CSM) drop-out (-ADE, -HIS, -LEU, -TRP, -URA) was used. Supplemented minimal medium (SMM) was prepared by adding adenine, histidine, leucine, tryptophan and/or uracil to SD medium according to the needs of the experiment. The final concentration of all supplements was 20 mg/L. Both SD and SMM were prepared as described in [223].

For the selection of ura- cells SD medium containing YNB and CSM was supplemented with 5-Fluoro Orotic Acid Monohydrate (Formedium) and glucose 2% to obtain plates containing a final concentration of 0.1% of 5-FOA.

All media used in this work were prepared with deionized water purified by the *Millipore* system (*Mili-Rho*). Sterilization of the media was performed in an autoclave at 121°C and 1.1 atm of pressure for 20 minutes. For the preparation of solid media, a final concentration of 2% of agar was used.

Yeast cell cultures were grown overnight in agitation at 220 rpm. When working with temperature-sensitive mutants a temperature of 25°C was used to allow cell growth. A restrictive temperature of 32°C or 33°C was used for the inactivation of the temperature-sensitive proteins. Cell growth was measure by turbidimetry through the reading of the optical density (OD) at 600 nm using the spectrophotometer Genesys 30 (Thermo Scientific) and the yeast concentration was calculated using the formula: OD_{600} of 1.0 = $2x10^7$ cells/ml. All overnight yeast cultures were diluted to OD = 0.4 before starting any experimental approach.

Homothallic switching endonuclease (HO) expression was attained by supplementing media with galactose to a final concentration of 2%. In some repair experiments, double-strand break (DSB) repair and cell recovery was promoted by repression of the HO by adding 2% of glucose to the medium. Samples were collected for Western blot, Southern blot or flow cytometry at different time points before and after the addition of galactose/glucose. For culture synchronization in G1 or G2/M, the pheromone alpha factor (Insight Biotechnologies) and nocodazole (Cayman Chemical Company) were used to a final concentration of 5 µg/ml and 15 µg/ml respectively. Cells were released from the blockage by washing them with fresh media. The storage of yeast strains was attained by dissolving cells in 16% glycerol and kept at -80°C.

The *Escherichia coli* strain used for all the molecular biology techniques performed in this work is DH5α. Bacteria were grown at 37°C in Luria Bertani (LB) (0.5% yeast extract, 1% tryptone and 1% NaCl). The selection of resistant clones of *E. coli* after plasmid transformation was attained by growing the cells in LB supplemented with ampicillin (Sigma) or kanamycin (Formedium) at a final concentration of 100 µg/ml and 50 µg/ml respectively. Maintenance of *E. coli* strains was done in 50% glycerol at -80°C.

GENETIC METHODS

S. cerevisiae transformation

Transformation of budding yeast was performed by following the lithium acetate protocol and using single-stranded nucleic acids as carrier [224]. An overnight culture grown in rich medium (YPD) was diluted to an OD_{600} = 0.25. Cell cultures were incubated at the corresponding temperature until they reached the logarithmic phase (OD_{600} = 1, equivalent to $2x10^7$cells/ml). Cells were harvest by centrifugation at 6,000 rpm during 1 minute and washed with sterile water. Cell pellet was then washed with SORB (100 mM LiOAc, 10 mM Tris-HCl pH8.0, 1 mM EDTA and 1 M Sorbitol) and resuspended in a final volume of 400 µl containing denatured salmon sperm as DNA carrier (Roche) at 1 mg/ml. For transformation, 20 µl of competent cells and 0.1-10 µg of plasmid or cassette were incubated with PEG (40% PEG3350, 100 mM LiOAc, 10 mM Tris-HCl pH8.0 and 1 mM EDTA) during 30 minutes at room temperature. After incubation, samples were subjected to heat shock at 42°C for 15 minutes and centrifuge during 1 minute at 6,000 rpm. Cells were grown in selective medium or YPD supplemented with antibiotics at 25°C or 28°C.

E.coli transformation

DH5α strain was transformed with purified plasmids or ligation products. The protocol consisted in a heat shock during 1 minute at 42°C and cell recovery for 1 hour in LB at 37°C. After incubation, cells were grown in LB plates supplemented with ampicillin or kanamycin.

MANIPULATION OF NUCLEIC ACIDS

DNA cloning

DNA digestion with restriction endonucleases, DNA electrophoresis and plasmid construction were performed as described in [225]. High quality plasmid purification from *E. coli* was performed by using the NucleoSpin Plasmid kit (MACHEREY-NAGEL), following the manufacturer's instructions. All the restriction enzymes, T4 DNA ligase and Lambda protein phosphatase used in this work were provided by *New England BioLabs*.

DNA quantification

DNA concentration was determined by measuring absorbance at 260 nm in a Nanodrop ND-1000 (Thermo Scientific) spectrophotometer. To assess the level of DNA purity the absorbance ratio 260/280 was considered (1.8-2 for pure DNA). To further confirm the quality of DNA, visualization of fluorescence after UV-light exposure was performed in agarose gels containing ethidium bromide (0.5 mg/ml) (Sigma).

Polymerase chain reaction (PCR)

PCR reactions were performed in a C1000 Touch thermal cycler (BIO-RAD) using specific conditions depending on the nature of the sample and the amplification procedure. For conventional amplifications Taq polymerase was used (GoTaq® G2 Flexi DNA polymerase, Promega). When amplifying longer fragments of DNA, the Expand Long Template PCR System (Roche) was employed. To amplify DNA with higher fidelity, KAPA HiFi DNA polymerase (KAPA Biosystems) was used. All the reactions were performed following the manufacturer's recommendations.

DNA visualization was attained by using 0.7% agarose (CONDA) gels prepared with TAE (Tris Acetate 0.04 M, EDTA 0.1 mM) and stained with ethidium bromide or Midori Green (NIPPON Genetics Europe). Genomic DNA or PCR products were observed in a Bio Rad Universal Hood

II Gel Doc (Bio Rad) UV-transilluminator. DNA purification from agarose gels was performed by using the GeneJET Gel Extraction kit (Thermo Scientific) and eluted in 50 µl of sterile water.

Analysis of yeast colonies by PCR

The correct construction of different yeast strains was confirmed by single colony PCR. Individual colonies were resuspended in 10 µl of sterile water and boiled for 10 minutes at 95°C. The reaction mix was added to a final PCR volume of 25 µl and a high amplification protocol was followed (95°C 30 sec, 55°C 30 sec, 72°C 2 min; 40 cycles). Samples were loaded into an agarose gel to confirm the presence of the expected PCR product.

DNA extraction and Southern blot

Optical density was measured as described in the previous section. For DNA extraction, 10 ml of cell culture at OD_{600} = 0.4 were harvested at 6,000 rpm at indicated time points. Cell pellet was washed with PBS 1X and stored at -80°C. Cell lysis was performed by incubating the samples with 40 units of lyticase (Alfa Aesar) in DNA preparation buffer (1% SDS, 100 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA) containing RNase (2 µl/sample) (Qiagen) and ß-mercaptoethanol (4 µl/sample) (Sigma) for 10 minutes. DNA was isolated by mixing the samples with phenol:chloroform:isoamylalcohol (25:24:1) in MaXtract™ High Density 1.5 ml tubes (Qiagen) for 10 minutes. After centrifugation, the soluble fraction was recovered; DNA was precipitated with 2 vol. of ethanol 100% and washed with ethanol 70%. Purified DNA was then resuspended in 100 µl of TE (Tris 10 mM and EDTA 1 mM) buffer and digested with the appropriate restriction enzyme.

DNA-DNA hybridization was performed following the protocol described by Southern [226]. After digestion, DNA was separated on 0.7%-1% agarose gels and subjected to Southern blotting. Gels were washed with depurination solution (0.125 M HCl) during 10 minutes. DNA denaturation was performed by using a solution containing 1 M NaCl, 0.4 M NaOH during 30 minutes, and neutralization was attained with 0.5 M Tris-HCl pH 7.4, 1.5 M NaCl for 30 minutes.

DNA was transferred into Amersham Hybond™-N^+ membrane (GE Healthcare) during 12 hours in the presence of 20X SSC (3 M NaCl, 0.3 M sodium citrate). The fixation of the DNA was performed by irradiation with UV light (120 mJ/cm^2) in a UV Stratalinker 2400 apparatus. Prehybridization was performed by incubating the membrane for 2 hours at 65°C with 5X SSC, 0.1% SDS and 5.5 mg/ml of dextran sulphate supplemented with PerfectHyb™ Plus

hybridization buffer (Sigma) at final concentration of 10% (v/v). For probe hybridization, the corresponding fluorescein-labeled probe previously denatured (30 minutes at 100°C and then 30 minutes on ice) was added. After at least 6 hours of incubation, membranes were washed to remove the excess of probe (2x15 min in 1X SSC, 0.1% SDS and 2x15 min in 0.5X SSC, 0.1% SDS).

Before detection, membranes were incubated for 1 hour at room temperature with a blocking solution containing 100 mM Tris-HCl, 150 mM NaCl, 1% milk powder. Signal amplification was achieved by incubating membranes for 1 hour at room temperature with an anti-Fluorescein antibody conjugated with alkaline phosphatase (1:125000) (Anti-Fluorescein-AP Fab fragments, Roche). After three washes with 100 mM Tris-HCl, 150 mM NaCl, 0.2% Tween®20 (Sigma) membranes were incubated for 15 minutes at room temperature with the alkaline phosphatase substrate CDP Star (GE Healthcare). Once incubated, membranes were exposed to medical X-ray films (Agfa) at different exposition times. FIJI software was used to analyse and quantify the images.

Probing was performed by labelling a PCR-amplified DNA fragment with a mix of nucleotides containing Fluorescein-12-dUTP (Fluorescein-High Prime, Roche). All probes were purified by centrifugation using the illustra™ MicroSpin™ G-50 columns (GE Healthcare). The oligonucleotides used to generate all probes employed in this work are listed in Table 4.

Table4. Name and sequence of oligonucleotides used to generate the probes for Southern blot experiments.

Name	Sequence (5' → 3')	Description
Mata distal 1	CATGCGGTTCACATGACTTTTGAC	Forward primer to create *MAT*a distal probe
Mata distal 2	AGGATGCCCTTGTTTTGTTTACTG	Reverse primer to create *MAT*a distal probe
Mata only 1	TTTGTTCTTTCGGGGAAACTG	Forward primer to create *MAT*-specific probe
Mata only 2	GTACAAACACATCTTCCCAATA	Reverse primer to create *MAT*-specific probe
-5kb HO Fwd	ACACCCAACAAAACACCTGTG	Forward primer to create 3kb probe
-3.5kb HO Rev	CCTTTTGCTTCTTGTACGCTC	Reverse primer to create 3kb probe
-7.5kb HO Fwd	TGGCCAGAACAATCATGAAGC	Forward primer to create 6kb probe
-6.25kb HO Rev	TCTTGCTATGGGTGGTATAGC	Reverse primer to create 6kb probe
14kb HO Fwd	TTATGTTGCCAACGGGAGTTC	Forward primer to create 10kb probe

-12.5kb HO Rev	TCTTCAGGACTTCTTAAGCCG	Reverse primer to create 10kb probe
21kb HO Fwd	TATCTGGGTATATTACCCGGC	Forward primer to create 21kb probe
21kb HO Rev	TTGTATCCATCGTTTCGGCTG	Reverse primer to create 21kb probe
27kb probe Fwd	GGGGACCATACCTACAATGTA	Forward primer to create 27kb probe
27kb probe Rev	CATAATTTATGGAGGTGGCGC	Reverse primer to create 27kb probe
-1.5kb HO Fwd	GTCTGTGATGTTGGAGATATG	Forward primer to create left probe
HO -1kb Rev	GGATTCTTATCTAGGGCCAAC	Reverse primer to create left probe
+1.5kb HO Fwd	CTTTCCCTGGAAATATCTCCG	Forward primer to create right probe
+2.5kb HO Rev	ACGAATGGGTATATGGGTCTG	Reverse primer to create right probe
Act1 probe 1	CGAACAAGAAATGCAAACCGC	Reverse primer to create probe against actin gene
Act1 probe 2	CTTGTGGTGAACGATAGATGG	Forward primer to create probe against actin gene
His3 probe 1	GAGCAGAAAGCCCTAGTAAAG	Forward primer to create probe against his3 gene
His3 probe 2	TAAGAACACCTTTGGTGGAGG	Reverse primer to create probe against his3 gene

PROTEIN EXTRACTION AND WESTERN BLOT

For protein extraction, 5 ml of culture at OD_{600} = 0.5 were collected for each time point. Cells were harvested by centrifugation for 1 minute at 6,000 rpm. After washing with 1 ml of 20% trichloroacetic acid (TCA) (Sigma) cell pellets were resuspended in 100 µl of 20% TCA and frozen. Cell breakage was performed with glass beads in a FastPrep machine (MPBio) (3x20 seconds cycles at power setting 5.5). Protein precipitation was attained by centrifugation at 5,000 rpm for 5 minutes at 4ºC. Upon precipitation, cell pellet was resuspended in 70 µl of 1 M Tris-HCl pH 8 and 70 µl of 2X SDS-PAGE loading buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.2% bromophenol blue). After boiling samples for 10 minutes at 95°C, insoluble material was separated by centrifugation and 5-12 µl of supernatant were loaded into 6%-8% acrylamide gels. When separating Net1 and Dna2 phospho-bands, 10 µM of Phos-Tag and 20 µM of $MnCl_2$ were used. When working with Phos-Tag gels, 5 µl of protein extract were loaded. The electrophoresis system used was a mini-PROTEAN® Tetra (Bio Rad) and all the manufacturer's recommendations were followed. The electrophoresis of the samples were performed in buffer containing Tris-Glycine (25 mM Tris, 192 mM glycine, 0.1% SDS)

Proteins were transferred into a PVDF membrane (Hybond-P, GE Healthcare) using a mini-Trans-Blot® system (Bio Rad) for 75 minutes at constant 280 mA in 1X transfer buffer (25 mM Tris, 192 mM glycine). Membranes were blocked with 5% milk in PBS-0.1% Tween®20. Phos-Tag gels were washed for 10 minutes with 1X transfer buffer containing 20% HPLC gradient grade methanol (Fisher Scientific) supplemented with 1 mM EDTA. A second wash with 20% methanol diluted into transfer buffer was performed before transferred. Proteins were transferred into PVDF membranes for 75 minutes at constant 400mA. Primary anti-Rad53 (Santa Cruz) antibody was used at a 1:2,000 dilution and the secondary anti-goat antibody was used at a concentration of 1:5,000 (Santa Cruz). Primary anti-HA (12CA5, Roche) and Anti-Myc (C3956, Sigma) antibodies were used at a 1:2,500 dilution. Secondary anti-mouse (GE Healthcare) and anti-rabbit (GE Healthcare) were used at a concentration of 1:25,000 and 1:5,000, respectively. After incubation with the primary antibody, membranes were washed 1x15 minutes and 2x5 minutes with PBS-0.1% Tween®20 at room temperature. After incubation with the secondary antibody, membranes were washed 1x15 minutes and 2x5 minutes with PBS-0.1% Tween®20, and 2x10 minutes with PBS at room temperature. Detection of antigen-antibody complexes was attained by incubating membranes with SuperSignal® West Femto (Thermo Scientific) or Pierce™ ECL Plus Western Blotting substrate (Thermo Scientific) for 5 minutes at room temperature and exposed to medical X-ray films.

MICROSCOPY

Live-cell fluorescence imaging of GFP, yellow fluorescent protein (YFP), cyan fluoresce protein (CFP), red fluoresce protein (RFP) and 4´-6-diamidino-2phenylindole (DAPI) staining was performed in a Deltavision microscope (PersonalDV, Imsol) equipped with a Cool Snap HQ CCD camera (Photometrics). For the visualization of living cells, 1ml of cell culture was centrifuged at 6,000 rpm for 1 minute. Cell pellet was washed with PBS and resuspended in 30-50 µl of PBS. Microscope slides containing 4 µl of cell suspension were employed to take images at different time points. Images were taken in 9 z-planes, with an interval of 0.4 µm between each plane. The total thickness of the sample was 3.6 µm. Differential interference contrast (DIC) images were used as reference. Images were analysed by using the SoftWoRx Suite visualization and analysis software. For image processing and analysis ImageJ and FIJI software were used.

MASS SPECTROMETRY

Protein extracts from 10^7 cells were prepared by extracting with urea buffer. Cells were collected by centrifugation and washed in 8 M urea buffer (8 M Urea, 0.1 N NaOH, 0.05 M EDTA, 2% 2-mercaptoethanol). Two volumes of glass beads and urea buffer were added to the pellet prior to breaking the cells in a FastPrep machine (MPBio) (3x20 seconds cycles at power setting 5.5). Solutions were made up to 0.5 ml with fresh urea buffer and centrifuged at 12,000 rpm for 10 minutes at 4ºC. Supernatant was transferred to a fresh tube, SDS-PAGE loading buffer was added, and boiled for 10 minutes. Proteins were separated in a 8% acrylamide gel. Gel lanes were excised into 6 equal regions and destained with 50 mM ammonium bicarbonate in 10% acetonitrile. Protein disulfite bonds were reduced using 10 mM dithiothreitol (incubated at 56°C for 30 minutes) and then alkylated with 25 mM iodoacetamide (incubated at room temperature in the dark for 20 minutes). 500 ng of Promega Gold trypsin was added to each sample and incubated overnight at 37°C. Peptides were extracted using 10% acetonitrile containing 5% formic acid and the solution was dried in a speedvac centrifuge. Dried peptide samples were solubilised in 1% acetonitrile, 0.1% trifluoroacetic acid (TFA) and desalted using Oasis HLB (Waters Corporation). Desalted peptides were eluted using 1 M glycolic acid in 80% acetonitrile and 5% TFA.

Phosphoproteomic analysis was performed essentially as described before [227]. Briefly, phosphopeptides were enriched using TiO_2 chromatography and dried up. Phosphopeptides were solubilised in 10 μl of 0.1% TFA prior loading on to an Ultimate 3000 nanoflow HPLC. Peptides were on a 90 minutes gradient, 4-45% mobile phase B (80% acetonitrile, 0.1% formic acid) and electrosprayed directly into an LTQ XL Orbitrap mass spectrometer. The mass spectrometer was operated in positive ionisation mode and in data-dependent acquisition (DDA) mode. The top 7 most abundant ions were subjected to multistage activation (MSA) collision-induced dissociation fragmentation.

Mascot was used for peptide identification from the MS/MS data by searching against the Swissprot *S. cerevisiae* database. Fixed modification of serine, threonine and tyrosine were used in Mascot Daemon. MS1 tolerance of 10 ppm and MS2 fragmentation tolerance of 600 mmu were used. A decoy database was also searched so as to report results with a false discovery rate of 1%.

Pascal software was used for quantification of the phosphopeptides using the peak heights of the extracted ion chromatograms. Replicates were averaged and statistical significance of

differences across conditions assessed by unpaired t-test of log transformed data. P-values were adjusted using the Benjamini-Hochberg FDR method.

FLOW CYTOMETRY

Cells were collected at each time point, fixed in 70% ethanol and stored at 4ºC. Previously fixed cells were harvest by centrifugation at 6,000 rpm for 1 minute and incubated overnight or 2 hours at 37°C with 1X SSC containing 0.3 mg/ml RNase A. After incubation, proteinase K (Sigma) was added to a final concentration of 0.05 mg/ml and samples were incubated for 1 hour at 50°C. Sonication of samples was performed in a Bioruptor®Plus sonication device (Diagenode) and immediately after, DNA was stained with 2 µg/ml propidium iodide during 60 minutes. Analysis was carried out using a FACSCalibur™ machine (BD Biosciences). Samples were analysed using the CellQuest Pro software. Each histogram corresponds to 10,000 events/cells.

SENSITIVITY ASSAY ON PLATES

Sensitivity assays were performed in drops by making serial dilutions from cells growing in YPD plates. To this end, 100 µl of cells were adjusted to an OD_{600} = 1 ($2x10^7$ cells/ml) and five tenfold serial dilutions were prepared in a 96-well plate using sterile water.

Replicates were attained by using a replica plater for 96-well plate (Sigma) and 5 µl of each dilution were seeded on YPD plates containing the indicated genotoxic compound. Cells were incubated at the corresponding temperature for 48-72 hours.

SEQUENCING

Confirmation of strains used in this work

DNA sequencing was carried out at GATC Biotech. Sample processing was performed following the company's instructions and recommendations. For purified plasmids/PCR products, 80 and 50 ng/µl of sample was used, respectively. In both cases, a total reaction of 10 µl was employed including 5 µl of the primer (5 µM).

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Publications derived from this work

1- **Ramos F**, Villoria MT, Alonso-Rodríguez E, and Clemente-Blanco A. Role of protein phosphatases PP1, PP2A, PP4 and Cdc14 in the DNA damage response. Cell Stress. 2019 Mar 3(3):70-85.

2- Villoria MT*, **Ramos F***, Dueñas E, Faull P, Rodríguez-Cutillas P and Clemente-Blanco A**.** Stabilization of the metaphase spindle by Cdc14 is required for recombinational DNA repair.* **These authors contributed equally to the work**. The EMBO Journal. 2017 Jan 4;36(1):79-101

3- **Ramos F**, Leonard J, Clemente-Blanco A and Aragón L. Cdc14 and chromosome condensation: Evaluation of the recruitment of condensin to genomic regions. Methods in Molecular Biology. 2017;1505:229-243.