Investigating the role of DDK in replication associated recombination

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Dbf4-dependent kinase (DDK) is an essential Serine/Threonine protein kinase well-known for its function in DNA replication initiation. DDK also functions in various other cellular processes including DNA repair mechanisms, such as mutagenesis. However, DDK roles in DNA damage tolerance remain elusive.

Using S. cerevisiae as model system, here we investigated DDK roles in replication associated recombination, a process known to be induced by DNA damage and important for error-free replication. Using different mutant alleles of DDK and conditions that do not affect its function in origin firing, we found that DDK dysfunction reduces the levels of recombination intermediates forming proximal to replication forks. The effect of DDK mutations in recombination was overlapping with the window of PCNA polyubiquitylation and largely epistatic with mms2 or rad18 mutations, affecting (poly)ubiquitylation of the replication processivity factor PCNA and error-free template switching. Moreover, the effect of DDK manifested also in mutant backgrounds in which a salvage pathway of recombination independent of PCNA poly-ubiquitylation operates. DDK mutants not only affect the sister chromatid junctions but also the inter-homolog junctions that rely on recombination events to the homologous chromosomes. Notably, we found that certain DDK mutants were specifically contributing to template switching, and not to the salvage pathway, and that the recombination function of DDK is separable from its roles in origin firing.

By ChIP quantitative analysis, we uncovered that the recombination defect of DDK mutants correlates with decreased local recruitment of Rad51 to the damaged forks, potentially caused by stabilization of the Srs2 anti-recombinase. Importantly, we also found that two different ddk mutants with shared defects in template switching but differential effects on the salvage pathway and checkpoint activation accumulate long single-stranded DNA (ssDNA) gaps at the replication fork junction and broken forks. The recombination defect of ddk mutants was not rescued by downregulation of the Mus81-Mms4 nuclease, differently from what observed in checkpoint mutants, nor by artificial local restoration of cohesion. Taken together, our results uncover roles for DDK in supporting replication-associated recombination by regulating Rad51 availability at sites of perturbed replication.
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Thank you!
LIST OF ABBREVIATIONS

DDR                    DNA Damage Response
DDT                    DNA Damage Tolerance
ROS                    Reactive oxygen species
GCRs                   Gross chromosomal rearrangements
ssDNA                  single stranded DNA
dsDNA                  double stranded DNA
DSB                    Double stranded break
HR                     Homologous Recombination
NHEJ                   Non Homologous End Joining
PRR                    Post replication repair
SCJ                     Sister Chromatid Junction
IHJ                  Inter homologous Junction
dHJ                     Double Holliday Junction
SSDA                   Synthesis dependent strand annealing
SSA                    Single Strand Annealing
STR                     Sgs1-Top3-Rmi1
SUMO                   Small Ubiquitin-like Modifier
STUbl                   SUMO targeted Ubiquitin ligases
PCNA                   Proliferating Cell Nuclear Antigen
RPA                    Replication Protein A
HMGB                   High Mobility Group Box
CDK                     Cell cycle dependent kinase
DDK                     Dbf4 dependent kinase
TLS                     Trans-lesion synthesis
TS                      Template Switching
2D                     Two-dimensional agarose gel electrophoresis
RT                     Room temperature
ARS                   Autonomous Replicating Sequence
NPC                    Nuclear Pore complex
APC/C                  Anaphase Promoting Complex
ChIP                  Chromatin Immunoprecipitation
BrdU                   5-bromo-2’- deoxyuridine
MMS                  Methyl methanesulfonate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CPT</td>
<td>Camptothecin</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxy nucleoside triphosphate</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium Bromide</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>TCA</td>
<td>Tri chloroacetic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>WT/wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast Peptone Dextrose</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
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1. INTRODUCTION

Eukaryotic cells are highly susceptible to different kinds of lesions that threaten the molecular structure of the DNA. It has been estimated that an average estimate of 70,000 lesions (DNA damages) are generated per cell in humans each day (Lindahl & Barnes, 2000). These lesions can occur through either exogenous or endogenous sources. Exogenous sources are different environmental DNA damaging agents that include ultraviolet radiation (creating pyrimidine dimers), ionizing radiations and various base-damaging chemicals, such as the ones used in cancer chemotherapy. On the other hand, endogenous DNA damage is caused by the generation of cellular byproducts such as reactive oxygen species (ROS). This often leads to various metabolic processes like oxidation, de-purination and de-pyrimidation in the DNA (Cadet, 2003). Moreover, DNA replication within the cell can also challenge the genomic integrity by introducing mismatches and topological constraints that can ultimately lead to DNA breaks and genome rearrangements (Branzei & Foiani, 2005). Ultimately, unrepaired lesions of DNA after subsequent rounds of replication, get converted into mutations that can adversely affect genomic stability. The genetic mutations created by these DNA lesions can lead to developmental disorders or cancer predisposition, but they can also promote long-term adaptations to environmental changes (Cadet, 2003). Eukaryotic cells have evolved several repair pathways that can recognize various lesions and use a series of specialized enzymes to mitigate the deleterious effects of DNA lesions on replication and genome stability. The choice of the repair pathway to be used is mainly determined by the type of DNA damage and the cell-cycle stage (Branzei & Foiani, 2008). Although cells are equipped with robust repair pathways for faithful restoration of the original sequence and structure of its DNA, at times they require distinct mechanisms to temporarily tolerate these damages before a complete repair can take place. When cells encounter DNA damage during replication, DNA damage response (DDR) and DNA damage tolerance (DDT) pathways become activated. These pathways ensure bypass of DNA damage and slowing down of replication. Dysfunctions in these pathways have shown to cause several human disorders like microcephaly and ataxia, and predispose to cancer (O’Driscoll & Jeggo, 2008).

1.1 DNA damage tolerance (DDT) pathways

Eukaryotic replication often encounters obstacles, such as damaged nucleotides and DNA adducts that can cause stalling of the replication fork. These events, if not properly
Dealt with, can lead to replisome disassembly and genome rearrangements (GCRs) which ultimately result in genome instability (Branzei & Foiani, 2010). In order to handle such situations, eukaryotic cells have evolved various mechanisms that allow a smooth completion of replication and rescheduling of DNA repair to later stages of the cell cycle. These bypass mechanisms are collectively referred to as DNA damage tolerance (DDT) pathways. DDT comprises 2 modes, Error-prone DDT and Error-free DDT pathways. These two pathways are highly conserved among different species of eukaryotes (Vanoli et al., 2010). The error-prone DDT pathway often referred to as the Trans-lesion synthesis (TLS) pathway is mediated by the use of specialized trans-lesion polymerases which are different from the normal replicative polymerases and are generally used to replicate across templates containing bulky lesions. This pathway is known to generate several mutations as it incorporates wrong nucleotides and is considered to be mutagenic (Waters et al., 2009). The error-free pathway involves a recombination mechanism where a homologous template, usually the sister chromatid is used as template for synthesizing over the damaged strand (Branzei, 2011; Giannattasio et al., 2014; Vanoli et al., 2010). Since this pathway involves a switch of templates through a recombination mediated mechanism (Higgins et al., 1976; Rupp et al., 1971), it is also called Template Switching (TS) (Fig. 1.1)
Figure 1.1. Overview of DNA damage tolerance pathways

Upon replication fork stalling during DNA damaging conditions, cells can activate certain DNA damage tolerance mechanisms which allows the bypass of replication blocking lesions. This bypass can occur either through replicating over the damaged DNA (Translesion synthesis, Left) that is often error-prone in outcome or through a recombination mechanism which involves the usage of undamaged sister chromatid as template (Template Switching, Right) which is generally error-free in outcome.

Several conserved proteins throughout the domain Eukaryota have been shown to exert roles in regulating DDT. It was revealed that a major fraction of DDT depends on the Rad6-Rad18 epistasis group of proteins that are involved in the so-called Post-replication repair (PRR) pathway. Another important factor that determines the choice of DDT pathway is the homotrimeric DNA polymerase clamp, PCNA (Proliferating cell nuclear antigen), which can undergo post-translational modifications by ubiquitylation and SUMOylation. Mono-ubiquitylation of PCNA accounts for error-prone DDT pathway while, PCNA polyubiquitylation mediates Template switching (Branzei et al., 2008; Hoege et al., 2002). Homologous recombination (HR) that involves the exchange between homologous sequences, is often studied in the context of DNA double-stranded break (DSB) repair. For this reason, the relationship of HR with recombination mediated template switching is still unclear. However, in recent years, interaction between several recombination factors and various players of PCNA polyubiquitylation pathway has also been revealed (Branzei et al., 2008).

Different models have been proposed on how the DDT pathway acts on DNA lesions. In one scenario, it is speculated that DDT factors work at the replication fork to bypass the lesion and allow continuous progression of stalled replication forks. While another speculation is that the re-priming is activated at the stalled/uncoupled replication fork, allowing the lesion to be confined in ssDNA gaps behind the moving replication fork. This latter model is known as the ‘gap-filling’ model (Fig.1. 2). Nevertheless, it has always remained controversial whether DDT events are occurring at the fork (that is, at the site of DNA damage) or behind the fork on the single stranded DNA (ssDNA) gaps containing lesions. Perhaps both scenarios happen depending on the lesion and chromatin context.
Figure 1.2. Proposed models for DNA lesion bypass during replication
A) Represents the model where DDT factors is speculated to act at the replication fork to bypass the lesion either through the direct action of TLS to replicate over the lesion or through replication fork reversal (four-way junctions).
B) Indicates the ‘gap-filling model’ where re-priming is activated leaving ssDNA gaps. This gap will be later filled either through TLS pathway (Error-prone) or through Template switching pathway (Error-free).

1.2 Regulation of DDT by PCNA
Error-free DDT is mediated largely by two genetic pathways: Rad51- and Rad52-mediated homologous recombination pathway as well as the so-called Post-replication repair (PRR) pathway (Branzei, 2011). The PRR pathway depends on ubiquitin conjugating and ligating enzymes, Rad6 and Rad18, respectively that are well conserved throughout the domain Eukaryota.

As previously mentioned, several regulators have been identified till date that can modulate the choice of DNA damage tolerance pathways. One such regulator of DDT pathway choice in eukaryotic cells is the Proliferating cell nuclear antigen (PCNA). PCNA is a DNA clamp known to act as a DNA polymerase processivity factor in eukaryotes. PCNA is known to be ubiquitylated as well as SUMOylated. During DNA damaging conditions, mono-ubiquitylation of PCNA happens at a conserved residue (K164), mediated by Rad18 and Rad6. This modification recruits trans-lesion
polymerase to the site of DNA damage (Kannouche et al., 2004). Upon recruitment of another E3 ubiquitin ligase, in budding yeast, Rad5, which acts in concert with the E2 heterodimer, Mms2-Ubc13, a K63-linked polyubiquitin chain can be attached to this already bound mono-ubiquitin at the K164 residue in creating poly-ubiquitylated PCNA. While PCNA mono-ubiquitylation promotes error-prone DDT and facilitate TLS, poly-ubiquitylation of PCNA mediate recombination associated template switching (Branzei et al., 2004).

Apart from ubiquitylation, PCNA is also shown to be modified by SUMOylation at K164 residue and also at K127 residue to a lesser extent by Siz1 (E3 enzyme) and Ubc9 (E2 SUMO ligase) enzymes, respectively (Hoege et al., 2002). Previous studies have shown that SUMOylated PCNA recruits the anti-recombinase Srs2 to the damaged DNA. The anti-recombinase Srs2 is a helicase that disrupts Rad51 protein filaments (Pfander et al., 2005). This is shown to favor the Rad5/Mms2/Ubc13 mediated TS recombination pathway, while preventing another canonical recombination pathway (known as ‘salvage pathway’ that will be explained below) that functions at later stages of DNA replication. However, Esc2, a SUMO-like domain containing factor facilitates local assembly of Rad51 filaments to the damaged sites by binding to SUMOylated PCNA readers Elg1 and Srs2, and thereby downregulating and facilitating the turnover of Srs2 anti-recombinase (Urulangodi et al., 2015)(Fig. 1.3).
Figure 1.3. Schematic representation of PCNA regulation by Ubiquitin and SUMO modifications

PCNA is the homo-trimeric DNA polymerase clamp that can undergo ubiquitin and SUMO modifications. PCNA undergoes mono-ubiquitylation which is mediated by Rad6 and Rad18 that favors TLS pathway. Mono-ubiquitylated PCNA can be extended to a polyubiquitylation chain with the help of Rad5 together with Ubc13 and Mms2 which facilitates Template Switching pathway. Apart from Ubiquitylation, PCNA can also undergo SUMOylation which is mediated by Ubc9 and Siz1 that can recruit the anti-recombinase enzyme Srs2 to the damaged forks. PCNA SUMOylation is shown to favor Template switching pathway while preventing another recombination pathway called ‘salvage pathway’.

1.3 Trans-lesion synthesis (TLS) mediated DDT

In order to carry out accurate and faithful chromosomal replication and to minimize the accumulation of mutations, replicative DNA polymerases are endowed with high fidelity and processivity. However, eukaryotic cells also possess another set of replicative polymerases which are referred to as trans-lesion polymerases (TLS) that can by-pass lesions encountered during DNA replication by the incorporation of nucleotides opposite to the lesions. However, unlike the usual replicative polymerases endowed with proofreading ability, the action of TLS polymerases can promote generation of mutations. In *Saccharomyces cerevisiae*, the error-prone DNA damage tolerance pathway is dependent on a set of specialized polymerases called TLS that can bypass the lesions, by filling the gaps while incorporating wrong nucleotides. This pathway often leads to reduced fidelity of DNA replication and thereby the introduction of mutations (S. Prakash et al., 2005; Waters et al., 2009). In budding yeast, two specialized trans-lesion polymerases employed in this mutagenic pathway are Pol ζ and Rev1 enzymes (Lawrence & Christensen, 1982; Lemontt, 1971). Pol ζ is composed of 2 main subunits: Catalytic subunit Rev3 and a regulatory subunit Rev7 (Nelson et al., 1996). Unlike replicative polymerases, Pol ζ lacks proofreading activity and possess poor processivity which give rise to spontaneous and induced mutagenesis. In addition to Pol ζ and Rev1, budding yeast carries Pol η encoded by the *RAD30* gene. Unlike the other two trans-lesion polymerases, this enzyme is considered to be error-free in nature when it operates on lesions induced by UV damage, such as CC and TC photoproducts (Johnson et al., 1999).
1.4 Homologous Recombination

Double stranded breaks (DSBs) are considered to be one of the most deleterious forms of DNA damage. For repairing DSBs, eukaryotic cells have evolved a repair machinery that consists of two major pathways: non-homologous end joining (NHEJ) and homologous recombination (HR). Activation of the NHEJ pathway can lead to the rejoining of both ends of a single DSB without any requirement of sequence homology. This pathway is shown to be active all-throughout the cell cycle with its highest activity occurring in the G1 phase. On the other hand, homologous recombination requires an intact homologous template to direct the repair and is active only in the S/G2 phase (Kowalczykowski, 2015; J. Li et al., 2019). Recombination-dependent repair refers to the exchange or transfer of information between 2 molecules. The “homologous” part refers to the usage of a sister chromatid or any other homologous region or chromosome that has similar sequence homology to the template chromosome, to ensure the accuracy of repair. Over the years, homologous recombination-based DNA damage repair has been extensively studied in different contexts in different eukaryotes ranging from yeast to humans. HR is important for telomere maintenance and is also required in meiosis where it plays several roles (Eckert-Boulet & Lisby, 2010; L. Prakash, 1981). In the model organism *Saccharomyces cerevisiae* (budding yeast), HR is the main DSB repair pathway, while in higher eukaryotes, NHEJ is equally, if not more frequently employed than HR, to repair DSBs. As in this thesis we are interested in studying and discussing about homologous recombination and its regulation in budding yeast, we will not be going into details into the NHEJ pathway.

In budding yeast, recombination-dependent DSB repair is mediated by a highly conserved group of proteins that comes under the *RAD52* epistasis group and its accessory components. During DNA damage, a single stranded DNA (ssDNA) gap can act as trigger for HR-mediated repair. This ssDNA is generated by a process called DSB end resection, which is mediated by an evolutionary conserved protein complex, Mre11-Rad50-Xrs2 (MRX) in yeast or Mre11-Rad50-Nbs1 (MRN) in mammals. The complex acts to process 5’ strands to generate ssDNA that will be further recognized by other long range resection machinery harboring Exo1(5’-3’ exonuclease) or a RecQ helicase (Sgs1 in budding yeast) along with the 5’ flap endonuclease and helicase Dna2 (Cejka et al., 2010; Mimitou & Symington, 2008). Cell cycle dependent kinases (Cdks) play important roles in promoting resection in the S phase as well as G2/M phases. They do so by phosphorylating various proteins like Exo1, Sae2 and Dna2 (X. Chen et al., 2011; Huertas et al., 2008; Tomimatsu et al., 2014). Exposure of ssDNA attracts the ssDNA-
binding protein, RPA (Replication protein A). RPA protects ssDNA from nuclease attack, assists to prevent the formation of secondary DNA structures and activates checkpoint signaling to arrest the cells in G2/M phase. RPA is then substituted by a RecA homologue, Rad51 recombinase to form a nucleoprotein filament. This action is mediated by the Rad52 homo-multimer which displaces RPA in favor of the binding of Rad51 (Sugiyama & Kowalczykowski, 2002; Zhao et al., 2017). Invasion of the Rad51 filament into the homologous duplex DNA initiates the search for a homologous sequence. Once homology is found, the strand invasion reaction proceeds to form a displacement loop (D-loop) structure (Patrick Sung & Robberson, 1995)(Fig. 1.4).

Strand invasion requires the participation of other factors in addition to Rad51, including Rad54, Rad55 and Rad57. Rad54, a member of the Rad52 epistasis group and a Swi2/Snf2 like factor, acts to overcome negative supercoils and is required for D-loop extension (Petukhova et al., 1998; Van Komen et al., 2002). In addition, Rad51 paralogs Rad55 and Rad57 function to stimulate DNA strand exchange by the Rad51 recombinase (P Sung, 1997).

DSB repair is initiated by the invasion of one end to the donor sequence and the 3’ end of the resulting invading strand is extended via DNA synthesis. Further ligation creates a stable double Holliday junction (dHJ), which is later resolved by the generation of crossover and non-crossover products (Szostak et al., 1983). There is also another model, synthesis-dependent strand annealing (SDSA) in which the invading strand is dismantled from the D-loop by a helicase to re-anneal to the other end of the original template. SDSA leads to gene conversions (GC) and generates exclusive non-crossover products. In addition, the presence of direct or inverted repeats at the ssDNA gap can result in the direct annealing of micro-homology segments or repeat elements that can lead to the repair of DNA lesion even in the absence of the Rad51 protein. This process is termed Single Strand Annealing (SSA) and is dependent on RPA, Rad52 and Rad59 proteins (Fishman-Lobell et al., 1992; Ivanov et al., 1996; Sugawara et al., 2000). This process is also considered to be largely error-prone and can lead to deletions or duplications.

Finally, to ensure proper chromosome segregation in mitosis, dHJ intermediates have to be resolved. Several mechanisms have been shown to process these structures in eukaryotes. Dissolution of this structures is initiated by the action of the Sgs1-Top3-Rmi1 (STR) complex, which generates non-crossover products. Incomplete dissolution or those intermediates that escape the action of STR complex are resolved by the structure-specific endonucleases Mus81-Mms4 and Yen1, activated in mitosis, which create both crossover and non-crossover products. The choice of which resolvase should function is
regulated in a cell-cycle specific manner to coordinate proper DNA transactions and chromosome segregation (J. Li et al., 2019).

Figure 1.4. Schematic representation of homologous recombination dependent double-stranded break repair (Ferretti et al., 2013)

Induction of a double stranded break leads to the rapid localization of MRX/N complex to the damaged site. Short range resection is carried out by MRX/N complex along with Sae2/CtIP to generate ssDNA which is coated by RPA. This processed double stranded breaks are no longer available for Ku (Ku70-Ku80) complex to bind thereby preventing repair by non-homologous end joining. Next, long range resection is then catalyzed by Sgs1 or Exo1 in conjunction with Dna2 endonuclease. Subsequently, RPA is replaced by Rad52 dependent Rad51. After a successful search for a homologous template, strand invasion occurs which is mediated by factors like Rad54 to form a nascent D-loop structure. It has to be noted that both short-range and long-range resection factors are potentially regulated by CDK phosphorylation.

1.5 Recombination mediated Template switching

In addition to the TLS pathway, cells can also employ template switching (TS) to deal with DNA lesions by filling DNA gaps with the help of a homologous DNA template, usually the new sister chromatid. Unlike trans-lesion synthesis, this pathway is
considered to be error-free as ideally it does not generate mutations. When damage is encountered during DNA replication, ssDNA is exposed in the vicinity of the DNA damage. The ssDNA is coated by RPA (Replication protein A), which prevents the DNA breakage and the formation of secondary structures. Rad52, which has the ability to bind to both Rad51 and RPA, removes RPA from the strand and exchanges it with Rad51 nucleoprotein to catalyze strand invasion (Vanoli et al., 2010). Rad52 and Rad55/Rad57 heterodimer facilitate strand invasion along with Rad51 (P Sung, 1997) and TS (Vanoli et al., 2010). On the other hand, RPA recruits the Rad18 ubiquitin ligase on to the ssDNA (Davies et al., 2008), thereby facilitating DDT and TS (Branzei et al., 2008). Previous reports in budding yeast have also revealed the role for a chromatin-remodeling complex INO80 in DDT in regulating both Rad51-mediated recombination as well as Rad18-mediated PCNA polyubiquitylation (Falbo et al., 2009). From our lab, studies have reported a role for the high mobility group box (HMGB) protein, Hmo1, in channeling lesion towards TS via its DNA bending activity, while preventing mutagenesis and toxic recombination (Gonzalez-Huici et al., 2014). The lab has also uncovered a role for sister-chromatid cohesion in facilitating error-free DDT by TS where both cohesin and replication-fork associated cohesion factors support this process, albeit via different mechanisms (Fumasoni et al., 2015).

In the post-replicative model of template switching, the ssDNA containing the lesion, is thought to anneal to the homologous duplex forming a three stranded DNA structure. Later, the newly synthesized strand from the duplex DNA is used as a template for gap filling. This leads to the formation of a D-loop structure that is further extended by the replicative polymerase Pol δ into pseudo-double holliday junction like intermediates (Giannattasio et al., 2014; Vanoli et al., 2010). Dissolution/Resolution of these cruciform structures are primarily carried out by the Sgs1-Top3-Rmi1 complex that convert them to hemicatenanes. Later, the full resolution takes place by the action of Top3 topoisomerase (Branzei & Szakal, 2016). In case of DDT recombination structures persisting till mitosis, cells activate an alternate resolution pathway mediated by the Mus81/Mms4 endonuclease complex whose action is restricted to G2/M phase when activation occurs via DDK/Cdk1/Cdc5-dependent Mms4 phosphorylation. The mitotic kinases CDK and Cdc5 facilitate resolution of persistent TS intermediates, using Mus81/Mms4 nuclease, but this action can lead to formation of crossover products (Gallo-Fernández et al., 2012; Matos et al., 2011; Szakal & Branzei, 2013).
1.6 Recombination mediated salvage pathway

In addition to the Rad5-mediated pathway of template switching, another recombination-dependent pathway was described. This latter pathway, also known as the salvage pathway, is active in backgrounds defective in PCNA SUMOylation. The salvage pathway is counteracted by the Srs2 helicase and SUMOylated PCNA (García-Rodríguez et al., 2016; Urulangodi et al., 2016). This pathway relies on the action of HR proteins, but is independent of the action of the Rad5/Mms2/Ubc13 pathway of PCNA polyubiquitylation (Branzei et al., 2008; Karras et al., 2013). The salvage pathway preferentially occurs in late S and G2/M, when the window of PCNA SUMOylation and polyubiquitylation begins to curb down. However, the choice and interplay between these recombination pathways remains a matter of further investigation (Branzei et al., 2008; Karras et al., 2013). It has been shown that one of the crucial functions of PCNA SUMOylation in error-free template switching may involve the ability of the Srs2 helicase to disrupt Rad51 filaments (García-Rodríguez et al., 2016; Krejci et al., 2003; Papouli et al., 2005; Pfander et al., 2005). When PCNA SUMOylation is disrupted, the canonical TS pathway is inhibited while a salvage pathway of recombination (which preferentially occurs in G2/M) takes over to promote damage bypass via the formation of sister chromatid junctions (SCJs) (Branzei et al., 2008)(Fig. 1.5).
Figure 1.5 Model of Recombination mediated Template Switching (TS) and Salvage pathway (Bi, 2015)

Upon replication fork stalling during a DNA damage, activation of DNA damage tolerance pathways occurs. Once of the central regulator of DDT pathway choice, PCNA can undergo polyubiquitylation that favors Rad5 and Rad18 mediated error-free Template switching (TS) pathway. Gap filling is carried out by the recombination proteins Rad51, Rad52, Rad54, Rad55/Rad57 and the repair is mediated by Pol δ. This is followed by the formation of SCJs and resolution of this SCJs is initially taken care by Sgs1/Top3/Rmi1 complex. Salvage pathway of recombination which is thought to occur in the later stages of replication, also produces SCJ intermediates. But unlike TS pathway, Salvage pathway is normally inhibited by SUMOylated PCNA and is also considered to be error-prone in function.
1.7 Stabilization of Stalled Replication forks

Replication fork progression in S-phase can be challenged by several reasons such as protein-DNA complexes, nucleotide pool depletion, DNA repeats, late replication DNA regions and encounters with the transcription machinery. These obstacles can impede the progression of replication forks thereby resulting in aberrant recombination events and also head-on and in-line transcription collisions between replisome and the RNA polymerase (Branzei & Foiani, 2010). Also, tethering of transcribed genes by the nuclear pore complex (NPC) can generate topological impediments increasing the chance of fork collapse (Bermejo et al., 2011). This fork collapse can ultimately result in the failure of maintenance of genomic stability.

The replication checkpoint stands out as a major regulator of the integrity of stalled replication forks. In budding yeast, it has been reported that during replication stress, mutations in checkpoint proteins Rad53 and Mec1 lead to replisome dissociation from stalled replication forks (Cobb, 2003; Lucca et al., 2004). While a later study showed that the phosphorylation of replisome components by checkpoint kinases is likely to control the function of replisome itself rather than its stability (De Piccoli et al., 2012). This stalled replication forks can further results in the formation of reversed forks that may also function to release topological constraints (Fierro-Fernandez et al., 2007; Postow et al., 2001). Persistence of reversed forks can in turn lead to their processing and formation of double-strand breaks as well as large regions of ssDNA proximal to the replication fork junction. Even though homologous recombination is a repair pathway that is employed by the cells to deal with collapsed or reversed forks (Branzei & Foiani, 2010), aberrant recombination can be deleterious to the cells. The recombination intermediates can be processed by the action of helicases such as Sgs1 (BLM in humans) together with Top3 topoisomerase or other helicases, such as Srs2 and Mph1 that can disrupt D-loops. These helicases are recruited to the stalled replication forks by the action of checkpoint kinases. In the absence of these helicases, recombination structures become targets of endonucleases like EXO1 and MUS81-EME1 that can initiate deleterious fork cleavage and potentially excessive recombination (Branzei & Foiani, 2010; Hanada et al., 2006).

1.8 Introduction to DDK (Dbf4 dependent kinase)

DDK or Dbf4 dependent kinase is an essential Serine/Threonine kinase that is conserved from yeast to humans. DDK is comprised of 2 subunits. Cdc7 (cell division cycle 7), which is the catalytic subunit, and Dbf4 (dumbbell former 4), which is the regulatory subunit that binds to the DNA (S. P. Bell & Labib, 2016). These 2 subunits are bound to
each other in a 1:1 stoichiometry. Both subunits of DDK were initially identified in budding yeast by a genetic screen. Cdc7 and Dbf4 temperature sensitive mutants were shown to have a dumb-bell phenotype and cause cell-cycle arrest immediately before the onset of S-phase (L. H. Hartwell, 1974; Leland H. Hartwell, 1971, 1973). This is due to the fact that DDK is important for the late step of DNA replication initiation (Hollingsworth & Sclafani, 1990). It was also previously reported that Cdc7 and Dbf4 subunits of DDK interact to permit initiation of chromosomal replication. It is also suggested that Dbf4 can modulate the activity of Cdc7 as overexpression of Dbf4 rescues the temperature sensitivity of Cdc7 (Kitada et al., 1992).

1.9 CDC7-DBF4 homologs in other eukaryotes
Identification of DDK kinase in budding yeast and its prominent roles in replication initiation paved the way to discover its orthologues in other eukaryotes. In *Schizosachharomyces pombe*, Hsk1 was identified as a gene related to Cdc7 important for chromosomal replication (H. Masai et al., 1995). Him1, a homolog of Dbf4 was also later characterized in *S. pombe* (Brown & Kelly, 1999)(Table 1.1). Shortly after the discovery of Hsk1 in fission yeast, Cdc7 and Dbf4 homologues were discovered in humans, *Xenopus* and mouse (Faul et al., 1999; Hess et al., 1998; Sato, 1997). A study conducted in budding yeast using yeast two-hybrid system showed that DDK subunits Cdc7 and Dbf4 interact directly with each other and this interaction creates a functionally active complex (Dowell et al., 1994; Harkins et al., 2009; A. L. Jackson et al., 1993).

In *Homo sapiens*, huCDC7 (574 aa) is the yeast *CDC7* homologue discovered in 1997, while huDRF1 (615 aa) and huASK/huDBF4 (674 aa) are the human homologues of yeast Dbf4 identified in a yeast two-hybrid screen in 1999 (Lepke et al., 1999; A. Montagnoli, 2002; Sato, 1997) (Table. 1.1). It was also reported that the essential function of DDK in DNA replication is evolutionarily conserved. Human DDK can complement Cdc7 and Dbf4 deletions in yeast if human CDC7 and DBF4 cDNAs are co-expressed respectively (Davey et al., 2011).
Table 1.1. Homologues of CDC7 and DBF4 (adapted from (Rossbach & Sclafani, 2016))

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Organism</th>
<th>Catalytic subunit</th>
<th>Regulatory subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>S. cerevisiae</em></td>
<td>CDC7 (507 aa)</td>
<td>DBF4 (704 aa)</td>
</tr>
<tr>
<td>2.</td>
<td><em>S. pombe</em></td>
<td>HSK1 (507 aa)</td>
<td>DFP/Him1 (545 aa)</td>
</tr>
<tr>
<td>3.</td>
<td><em>Xenopus laevis</em></td>
<td>XeCDC7 (564 aa)</td>
<td>XeDBF4 (661 aa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XeDrf1 (772 aa)</td>
</tr>
<tr>
<td>4.</td>
<td><em>Mus musculus</em></td>
<td>muCdc7 (564 aa)</td>
<td>muASK (664 aa)</td>
</tr>
<tr>
<td>5.</td>
<td><em>Homo sapiens</em></td>
<td>Hu Cdc7 (574 aa)</td>
<td>huASK/huDbf4 (674 aa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>huDrf1 (615 aa)</td>
</tr>
</tbody>
</table>

The table depicts the different homologues of Cdc7 and Dbf4 subunits identified in different eukaryotic species.

1.6 Structural organization of DDK

In budding yeast, Cdc7 is a 58 kDa protein with 507 aminoacids, while Dbf4 is an 81 kDa protein with 704 aminoacids (Leland H. Hartwell, 1971; Johnston et al., 1982). Several kinase motifs are present in the Cdc7 catalytic subunit of DDK (I-XI), which are interrupted by kinase-insert (KI) sequences. Budding yeast contains three such KI sequences while higher eukaryotes possess just two of them (Faul et al., 1999; Guo & Lee, 1999; H. Masai et al., 1995; Sato, 1997). However, the length and amino acid sequences of these motifs vary among different Cdc7 orthologs. Further, *in vitro* and crystallographic studies revealed that not every section of these KI sequences is essential for its enzymatic activity (Hughes et al., 2012; Kitamura et al., 2011). Some of these KI sequences are involved in the nuclear export and import of DDK, highlighting their roles in the localization and spatial regulation of Cdc7 (B. J. Kim et al., 2007; B. J. Kim & Lee, 2006).

Crystallographic studies of DDK revealed how the two subunits of DDK are held together resulting in kinase activation. The N-terminal segment of Cdc7 is composed of anti-parallel β-sheets that contain the active ATP binding domain. While the C-terminus is composed of an α-helical phosphor-acceptor domain encasing a Mg$^{2+}$ ion along with a catalytic loop domain. The Dbf4 subunit of DDK possess three motifs, M, C and N which
are conserved among different eukaryotic species (Fig. 1.6). Motif M contains β-strands that bind to the β-strands of the KI domain of Cdc7 to form an anti-parallel sheet. Motif C of Dbf4 contains α-helices and a β-strand that form a zinc finger for binding with the N-terminus of Cdc7. Dbf4 wraps around Cdc7 by binding through these 2 motifs in a bipartite manner. The M and C motifs of Dbf4 are required for the binding of Dbf4 to Cdc7 and for Cdc7 enzymatic activity, while the N motif of Dbf4 is not required for binding to Cdc7 or Cdc7 enzymatic activation (Hughes et al., 2012). Nevertheless, this N motif of Dbf4 plays a role in interacting with the replication machinery (Pasero et al., 1999).

**Figure 1.6. Structural motifs of DDK subunits in S. cerevisiae**

DDK is composed of two subunits. The catalytic subunit is called Cdc7 (507 amino acids) and the regulatory subunit is called Dbf4 (704 amino acids). Cdc7 in budding yeast is composed of three kinase domains and three kinase insert sequences while Dbf4 consists of three motifs: N, M and C.

### 1.11 DDK regulation during cell cycle

DDK subunits are differentially regulated throughout the cell cycle. In *S. cerevisiae*, Cdc7 protein levels remain stable and are bound to the chromatin throughout the cell cycle (Weinreich, 1999). However, Dbf4 protein levels oscillate throughout the cell cycle depending on the protein stability. Dbf4 protein stability mainly relies on the anaphase promoting complex (APC/C) that targets the protein for degradation in G1 phase of the cell cycle. Dbf4 protein level increases at G1-S transition and it remains so throughout the S phase until the activity of APC/C is inhibited and gradually decreases upon completion of S phase (L. Cheng et al., 1999). Dbf4 and Cdc7 are SUMOylated during replication, with this modification triggering STUbL-mediated turnover of DDK if SUMO chains are not counteracted by the Ulp2 SUMO protease (Psakhye et al., 2019). Even though Cdc7 protein expression is quite stable throughout the cell cycle, its enzymatic activity is highly dependent on Dbf4. For this reason, the kinase activity of Cdc7 remains low in the G1 phase when Dbf4 is degraded, but increases as cells transit
from G1 to S phase and remains high all throughout S phase. The activity reduces according to the completion of the S-phase (Oshiro et al., 1999). This dependency of DDK enzymatic activity on Dbf4 levels stays conserved among different eukaryotes, including humans and *Xenopus* (Furukohri, 2003; Jiang & Hunter, 1997). In humans and *Xenopus*, besides Dbf4, DDK is also regulated by another Dbf4 related factor called Drf1 (Dbf4 related factor-1) (A. Montagnoli, 2002). In *Xenopus*, it was shown that unlike Dbf4, regulation of DDK by Drf1 is required for the early stages of embryonic development in promoting DNA replication (T. S. Takahashi, 2005). This indicates that the regulation of DDK’s enzymatic activity by Dbf4 or Drf1 is essential at different stages of vertebrate development.

Previous studies performed in budding yeast have also shown that besides a role in replication initiation, DDK also plays prominent roles in cell cycle progression (Bousset & Diffley, 1998; Donaldson et al., 1998). More recent evidence showed that DDK controls the spatial and temporal regulation of S-phase progression and also regulates origin firing efficiency. It does so by controlling the phosphorylation activity of a telomere binding protein, Rif1 (Rap-interacting factor 1). Rif1 phosphorylation by DDK ultimately results in timely regulation of replication across different origins of replication (Davé et al., 2014; Hiraga et al., 2014; Yamazaki et al., 2012). Similar observations were also made regarding the fission yeast ortholog, Hsk1-Dfp1, where Hsk1 was shown to regulate origin firing efficiency (Hayano et al., 2012).

### 1.12 DDK and replication initiation

In eukaryotes, DNA replication is a tightly controlled and regulated process. This ensures the complete duplication of the genome precisely once per cell cycle. DNA replication in eukaryotes occurs in three different stages, initiation, elongation and termination. The task of replication initiation is conducted by a multi-protein machinery called the replisome complex. The replisome complex is composed of several proteins that are directly or indirectly involved in the replication initiation process. The DNA duplication process is usually initiated by replicative helicases, ring-shaped motors that have the ability to unwind the duplex DNA and pave the way for the replication machinery to pass smoothly along the DNA (Costa et al., 2013; O’Donnell et al., 2013).

Replication initiation is carried out in two main stages:

a) Origin licensing: occurs in the late M and G1 phases of the cell cycle where the core of the eukaryotic replicative helicase, which is the mini-chromosome maintenance 2-7 (Mcm2-7) complex, is loaded onto the replication origins of the duplex DNA. They do
so with the help of the Origin Recognition Complex (ORC) along with the co-factors, Cdc6 and Cdt1 (S. P. Bell & Stillman, 1992; Bleichert et al., 2017; Deegan et al., 2016).

b) Helicase activation: conversion of the MCM helicase into an active Cdc45-Mcm2-7-GINS (CMG) helicase occurs in S-phase with the help of few other initiator proteins (Deegan et al., 2016).

a) Origin licensing

Replication begins at specific regions within the DNA called origin of replication initiation (ori). Origin licensing is initiated by the loading of the Origin recognition complex (ORC) at ori. In budding yeast, these origins of replication are referred to as Autonomously Replicating Sequences (ARSs), which are in vivo replication initiation sites (Brewer & Fangman, 1987). ORC binds to the DNA in an ATP-dependent manner which further acts as a scaffold for the recruitment of Cell division cycle 6 (Cdc6) protein which stabilizes ORC-DNA interaction. This interaction triggers the loading of the inactive Mcm2-7 hexamer along with its pre-bound co-factor Cdc10 dependent factor 1 (Cdt1) (Speck et al., 2005; Tanaka & Diffley, 2002) in the G1 phase of the cell cycle. This whole complex is known to form the pre-replication complex (pre-RCs).

b) Helicase activation

Activation of the loaded MCM helicase occurs at the G1/S cell-cycle phase boundary and persists all-throughout the S-phase. The important players that regulate origin firing through molecular transitions is kinases S-CDK(Clb5,6/Cdc28) and DDK (Dbf4-dependent kinase). The initiation of replication at pre-RCs is triggered by the action of these two kinases. S-CDK and DDK phosphorylate and activate initiation factors that stimulate their interaction with the pre-RC complex leading to the full activation of Mcm2-7 helicase(Gambus et al., 2006). The important role of CDK (Cell-cycle dependent kinase) in budding yeast is its ability to phosphorylate the initiation factors Sld2 and Sld3. This further results in priming the interaction with the scaffolding protein Dpb11(DNA polymerase B possible subunit 11) (Tanaka et al., 2007; Zegerman & Diffley, 2007). Dpb11 loads the hetero-tetrameric GINS (go-ichi-ni-san or 5-1-2-3 referring to its subunits Sld5, Psf1, Psf2, and Psf3) and DNA polymerase ε to the origin. All this origin firing factors together constitute pre-Landing complex (pre-LC). DDK (Dbf4 dependent kinase) promotes replication initiation by its phosphorylation of Mcm subunits. The crucial function of DDK (Dbf4 dependent kinase) is its phosphorylation of the N-terminal tail of Mcm2/4/6 subunits which induces a conformational change for MCM2-7 complex (Hardy et al., 1997; Lei et al., 1997;
Weinreich, 1999). In vitro studies have shown that DDK can also phosphorylate Cdc45 (Nougarède et al., 2000). This further stimulates the recruitment of Cdc45 to the MCM helicase complex leading to the formation of an active CMG (Cdc45-Mcm2-7-GINS) complex (Gambus et al., 2006; Ilves et al., 2010; Moyer et al., 2006). This is further called as the pre-initiation complex (pre-IC). In a breakthrough study conducted by Dr. Diffley and his colleagues, they were able to develop an in vitro reconstituted DNA replication initiation system 16 purified replication components (Yeeles et al., 2015). A schematic of origin firing is shown below (Fig 1.7)

Figure 1.7. Representative model of DDK’s role in replication initiation in budding yeast (adapted from (Karim Labib & Gambus, 2007))

Origin licensing occurs in the G1 phase of the cell cycle by the loading of inactive MCM helicases at replication origins to form a pre-RC complex. Upon G1/S phase transition, CDK phosphorylates Sld2 and Sld3, which then primes the interaction with Dpb11 and
further promotes loading of other initiation factors like GINS, Cdc45 and DNA polymerase ε. DDK, on the other hand, phosphorylates the N terminal tail of Mcm2,4 and 6 subunits. Phosphorylation and loading of all this origin firing factors by the combined action of CDK and DDK results in the full activation of MCM helicase forming an active complex called CMG complex together with Cdc45 and GINS proteins.

DDK is known to phosphorylate serine/threonine amino acid residues that are adjacent to acidic amino acids or phospho S/T residues in +1 position (Cho et al., 2006). *In vitro* studies have shown that MCM 2, 3, 4, 6 and 7 subunits are DDK targets (K. Labib, 2010). Mcm2 has been identified as an important DDK target that gets phosphorylated at serine residues, S164 and S170 (Bruck & Kaplan, 2009, 2015). *In vivo* studies have shown that phosphorylation of MCM helicase by DDK induces a conformational change thereby favoring interaction with other origin firing factors. Some insights into the molecular mechanism of DDK in origin firing came from the finding of a P83L mutation in Mcm5 subunit that can by-pass the requirement of DDK for viability. This presumably happens due to the mimicking of conformational change that facilitates replication initiation (Hardy et al., 1997; Hoang et al., 2007). It was seen that removal of 74-174 residues of Mcm4 allows DNA replication *in vivo* even without DDK (Hardy et al., 1997; Sheu & Stillman, 2010). Thus, Cdc7-Dbf4 can have an inhibitory action of N-terminus of Mcm4 and Mcm6 to promote origin firing by phosphorylation. Both *in vitro* and *in vivo* studies revealed that DDK activity is also required for the recruitment of origin firing factors like Sld3/7 and Cdc45 to replication origins along with multiple phosphorylation sites on the tail of Mcm 2-7. This is important for successful loading of Sld3 (Deegan et al., 2016).

### 1.13 DDK and Cohesion

Proper chromosomal segregation is crucial for genomic integrity. During mitosis, the two copies of each chromosome have to be distributed equally to the daughter cells. Separation of two sister chromatids takes place at the early onset of anaphase. The chromatids are pulled towards opposite spindle poles by the cleavage of the chromosomal cohesin complex that is wrapped around the sister chromatids. One of the key findings related to chromosome cohesion was the assistance provided by the Scc2-Scc4 complex in loading the cohesin complex during the G1-phase of the cell-cycle (Lengronne et al., 2006; Uhlmann & Nasmyth, 1998).

Cdc7 kinase is shown to play important roles in sister chromatid cohesion among different eukaryotes. In budding yeast, DDK accumulates at the kinetochores in the
telophase stage of mitosis (Natsume et al., 2013). Ctf19 kinetochore complex interacts with DDK and this interaction is required for its recruitment to the kinetochores. This in turn allows for the loading of Sld3-Sld7 initiator proteins to the pericentromeric replication origins for early origin firing. Kinetochore associated DDK was also shown to promote robust sister chromatid cohesion at pericentromeres by locally promoting the recruitment of Scc2-Scc4. This function of DDK is independent from its role in origin firing (Natsume et al., 2013).

Similar function of DDK in cohesion was also reported in the case fission yeast, S. pombe. Here, cohesin recruitment to the heterochromatin region is dependent on Swi6, a conserved heterochromatin protein that is equivalent to mouse homologue HP1. This process is essential for accurate chromosome segregation. Both in vivo and in vitro studies have shown a role for Hsk1 (fission yeast ortholog of Cdc7) in interacting and phosphorylating the Swi6 protein. Studies have also highlighted the importance of this interaction between Hsk1 and Swi6 in maintaining proper sister chromatid cohesion in fission yeast (Bailis et al., 2003; Bernard, 2001; Nonaka et al., 2002).

Studies in Xenopus egg extracts have shown that the recruitment of Scc2 and cohesin to chromatin is dependent on the loading of the pre-replication (pre-RC) complex. It was also shown that coupling of origin licensing with sister chromatid cohesion is facilitated by the Scc2 subunits of the cohesin complex. DDK kinase which is shown to bind to the pre-RC complex is required to tether Scc2-Scc4 complex to the pre-RCs and to activate them for DNA replication, underscoring a link between DNA replication initiation and chromosome cohesion. Conversely, immunodepletion of DDK in Xenopus egg extracts have shown to significantly reduce the association of Scc2-Scc4 with chromatin (Gillespie & Hirano, 2004; N. Takahashi et al., 2004; T. S. Takahashi et al., 2008).

1.14 DDK and meiosis

In eukaryotic cells, two types of cell divisions take place: mitosis and meiosis. Mitosis produces two genetically identical daughter cells that are segregated from the sister chromatids after the replication of chromosomes. In contrast, meiosis generates two haploid gametes from diploid germ cells after a single round of DNA replication followed by two consecutive chromosome segregation rounds. The two segregation rounds in meiosis is defined as meiosis I and meiosis II. While meiosis I results in the separation of homolog chromosomes to the opposite poles, meiosis II resembles mitosis in which the sister chromatids are segregated to opposite poles (Sakuno et al., 2009).
Cdc7 kinase in yeast has been shown to regulate initiation of meiotic recombination as well as chromosomal segregation (Buck et al., 1991; K. Ogino et al., 2006; Sasanuma et al., 2008). Cdc7 inactivation in yeast is reported to show a delay in replication and also leads to a prophase arrest with no recombination. Meiotic recombination is initiated by the formation of programmed DNA double strand breaks (DSBs). In budding yeast, several factors have been identified in playing roles in DSB formation. Mer2 is one such factor. Mer2 is an accessory factor for the meiotic endonuclease Spo11 that is responsible for DSBs formation. Previously, it was demonstrated that cell-cycle dependent kinases (S-CDKs) regulate DSB formation by phosphorylating the Mer2 protein (Henderson et al., 2006). Later studies also reported a role for Cdc7-Dbf4 kinase in promoting meiotic recombination by phosphorylating Mer2 together with S-CDKs, which is essential for the further recruitment of Spo11 endonuclease (Sasanuma et al., 2008; Wan et al., 2008). Furthermore, by using the mcm5-bob1 allele that can bypass the need for Cdc7 in replication initiation in budding yeast, a novel role for Cdc7-Dbf4 in meiotic chromosomal segregation was uncovered. This is related to the transcription of NDT80, a global transcription activator that is involved in the execution of the first meiotic cell division in fission yeast (Lo et al., 2008; Matos et al., 2008; Valentin et al., 2006). Monopolin is a complex that is localized on kinetochores, which is required for segregation of homologous chromosomes during meiosis I. Additionally, Cdc7 is also important for the localization of the monopolin subunit, Mam1, onto the kinetochores to facilitate mono-orientation of sister kinetochores. Failure in any of these processes leads to mis-segregation of chromosomes and spore lethality (Lo et al., 2008; Matos et al., 2008).

In budding yeast, replisome associated components Tof1 and Csm3, physically interact with the Cdc7-Dbf4 complex, recruiting it to the replisome, where it phosphorylates Mer2 in the wake of the replication fork. This process spatially and temporally coordinates replication with subsequent formation of DSBs in meiotic recombination (Murakami & Keeney, 2014).

DDK is also reported to play direct roles not only in meiotic recombination, but also in the separation of chromatids during meiosis I. Chromatid separation is initiated by the cleavage of cohesion by separase during cell division. This is regulated through its phosphorylation. Cdc7-Dbf4 regulates the cleavage of the cohesion subunit Rec8 by phosphorylating it in concert with Casein kinase I (Katis et al., 2010).
1.15 DDK and mitosis

Polo kinases comprise a large family of proteins that function in regulating several cellular processes, including mitotic entry, chromosome segregation and mitotic exit. In *S. cerevisiae*, Cdc5, a polo-like kinase is shown to regulate several aspects of mitosis like mitotic progression and cytokinesis. Recently, another role of Cdc5 was elucidated in the removal of centromeric cohesion during mitosis through its association with the centromere (Mishra et al., 2016). A yeast two-hybrid analysis in budding yeast performed to identify the N-terminal interactors of Dbf4 revealed that Cdc5 is the only Polo ortholog to interact with DDK (Y.-C. Chen & Weinreich, 2010). DDK is shown to regulate mitotic exit by targeting Cdc5 kinase and inhibiting it. It does so by mediating the interaction of Dbf4 N-terminus with the polo-box domain (PBD) of Cdc5 which can alter the substrate binding of Cdc5 kinase. This further inhibits the activation of mitotic exit network (MEN) proteins without affecting the kinase activity of Cdc5. DDK mutants that shows defects in binding with Cdc5 have shown to support S-phase cell cycle progression. But this was further followed by aberrant chromosome segregation in mitosis. DDK through its Dbf4 subunit therefore acts as a Cdc5 inhibitor to ensure proper exit of cells from mitosis (Miller et al., 2009). Dbf4 was also identified to carry a non-consensus motif that binds to Polo kinase and this PBD region is distinct from the one that is used to bind other phosphoproteins (Y.-C. Chen & Weinreich, 2010). Therefore, DDK is an important regulator of mitosis that prevents chromosome mis-segregation, thereby preventing genomic instability.

Very recently, another important role of DDK in regulating homologous recombination during mitosis emerged. Cdc7-Dbf4 kinase was shown to target and activate Mus81-Mms4, an endonuclease resolving joint molecules. Mus81-Mms4 activation during mitosis is important for DNA damage response, especially to process recombination intermediates that arise from DSBs and stalled replication forks. Activation of the Mus81-Mms4 nuclease is mainly dependent on the action of CDK, Cdc5 and DDK kinases. DDK along Cdc5 bind and phosphorylate Mus81-Mms4 in an interdependent manner. DDK-mediated phosphorylation is critical for the activation of this nuclease, making it a novel regulator of homologous recombination. It was also reported that Rtt107, which is a scaffold protein, recruits both Cdc5 and DDK to the Mus81-Mms4 complex enabling its full activation that is required for efficient joint molecule (JM) resolution (Princz et al., 2017).
1.16 DDK and replication stress

In addition to its well-established role in S phase replication during unperturbed conditions, DDK has also been implicated in replication stress conditions. Upon exposure of cells to exogenous or endogenous stress conditions, replication forks stall, leading to the activation of the DNA damage response, including the DNA damage checkpoint. Checkpoint activation suppresses further origin firing and slows down the fork progression (Bartek et al., 2004).

In *S. cerevisiae*, Rad53 checkpoint kinase (Chk2 in humans) is reported to hyper-phosphorylate Dbf4 upon fork stalling. This hyper-phosphorylation results in attenuated kinase activity of DDK. Studies using *Xenopus* egg extracts also revealed that DDK activity is inhibited upon etoposide (DNA topoisomerase II inhibitor) treatment in a checkpoint dependent manner (Costanzo et al., 2003; Weinreich, 1999).

A later study conducted on *Xenopus* and mammalian cells contradicted this finding by showing that DDK functions as an upstream regulator of DNA damage checkpoint control in modulating the S-phase checkpoint signaling (Tsuji et al., 2008). Interestingly, Cdc7 was also reported to phosphorylate Rad53 in a manner that is required for its full checkpoint activation (Dohrmann et al., 1999; Kihara et al., 2000). Furthermore, it was indicated that Rad53 interacts with the N-terminal of Dbf4 and a deletion of this motif results in checkpoint defect upon HU conditions (Duncker et al., 2002; Keiko Ogino et al., 2001; Varrin et al., 2005).

There are several findings that support the notion that DDK is activated upon replication stress. Early evidence of the involvement of DDK in replication stress response came from findings that mutant yeast strains lacking Cdc7 (but having the *mcm5-bob1* allele) can bypass the need for Cdc7 in replication initiation and are hypersensitive to hydroxyurea (HU) treatment. Recent reports have also indicated that both the replication checkpoint signaling and the ubiquitin proteasome signaling are required for the stabilization of Cdc7-Dbf4 on chromatin, which is important for lesion bypass during replication stress in human cells (M. Yamada et al., 2013).

The activation of DDK upon replication stress is still an open question as there are conflicting results on this notion. There are also data indicating that Dbf4 phosphorylation by the checkpoint kinase does not affect the enzymatic activity of Cdc7. This indicates that the regulatory phosphorylation of Dbf4 may only affect the underlying pathways associated with the suppression of origin firing. It might also be possible that only a small fraction of DDK associated with the early pre-RC complexes is being phosphorylated by the checkpoint kinase. This phosphorylation will inactivate DDK or
promote its dissociation from chromatin and pre-RC complexes, rendering it unavailable for late origin firing (Masayuki Yamada et al., 2014).

1.17 DDK and checkpoint activation

The importance of DDK in checkpoint activation is shown in budding yeast as well as in higher eukaryotes. In human cells, it has been reported that Claspin, a critical mediator of the replication stress pathway, acts as a regulator of the vertebrate checkpoint kinase Chk1. Claspin is shown to recruit Cdc7 for initiation of DNA replication during normal S-phase in human cells. Cdc7 is shown to bind an acidic patch of Claspin which triggers Claspin phosphorylation through its Chk1-binding domain. This phosphorylation is shown to be crucial for the interaction of Claspin and Chk1 kinase (Yang et al., 2016, 2019). It was also shown that in Cdc7 depleted mouse embryonic stem (ES) cells during Hydroxyurea (HU) or UV induced DNA damaging conditions, there is decreased phosphorylation of Chk1 and also a reduced chromatin association (Chini & Chen, 2004; J. M. Kim et al., 2008). Moreover, studies in fission yeast showed that Hsk1(Cdc7) can phosphorylate the checkpoint clamp component Rad9 from the 9-1-1 complex in response to replication induced DNA damage. Here they propose that this phosphorylation is critical for the unloading of Rad9 from DNA damage sites to further facilitate repair (Furuya et al., 2010). In addition to this, recently, DDK was also proposed to play a role in initiating checkpoint signaling by promoting ssDNA accumulation at stalled replication forks. Their experimental analysis also suggests that DDK might function here through its regulation of Exo1 stability and/or activity (Sasi et al., 2018).

Even though DDK plays an important role in replication initiation, studies in various eukaryotes have provided evidence that the activity of DDK is functionally regulated at different replication origins to temporarily prevent origin firing during replication stress. Previous reports have demonstrated the importance of limiting DDK activity through the regulation of Dbf4 subunit of DDK, to temporarily regulate origin firing in yeast (Mantiero et al., 2011).

Under DNA damaging conditions or dNTP pool depletions (by HU treatment), Dbf4 is shown to be targeted and phosphorylated by the checkpoint effector kinase Rad53 which in turn leads to the removal of DDK kinase from the chromatin, thereby inhibiting further origin firing. It has also been shown that hyper-phosphorylation of DDK has a modest enzymatic activity (Duncker et al., 2002; Weinreich, 1999). Importantly, abrogation of Rad53-Dbf4 N-terminal interaction prevents Dbf4 phosphorylation and leads to firing of late origins during replication stress and subsequent checkpoint activation (Zegerman &
Diffley, 2010). This feature of the checkpoint in inhibiting origin firing is shown to be conserved among different eukaryotes. For instance, an early study conducted using *Xenopus* egg extracts also show that Cdc7 kinase activity is inhibited due to its dissociation from the DNA because of ATR dependent prevention of Dbf4 binding (Costanzo et al., 2003).

DDK is shown to have numerous Rad53 phosphorylation sites on the Dbf4 subunit and its phosphorylation by Rad53 can lead to a reduced enzymatic activity of DDK towards Mcm2 (Kihara et al., 2000). Rad53 consists of two fork-head associated domains, namely FHA1 and FHA2, which are centrally flanked by a kinase domain. Using a combination of structure-guided site-directed mutagenesis and yeast two-hybrid analysis, a group has identified the Dbf4 domain required for its interaction with Rad53. Dbf4 motif containing domain with 105-221 amino acids is required and is sufficient for the interaction of Dbf4 and Rad53. This motif consists of a unique N-terminal alpha helix that is an integral element for the binding of FHA1 domain of Rad53 kinase. This region also folds as a BRCA1 C terminus domain (BRCT) because of which this domain was designated as H-BRCT domain (Matthews et al., 2012). Other than DDK, the checkpoint kinases are also known to target other initiator kinases like CDK (Cell cycle dependent kinases) through its downstream substrate Sld3 (Zegerman & Diffley, 2010).

1.18 DDK and Trans-lesion synthesis mediated DNA damage repair

Trans-lesion synthesis is one DDT pathway that gets activated under various replication stress conditions. In human cells, 17 enzymes have been identified to be trans-lesion polymerases while only three are identified in budding yeast. These three trans-lesion polymerases identified in *S. cerevisiae* are Polζ, Polη, and Rev1. While Polζ is encoded by REV3-REV7 genes, Polη, and Rev1 are encoded by RAD30 and REV1, respectively. TLS polymerases have low processivity and fidelity in comparison with replicative polymerases. They also lack proofreading exonuclease activity (Brandão et al., 2014). The binding of trans-lesion polymerase during TLS to the sites of DNA damage is facilitated by the action of Rad18, an E3 ubiquitin ligase. Rad18 promotes the recruitment of DNA Polymerase eta (Polη) and also mono-ubiquitinates proliferating cell nuclear antigen (PCNA) that directs the TLS pathway.

Previous work in budding yeast indicated significant roles for DDK in the trans-lesion synthesis pathway. Rad18 ubiquitin ligase was identified as a novel substrate for DDK phosphorylation. A cluster of serine residues located in the Polη-binding motif of Rad18 undergoes DDK phosphorylation which positively regulates the formation of Rad18-
Polη complex thereby recruiting Polη and other trans-lesion polymerases to the stalled replication forks (Day et al., 2010; Vaziri & Masai, 2010).

DDK is also shown to regulate TLS pathway by its direct action on one of the subunits of trans-lesion polymerases. Previous reports have shown that DDK is required for the generation of complex frameshift events (CINS), which is also a distinct feature of Polζ trans-lesion polymerase. It was also shown that Cdc7 can bind to the Rev7 subunit of Polζ independently of the Dbf4 regulatory subunit. Therefore, DDK regulates Polζ by phosphorylating Rev7, which in turn facilitates Polζ loading onto chromatin. Once bound to chromatin, Rev7 would become attached to the Rev3 subunit forming an active Polζ complex (Brandão et al., 2014).

1.19 DDK and Cancer

For efficient cancer therapies, dysregulation of normal cell cycle progressions was considered a possible target. Given that DDK plays numerous roles in DNA replication, DNA damage repair and cell-cycle progression, it has drawn significant attention as a potential target for new cancer chemotherapeutics (Hisao Masai, 2008; Swords et al., 2010). Over-expression of Cdc7 is a hallmark for several cancers. Increased expression of Cdc7 in cancers tends to correlate with poor clinical outcomes (Bonte et al., 2008; A. N. Cheng et al., 2013; Clarke et al., 2009; Ghatalia et al., 2016; Hou et al., 2012; Kaufmann et al., 2008; Kulkarni et al., 2009; Melling et al., 2015; Nambiar et al., 2007). Knock-down experiments of Cdc7 in a variety of tumor cell lines were shown to cause accumulation of nuclear damage in S-phase which further leads to cell death or aberrant mitosis (Im & Lee, 2008; Alessia Montagnoli et al., 2004). Usage of the cell cycle indicator, Fucci, as well as similar fluorescent cell cycle indicators in cancer cell lines have revealed differential effects in p53 positive and negative cells on the pattern of cell cycle responses upon CDC7 depletions. p53 positive cells predominantly arrest in G2 phase and also accumulate mitotic regulator proteins like cyclin B1 which leads to aberrant mitotic entry and subsequent apoptosis at the post-mitotic state. On the other hand, p53 positive cancer cells die as soon as they enter S-phase upon CDC7 depletion. This knowledge on the differential mode of cell death provides insights into efficient combinatorial therapies where one can use known anti-cancer agents in conjunction with CDC7 inhibitors to achieve synergistic effects (Ito et al., 2012). Conversely, it was also shown that loss of CDC7 in tumor cells induces a p53 independent apoptotic cell death without eliciting a standard checkpoint response. In contrast, a normal healthy cell is
spared from such response by the activation of p53 dependent cell-cycle checkpoint (Alessia Montagnoli et al., 2004).

Considerable efforts have been made to create potential CDC7 inhibitors, with Nerviano Medical Sciences in Milan, Italy being the first to create a potent Cdc7 kinase inhibitor. They developed the small molecule ATP-competitors that belong to the pyrrolopyridones class, as CDC7 inhibitors (Vanotti et al., 2008). Among different compounds that were screened, PHA-767491 has emerged as a leading candidate for CDC7 inhibition. This compound blocks origin firing by impairing MCM2 phosphorylation, but without impeding fork progression or activation of the checkpoint cascade. This compound has been widely tested and used for its anti-tumor activity in Acute myeloid leukemia (AML), breast and colon cancer models. It has an IC50 value of 10 nM using purified kinase (Alessia Montagnoli et al., 2008). Treatment of cancer cell lines with this compound caused cell apoptosis and lead to shrinkage of tumor growth in pre-clinical models. This drug was also reported to be a promising candidate in treating pancreatic adenocarcinoma (Huggett et al., 2016). It was also indicated to be used in combination therapies along with 5-fluorouracil in the treatment of hepatocarcinoma (W. Li et al., 2015). The following years witnessed the emergence of another potent and specific inhibitor of CDC7, called XL413, and discovered by Exelxis (Koltun et al., 2012) which advanced into phase 1 clinical trials. But unlike the robust anti-proliferative and apoptotic effects seen with PHA-767491, XL413 was shown to exhibit limited activity in many cancer cell lines as its action was hampered because of its poor cell permeability. Many researches are ongoing now to identify a potential CDC7 inhibitor that can be readily taken up by the cell (Kurasawa et al., 2020; Sasi et al., 2018). Taken together, CDC7 inhibitors represent a promising class of anti-cancer drugs that could be widely used in cancer therapeutics.
2. MATERIALS AND METHODS

2.1 Yeast strain genotypes and media

2.1.1 Yeast strain genotypes

In this study, we mainly used derivatives of W303. The genotype of used strains are listed in Table 2.1 below.

<table>
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<td>Mata ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 Rad5+ srs2::pADH1-tc3-3xHA-Srs2 (KanMX)</td>
<td></td>
</tr>
<tr>
<td>HY10266</td>
<td>Mata ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 Rad5+ srs2::pADH1-tc3-3xHA-Srs2 (KanMX) cdc7-4</td>
<td></td>
</tr>
<tr>
<td>HY7689</td>
<td>Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+, RAD5+,srs2::3HA-SRS2.</td>
<td></td>
</tr>
<tr>
<td>HY7679</td>
<td>Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, ura3-1,can1-100, GAL, PSI+, cdc7-4, RAD5+,srs2::3HA-SRS2</td>
<td></td>
</tr>
<tr>
<td>FY1131</td>
<td>Mata, ade2-1, trp1-1, leu2-3112, his3-11,15, ura3, can1-100 RAD5+ smc6-P4-13Myc::HIS3</td>
<td></td>
</tr>
<tr>
<td>FY1898</td>
<td>Mata ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 RAD5+ tof1A::HIS</td>
<td></td>
</tr>
<tr>
<td>FY0119</td>
<td>Mata, ade2-1, ura3, trp1-1, leu2-3, leu2-112, his3-11, his3-15, can1-100, GAL, PSI+,RAD5+ rad53K227A::KanMX4</td>
<td></td>
</tr>
<tr>
<td>HY9123</td>
<td>Mata ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100 RAD5+ (LEU2)pCDC7-cdc7-K16R (HIS3)pDBF4-dbhf4-K6R,K14R,K432R(hphNT1)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1 List of strains used in this study**
2.1.2 Media used

2.1.2.1 Media for *E. coli* growth

Luria-Bertani medium (LB-from Difco™)

a) Difco™ LB Agar per litre (pH 7.0 ± 0.2)
   
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g (1%)</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5 g (0.5%)</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>10 g (1%)</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g (2%)</td>
</tr>
</tbody>
</table>

b) Difco™ LB broth per litre
   
   Ingredients mentioned above for LB agar without agar (2%)

c) Difco™ LB with Ampicillin (50 μg/mL)

2.1.2.2 Media for yeast (*S cerevisiae*) growth

a) YP agar Per Liter
   
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>10 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>20 g</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.33 g</td>
</tr>
<tr>
<td>Dextrose.</td>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g (2%)</td>
</tr>
</tbody>
</table>

b) YP media per litre
   
   Ingredients mentioned above for YP agar without agar (2%)

c) YPD agar per litre
   
   Ingredients mentioned above for YP agar + 2% glucose

d) YPD media per litre
   
   Ingredients mentioned above for YPD agar without agar (2%)

e) SC drop-out agar per litre
   
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Nitrogen Base (without ammonium sulfate or amino acids)</td>
<td>1.7 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g (2%)</td>
</tr>
<tr>
<td>Amino acids as required</td>
<td></td>
</tr>
</tbody>
</table>

f) SC drop-out media Per Liter
Ingredients mentioned above for SC drop-out agar without agar (2%)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COONa.3H₂O</td>
<td>1.36%</td>
</tr>
<tr>
<td>KCl</td>
<td>0.19%</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.12%</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.074%</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

2.2 Yeast Strain Construction

2.2.1 Plasmid DNA isolation from *E. coli*

Selected *E. coli* strain from glycerol stock was streaked into bacterial selection plate containing ampicillin to obtain single colonies the next day. The single colonies were then inoculated in 10 ml of LB broth supplemented with ampicillin (50 μg/mL) and were allowed to grow overnight at 37°C. The cells were then pelleted and plasmid DNA isolation was performed with Plus SV Minipreps DNA Purification System (Promega) following the manufacturer’s instructions. The isolated plasmids were eluted in 50 μl of double distilled water (ddH₂O).

2.2.2 Yeast Transformation

Desired yeast mutants were constructed with Lithium Acetate-based transformation of yeast cells with PCR amplification of a deleted gene/tagged cassette (Gietz et al., 1995). Specific primers were designed and used to amplify the region of interest. The amplified fragment is then purified using Wizard® SV Gel and PCR Clean-Up System (Promega).

For transformation, log phase culture that are grown overnight at 25°C were pelleted and resuspended in LiAc/TE buffer (0.1 M lithium Acetate; 1X TE buffer) to make a final concentration of 2x10⁹ cells/ml. The cells were then incubated at 28°C for 20 min. After incubation, 1x10⁸ of these competent cells were then transferred to 1.5 ml centrifuge tube containing 5 μg of DNA and 5 μl of denatured carrier DNA (salmon sperm DNA, Sigma-Aldrich). After 20 min of incubation at 30°C, 500 μl of 40% PEG/LiAc was added to this suspension. The mixture was then resuspended with a pipette and incubated at 28°C for 30 min. 50 μl of DMSO was added to this to make the final concentration of 10%. Cells were then given a heat shock at 42°C for 15 min, and were then allowed to recover at room temperature for 5 min. These cells were then centrifuged to remove PEG/LiAc mixture and washed with 1 ml of YPD. The cells were resuspended in 100 μl YPD and transferred to 15
ml centrifuge tube containing 1 ml of YPD. This mixture was then incubated at 30°C for 3 hrs in a shaker incubator. After 3 hrs, the cells were plated on selection medium and were incubated at 30°C for 2-3 days. The resulting transformants were later subjected by checking the markers, PCR and western blotting (if required) to confirm the correct integration of the cassette.

2.2.3 Yeast crosses
Other than transformation, we created mutants with more than one mutation by crossing 2 strains of opposite mating type followed by selecting the desired genotype combination from the product of meiosis. Mat A and Mat α strains were grown overnight and were mixed together next day on a YPD agar plate. This mixing helps them to recognize opposite sex and mate and thereby generating dumb-bell shaped cells called zygotes. These zygotes were separately picked up using a micromanipulator (Automatic Singer) and were allowed to grow on a new YP agar plate for the next 2-3 days. Once diploid colonies are formed, these colonies were then patched on VB sporulation media for meiosis induction. After 3-4 days, these colonies sporulate to form tetrads (as observed under microscope) which contain 4 haploid spores. The haploid spores of tetrad are dissected using a micromanipulator and incubated at a permissive temperature for the spores to grow and form colonies. The spores are individually checked for obtaining the desired mutants using selection markers, PCR and/or western blotting.

2.3 Yeast Culturing and arresting, Usage of DNA damaging agents and Conditional depletion
Yeast strains were grown at 25°C (temperature sensitive strains) or 28°C (conditional AID strains) in YPD medium. The strains were then arrested at desired cell cycle stages with the usage of α-factor (G1 arrest) or nocodazole (G2/M arrest).

2.3.1 G1 arrest
G1 arrest of yeast cells was achieved by using α-factor pheromone. This is a peptide that is composed of 13 amino acids that is being recognized by Mat A yeast cells. Once Mat A cells recognize this pheromone, those cells begin to have morphological changes and thereby resulting in G1 arrest. Exponentially grown cells were treated with 3 μg/ml α-factor (Genscript) for 2 hrs at 25°C with a second α-factor addition (half of the initial concentration) after an hour of first addition. If 95% of cells appeared to be in G1 with its characteristic morphology, they are said to be synchronized enough to proceed to the S phase. Cell were
then washed once with YP broth to wash away excess α-factor and then the cells were released into S phase.

2.3.2 Nocodazole arrest
G2 arrest of yeast cells were achieved by using Nocodazole. This is a tubulin binding agent that prevents mitosis by disrupting microtubule polymerization. Hence, upon addition, the cells will become arrested in M phase (Prometaphase to be specific). Here, cells are treated for 2.5 hrs with 10-20 μg/ml of nocodazole dissolved in DMSO (1% total). After synchronization, these cells were washed in YP + 1% DMSO and released to next stage of cell division.

2.3.3 Different drug treatments
Methyl Methane Sulphonate (MMS): a drug used in most of the 2D experiments below. The cells were treated with 0.033% of MMS during the experiments. For spot assays, much lesser concentrations of MMS were used as indicated in the figures.
Hydroxyurea: this drug was used at a concentration of 200 mM in 2D experiments.
BrdU: was used at the concentration of 200 μg/ml for the BrdU-qPCR assay.

2.3.4 Conditional depletion of proteins
Temperature sensitive mutants were inactivated completely by incubating cells at a non-permissive temperature (37°C). For most of the 2D experiments, a semi-permissive temperature was used to inactivate the gene (28°C).
For conditionally depleting tetracycline promotor tagged gene by inhibiting de novo translation, tetracycline (NZYTech) was used at a final concentration of 1 mM. For ensuring complete protein degradation, 0.5 mM mg/ml tetracycline was added again after 2 hr upon release.
1 μM of auxin was added after 20 min from G1 release to conditionally deplete AID-tagged target genes.

2.4 Protein Extraction and Electrophoresis
2.4.1 TCA protein extraction
Yeast protein extraction was carried out using Trichloroacetic acid (TCA) method as described in (Foiani et al., 2000). 15 ml of 1x10⁷ yeast cells were harvested by centrifugation and were resuspended in 100 μl of 20% TCA. Equal volume of acid-washed glass beads (Sigma-Aldrich) was added vortexed for about 20 min for cell lysis. Later, 200 μl of 5%
TCA was added to this mixture, to get a final concentration of 10% TCA. The cell lysate was then transferred to a fresh tube centrifuged at 5000 rpm for 10 min. 100 µl of 2X Laemli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl; pH 6.8) was added to the pellet and dissolved by vortexing. The acidity of the mixture was neutralized by the addition of 50 µl of 1 M Tris base. This mixture was boiled at 95°C for 10 min and then centrifuged at 15,000 rpm for 10 min. After centrifugation, the supernatant containing the proteins was then subjected to SDS-PAGE.

2.4.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The type of gel used for running was usually 4-15% or 4-20% gradient Bio-Rad gels (Criterion™ TGX™ Precast Midi Protein Gel). Proteins are loaded and the gel was run at 120 V for about 2 hrs.

2.4.3 Western blotting

Once the loading dye has passed into the buffer from the gel, the running is said to have completed. Proteins from the gel were transferred to a nitrocellulose membrane (Protran, Whatman59, 0.45 mm) in 1X Transfer buffer (1% glycine, 0.02 M Tris base, 20% methanol) at 50 V for 2 hrs. After the transfer, the membrane was stained with Ponceau for a rough qualitative protein estimation. The ponceau stain was removed by washing the membrane 2 times with 1X PBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20). The membrane was then blocked with 5% skim milk in 1X PBST for about 1 hr. The membrane was then subjected for overnight incubation in primary antibody. The next day, membrane was washed 3 times with 1X PBST; 10 min for each wash. The membrane was later incubated with horseradish peroxidase-conjugated secondary antibody diluted in skim milk. This incubation was then followed by 3 more washes with 1X PBST. Later the desired proteins were detected from this membrane by using Supersignal™ from West Femto Maximum Sensitivity substrate (Thermo Scientific™).

The main primary antibodies used are:
monoclonal HA antibody (Bio-legend), anti-Rad51 (y-180, Santa Cruz Biotechnology), anti-BrdU (MBI-11-13, MBL), anti-myc (9E10, sc-40, Santa Cruz Biotechnology), and Pgk1(22C5-A6457, Invitrogen).
2.5 Cell Based Assays

2.5.1 Fluorescence activated cell sorter (FACS) analysis

FACS analysis was performed according to the protocol described in Vanoli et al., 2010 using SYTOX green staining (Invitrogen). For FACS, 2 ml of 1-2x 10^7 cells were collected and centrifuged at 4000 rpm for 1 min. The cell pellet was then washed in 1 ml of 50 mM Tris-HCL, pH-7.5 and centrifuged again to recover the pellet. The cells were then treated with 200 µl of 50 mM Tris-HCl containing 2 mg/ml of RNase A (Sigma-Aldrich) for at least 3 hrs at 37°C. After RNase treatment, cells were pelleted again and resuspended in 200 µl of 50 mM Tris-HCl containing 1 mg/ml Proteinase K (Roche) and incubated at 50°C for 30 min. The cell pellet was recovered after this incubation by centrifuging again at 4000 rpm for 1 min. Cells were then resuspended in 500 µl of 50 mM Tris-HCl. 100 µl of this mixture was taken from the cell suspension and added to 1 ml of Tris-HCl pH7.5 50 mM along with 1 µl of 5 mM sytox green (final concentration of 1 mM). Samples are then sonicated for 3 sec and analyzed using FACSCalibur™ Flow Cytometer for FL1H fluorescence.

2.5.2 Spot assay

Yeast cells were grown at 25°C/28°C (based on the type of mutants) overnight to obtain an exponentially grown culture. Cells were counted and normalized to a concentration of 1x10^7 cells and then a serial dilution of 1:10 for 6 times was made and spotted on different drug containing plates with indicated concentrations (see RESULTS section) and were incubated for 3 days at 28°C. The plates were then scanned at the end of 3rd day.

2.5.3 Chromatin immunoprecipitation (ChIP)-qPCR

Protein-DNA complexes were stabilized by formaldehyde, and the DNA is sheared using sonication. The sheared DNA bound to a protein is immuno-precipitated using the protein-specific antibody or an antibody against a tag, and then this DNA fragment was PCR amplified to detect the amount of DNA bound to the protein.

Solutions used:

1) 1X PBS
- 137 mM NaCl
- 10 mM PO_4 (pH-7.4)
- 2.7 mM KCl

2) 1X TBS
- 20 mM Tris-HCl (pH 7.5),
- 150mM NaCl

3) 1X TE
- 10 mM Tris-HCl pH 8.0,
1 mM EDTA

4) 1XPBS with BSA. - 1X PBS + 5mg/ml BSA

These solutions were filter sterilized

5) Lysis buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes-KOH pH 7.5</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>140 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>Triton-X100</td>
<td>1%</td>
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<tr>
<td>Sodium deoxycholate</td>
<td>0.1%</td>
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</table>

6) Wash Buffer

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<td>LiCl</td>
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<tr>
<td>NP-40</td>
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<tr>
<td>Na-deoxycholate</td>
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<tr>
<td>EDTA</td>
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</tr>
</tbody>
</table>

7) Elution Buffer

<table>
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<tr>
<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>50 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
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</tbody>
</table>

8) TE -1% SDS

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
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<td>10 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
</tr>
</tbody>
</table>

DAY 1 : Inoculation of culture and preparation of magnetic beads with antibody

1. The selected strains are inoculated in 50 ml of YPD medium and incubated at 25°C overnight to get a final cell count of 1x10^7 cells/ml.
2. 1.7 ml prelubricated tube containing required volume of magnetic beads (Dynabeads protein G, Invitrogen) (15 µl/1 ml of sample) was prepared.
3. The tube was placed on a magnetic grid for the magnetic beads to get attached on to the surface of the tube. After attachment, the supernatant was removed with a vacuum pump.

4. The attached bead were then washed twice with 1ml of ice-cold 1X PBS/BSA and later removed again using vacuum pump.

5. The beads were resuspended in required volume of PBS with BSA along with adequate concentration of antibody.

6. The tube is then kept for overnight rotation at 4°C.

---

**DAY 2 : Sample collection, chromatin extract preparation and immuno-precipitation**

1. Exponentially grown cells were arrested in G1 with α-factor and released at 28°C in media containing 0.033% MMS. For each indicated time point 50 ml of the culture is then transferred to a falcon tube containing 1.350 ml of 37% formaldehyde to obtain a final concentration of 1%. The tubes are rotated at room temperature for 30 min for efficient cross-linking.

2. In order to stop the cross linking, 2 ml of 2.5 M glycine was added to this solution and then vortexed for another 15 min.

3. The cells are recovered by centrifugation and is then washed thrice with 20 ml of ice-cold 1X TBS followed by centrifugation at 1850 g for 3 min at 4°C.

4. The pellet obtained is further treated with 0.8 ml of cold lysis buffer that is further supplemented with EDTA-free Protease Inhibitor Tablets (Sigma-Aldrich).

5. Transfer each 0.4 ml of this lysis buffer to two separate 2ml O-ring screw-cap tubes. Add glass beads (Sigma-Aldrich) up to 1 mm below the buffer’s meniscus (1 ml of beads approximately).

6. Cells were lysed using FastPrep-24™ with the multi bead shoker under the following setting: speed-6.5, time-20 sec, Pause for 5 min after each cycle.

7. The cell extract was then recovered from the beads by making a hole in the bottom of the centrifuge tube using a needle. The collected extracts were then centrifuged at 0.8 g for 5 min at 4°C and the lysate is recovered.

8. 0.45 ml of supplemented lysis buffer is now added on to the pellet without resuspending it. The chromatin is sheared by applying 5 sonication cycles for 15 sec at 1.5 tune. After each round of sonication, the chromatin is pelleted by centrifugation at 2300 g for 1 min at 4°C.
9. At the end of 5 cycles, the sheared chromatin is obtained as a pellet by centrifugation at 16000 g for 5 min at 4°C.

10. 10 µl of this supernatant (now considered as input) is added into another 1.5 ml centrifuge tube containing 190 µl of TE buffer with 1% SDS.

11. The remaining supernatant is then transferred to another fresh 1.7 ml pre-lubricated tube. The pre-washed magnetic beads were then added into each pre-lubricated tube: 15 µl per tube.

12. This is then incubated overnight by rotation at 4°C.

DAY 3: Washing of beads and reverse crosslink:

1. The tubes containing magnetic beads are now placed on a magnetic stand. 5 µl of the supernatant is now transferred to another tube to be used as hybridization control (SUP). Another 5 µl is collected for WB for the analysis of IP efficiency.

2. Once the beads (with DNA) are attached to the magnet, the supernatant is cleared by vacuum pump and the beads are washed with the following reagents:

   a) Twice with 1 ml ice-cold lysis buffer

   b) Twice with ice-cold lysis buffer with 360 mM NaCl

   c) Twice with 1 ml ice-cold wash buffer

   d) Once with 1 ml ice-cold 1X TE pH-8

3. After washing the beads were centrifuged at 800 g for 3 min at 4°C. The tubes were placed back in the magnetic grid and the remaining liquid was removed using a vacuum pump.

4. The beads were then resuspended with 40 µl of elution buffer and were incubated at 65°C for 10 min at 1200 rpm.

5. The beads are then centrifuged for 1 min at 16000 g at room temperature. The tubes with beads are then kept back in the magnetic grid. 5 µl of this mix was taken for western blot analysis.

6. The remaining IP fractions are then added another tube with 35-40 µl of TE buffer with 1% SDS. The tubes are then incubated overnight at 65°C in order to reverse the crosslink.
DAY 4: DNA Purification

1. The samples were pelleted with a short spin.
2. The following reagents were added to Input and IP samples:
   For Input: TE buffer - 89.5 µl, Glycogen (20 mg/ml) – 3 µl, Proteinase K (50 mg/ml) – 7.5 µl
   For IP: TE buffer- 44.75 µl, Glycogen (20 mg/ml) – 1.5 µl, Proteinase K (50 mg/ml) – 3.75 µl
3. The samples were mixed well by manual vortexing and then incubated at 37°C for 2 hrs.
4. 20 µl of sodium acetate (3 M) and 500 µl of 100% ethanol were added to these tubes which was then vortexed and incubated at -20°C overnight.
5. The next day, samples were again centrifuged for 10 min at 4°C at 13000 rpm.
6. The supernatant was removed and the DNA was washed with 500 µl of 70% ethanol. This was then centrifuged and resuspended in 25 µl of 1X TE.
7. The samples were kept at 30°C for 30 min and is later stored in -20°C.
8. Quanti-Fast kit (SYBR green PCR kit, Qiagen) was used for setting up qPCR reactions and the instructions of the manufacturer was followed with additional changes:
9. Input DNA was diluted 10 times and the IP was diluted 5 times.
10. 20 µl of the reaction mix was added on to the qPCR plate along with the forward and reverse primers. 5 µl of the DNA is added into the plates.
11. The samples were placed in a thermal cycler under the following conditions:
    16°C for 20 min
    24°C for 20 min
    37°C for 20 min
    75°C for 5 min
    4°C hold
12. Each ChIP experiment was repeated 3 times and each qPCR was performed in triplicates using a Roche Light Cycler 96 system. Forward and reverse primers were used for the early replication of origin region, ARS305, ARS607 and for a region away from ARS609(Negative control).
13. The 2ΔΔC(T) method was used for qPCR analysis as previously described (Livak & Schmittgen, 2001).
2.5.4 BrdU-qPCR

For this technique, yeast cells were engineered in such a way that it is can incorporate a thymidine analog, 5-bromo-2’-deoxyuridine (BrdU) into the newly-synthesized DNA. The cells here are grown in YPD medium containing BrdU. Following this, genomic DNA extraction was carried out and BrdU labelled DNA was immunoprecipitated. This DNA was amplified by qPCR to detect the amount of BrdU incorporated. In this context, the BrdU incorporation corresponds to the origin firing efficiency. Here, we used anti-BrdU antibody-MBL M1-11-3 (MBL Lifescience) to detect the BrdU bound DNA.

Experimental protocol
1. The cells were grown overnight at 25°C in 150 ml URA medium up to 1x10^7 cells/ml.
2. The next day, cells were synchronized with α-factor and later released from G1-phase into YPD medium containing 0.033% MMS and 200 mg/ml BrdU.
3. Sample collection (150 ml) was carried out at 30 min, 60 min, and 90 min. The collected mixture was then mixed with 1.5 ml of 10% sodium azide and incubated in ice for 30 min.
4. The culture was then centrifuged using the Beckman centrifuge and the JA-14 rotor at 5000 rpm for 5 min at 4°C.
5. The pellet is then washed with sterile, ice-cold 1X TE buffer and is pelleted again by centrifuging at 3220 g, 5 min at 4°C. The excess water was removed using a vacuum pump.
6. Genomic DNA was extracted from this samples following the protocol described in QIAGEN Genomic DNA Handbook.
7. The cell pellets were resuspended in 50 ml falcon centrifuge tube with 5 ml of spheroplasting buffer (1 M sorbitol, 100 mM EDTA pH 8.0, 0.1% β-mercaptoethanol).
8. This suspension was then placed at 30°C until spheroplasts are visible under microscope.
9. The supernatant was then discarded and the pellet was then resuspended in 5 ml of G2 buffer of the QUIAGEN kit.
10. The suspension was then treated with 100 µl of RNase (10 mg/ml) which was then incubated for 30 min at 37°C.
11. Later, 100 µl of Proteinase K (20 mg/ml) was added to this suspension and then incubated for 60 min at 37°C.
12. A supernatant is obtained by centrifugation at 5000 rpm, 4°C, for 5 min.
13. This suspension is then mixed with 5 ml of QBT buffer and is applied it to the Genomic column of 100/G that was previously equilibrated with 4 ml of QBT buffer.
14. The columns were then washed two times with 7.5 ml of QC buffer.
15. Finally, the isolated DNA was eluted from the columns using 5 ml of pre-warmed QF buffer at 50°C.
16. DNA was recovered in an isopropanol-containing corex, tube which is then centrifuged for 10 min at 8100 rpm RT in a proper swing out rotor.
17. The pellet obtained is then washed in 1 ml of 70% ethanol which is again centrifuged for 5 min at 8100 rpm RT.
18. The pellet is kept for air drying and then dissolved with 250 μl of Tris HCl pH 8.10 mM and is then allowed for complete dissolving overnight at 4°C.

Protein A magnetic beads preparation
1. 20 μl of dyna-beads was used per sample and added to a Costar pre-lubricated tube.
2. The beads were washed twice with 1 ml of PBS 1X, 5 mg/ml BSA, 0.1% Tween 20
   The beads are then re-suspended in 20 μl of PBS, 5 mg/ml BSA, 0.1% Tween 20; into each IP 4 μg of anti-BrdU antibody is added.
3. The beads are then incubated overnight at 4°C, slow vortexing.

Chromatin shearing and BrdU immunoprecipitation
1. The BrdU containing DNA is sheared to 200-1000 bp fragments by sonication
2. The following parameters were used for the sonication:
   Power: 20%
   15 seconds/pulse
   5 sonication cycles
3. After each sonication cycle, the chromatin was pelleted by centrifuging at 2300 g for 1 min at 4°C.
4. Towards the end of sonication, sheared DNA was centrifuged for 5 min at 3000 rpm at 4°C
5. The antibody-beads complex that was kept for overnight incubation was washed twice with 1 ml of PBS containing 1.5 mg/mL BSA, 0,1% Tween20.
6. After the last washing step, they were then resuspended in 20 μl of 1X PBS containing 5 mg/ml BSA, 0.1% Tween20.
7. The antibody-beads complex was equally divided into two Costar pre-lubricated tubes with 10 μl per tube.
8. Sheared DNA obtained was quantified and normalized. This was boiled at 100°C for 10 min for denaturing and is then immediately plunged into ice.
9. The following solutions were added rapidly into each tube:
   - 100 μl of ice-cold 2X PBS
   - 200 μl of ice-cold PBS with 2% BSA, 0.2% Tween20

10. The DNA solution from each tube was added to 10 μl antibody-beads complex and is then incubated overnight at 4°C with vortexing.

Beads washes and DNA purification
1. The magnetic beads containing tubes were placed in a magnetic grid.
2. Once the beads got attached to the magnet, this leaves a clear supernatant. 2.5 μl + 2.5 μl of supernatant from each precipitation tube is collected and is transferred to a new 1.5 ml tube with 45 μl of 1X elution Buffer (Supernatant fraction).
3. This is then kept at room temperature.
4. The beads were then washed as follows:
   - 2X with 1 ml of ice-cold Lysis buffer
   - 2X with 1 ml of ice-cold Lysis buffer + 500 mM NaCl
     (add 72 μL of NaCl 5 M to 1 ml lysis buffer)
   - 2X with 1 ml of ice-cold washing buffer
   - 1X with 1 ml of ice-cold TE 1X pH 8
5. The tubes were placed back in the magnetic grid, the remaining TE buffer was removed using a micropipette to avoid bead aspiration.
6. The tubes were then centrifuged at 800 g 4°C for 3 min.
7. The tubes were placed back in the magnetic grid again and the remaining liquid was removed using a vacuum pump.
8. The beads are then resuspended in 50 μl of elution buffer; and then incubated at 65°C for 10 min by mixing 3 times during the incubation.
9. After incubation, the tubes were then centrifuged for 1 min at 16000 g at Room temperature.
10. The tubes were placed back in the magnetic grid and the eluted material was then transferred into new tubes.
11. Into the IP and SUP, following solutions are added:
   - 49 μl of 1X TE buffer
   - 1 ml of Proteinase K (stock 50 mg/mL)
     (The final concentration of the proteinase K is 0.5 mg/ml)
12. The tubes were then mixed without vortexing and was then incubated at 37°C for 1 hr.
13. The DNA from this mixture was purified by Qiagen PCR purification Kit. DNA was eluted with 50 μL of EB buffer.

14. The two identical IP samples were pooled together and precipitated by the addition of the following:
   - 5 μl of 3 M sodium acetate, 1 μl glycogen to the IP samples
   - 2.5 μl of 3 M sodium acetate, 0.5 μl glycogen to the SUP samples

15. 2.5 times volumes of cold 100% ethanol was added into this suspension.

16. The tubes were then incubated at -20 °C for overnight

17. The tubes were then centrifuged at ≥ 13400 g for 10 min at 4°C.

18. The supernatant was discarded using a pipette and spun again

19. The remaining ethanol was discarded using a gel loading tip.

20. The tubes were left at 37° C for 5 min and is then re-suspended in 10 μl of ddH2O

21. The precipitate was then recovered by vortex and pulse-spinning 3 times.

22. Proceed for qPCR as mentioned in the ChIP-qPCR protocol.

### 2.5.5 Electron microscopy

Sample collection, in-vivo psoralen crosslinking and DNA extraction was followed based on the 2D gel protocol which is described in the below section (2D DNA gel electrophoresis). Extracted genomic DNA was subjected for partial restriction digestion using PvuI (NEB) and the DNA fragments containing ssDNA were enriched by a column containing BND cellulose resin. Enriched DNA samples were spread on carbon coated metal grids (4-nm thickness) stained with uranyl acetate, which was further followed by platinum-based rotatory shadowing (0.4 nm without rotation and up to 8 nm with rotation). EM analysis was performed as described on (Neelsen et al., 2014).

### 2.5.6 2D DNA gel electrophoresis

For 2D methods, CTAB based DNA extraction was followed as described in (Branzei et al., 2006; Liberi, 2005; Lopes et al., 2001).

#### 2.5.6.1 Yeast DNA extraction using CTAB for 2D gel electrophoresis

Solution and Reagents to be prepared:
- a) 10% sodium azide, stored in 4°C
- b) 10 mg/ml zymolyase stock (1000U/ml)
- c) Spheroblasting buffer:
- 1 M sorbitol
- 100 mM EDTA pH 8.00,1%
- β-mercaptoethanol
- 10 mg/ml Zymolyase
d) Solution I
   - 2% w/v CTAB (FLUKA-cetyltrimethylammonium bromide)
   - 1.4 M NaCl
   - 100 mM Tris HCl pH 7.6
   - 25 mM EDTA pH 8.0
e) 10 mg/ml RNase (Sigma-Aldrich) in water
f) 20 mg/ml Proteinase K in water
g) 24:1 Chloroform/isoamyl alcohol
h) Corex glass tubes
i) Solution II
   - 1% w/v CTAB
   - 50 mM Tris HCl pH 7.6
   - 10 mM EDTA
j) Solution III
   - 1.4 M NaCl
   - 10 mM Tris HCl pH 7.6
   - 1 mM EDTA

Experimental protocol:

1) Exponential cells were arrested in G1 phase using alpha factor and synchronously released from G1 phase in 0.033% MMS containing YPD medium. 200 ml of culture was collected at the indicated time points in 250 ml centrifuge tubes containing 1% sodium azide.

2) The tubes were then kept in ice for about 15 min and then centrifuged at 5000 rpm for 5 min.

3) The cell pellet was washed in 30 ml of ice-cold water twice to remove the excess media. At the end of second washing, the cell pellet was transferred to 50 ml centrifuge tube and proceeded to perform psoralen crosslinking.
Psoralen crosslinking:
Psoralen is an efficient crosslinking agent that can intercalate into double stranded DNA upon irradiation with ultraviolet (UV) light (366 nm) by forming covalent crosslinks between pyrimidines of opposite strands. Trimethylpsoralen (TMP) is the most commonly used psoralen derivative for *in vivo* DNA crosslinking (Wellinger & Sogo, 1998).

Psoralen DNA crosslinking is done to block the branch migration of replication intermediates and thereby to preserve its original structure for further preventing 2D gel artifacts. In this way, we are able to visualize only certain structures (like template switching intermediates) while some other intermediates such as hemicatenanes is lost during UV treatment (Zardoni et al., 2020).

Solution required for psoralen crosslinking:
- 6-well plates (FALCON)
- UV stratalinker (Stratagene), 365 nm and 265 nm UV lamps

Protocol for psoralen crosslinking:
a) The collected cells in 15 ml centrifuge tube were resuspended in 5 ml of cold water and transferred to a 6-well plate (1 sample/well)
b) The 6-well plate was always kept in ice while performing psoralen-crosslinking.
c) 300 μl of psoralen solution was added in each well, mixed well using a 5 ml pipette and was then incubated for 5 min on ice itself (The plate was covered with aluminum foil to keep samples in the dark).
d) The cells are then irradiated for 10 min in a Stratalinker (Stratagene) with 365 nm UV lamps, at a distance of 3 cm from the lamp.
e) Step c) and d) is repeated for 3 more times.
f) Once the four rounds of psoralen crosslinking is done, the cells were then transferred back to 50 ml centrifuge tube and the wells of 6-well dish were washed with another 5 ml of cold water to collect all the traces of cells which was also then transferred to the same falcon tubes.
g) The pellet was recovered by centrifugation at 4000 rpm for about 2 min.

4) The psoralen crosslinked cells in the form of pellet were now re-suspended in 5 ml of spheroplasting buffer.

5) This mix was then incubated at 30°C for about 50 min. The spheroplasts are collected by centrifugation at 4000 rpm for 10 min at room temperature. The spheroplasts are also washed with 10ml of water and centrifuged again to remove the excess spheroplasting buffer.
6) The spheroplasts were then resuspended in 2 ml of water and then 2.5 ml of solution I and 300 μl of 10 mg/ml RNAse A were subsequently added to this solution. The suspension was gently mixed and incubated at 50°C for about 20 min.

7) 200 μl of 20 mg/ml Proteinase K was added to this mixture and re-incubated at 50°C again for about 1 hr 30 min. After 1.5 hrs, another 100 μl of Proteinase K was added to this mix and were incubated overnight at 30°C.

Day 2:

1) Next day, the solution was centrifuged at 4000 rpm for 15 min at room temperature. The obtained supernatant and pellet are processed separately as indicated below:

   Supernatant:

   2) After centrifugation, the supernatant was transferred to a 15 ml centrifuge tube containing 2.5 ml of 24:1 Chloroform/isoamyl alcohol.

   3) The suspension was mixed vigorously for about 30 sec and is separated by centrifugation at 4000 rpm for 10 min.

   4) After centrifugation, the clear upper phase was transferred carefully into a corex glass tube with a 5 ml pipette into which 10 ml of Solution II was added.

   5) This suspension was kept for at least 2 hrs for DNA precipitation (Note that the prolonged incubation (1-2 hrs) with Solution II might help with a better DNA precipitation.)

   6) After 2 hrs, the precipitated DNA was obtained by centrifugation at 9000 rpm for 10 min in a Beckman JS 13.1 swing out rotor.

   7) The supernatant is then discarded and the pellet is then dissolved in 2.5 ml of solution III.

   Pellet:

   8) The pellet was resuspended in 2 ml of solution III by vortexing, and then incubated at 50°C for 50 min.

   9) The solution was then transferred to a 15 ml centrifuge tube containing 2.5 ml of chloroform/isoamyl alcohol (24:1).

   10) The two phases are then separated again by centrifugation at 4000 rpm for 10 min. The upper phase was carefully transferred into the corex glass tube containing solution III obtained from the treatment of the supernatant (see treatment of “supernatant” step 7).
11) The DNA was now precipitated with 1 volume (5 ml) of isopropanol and centrifuged at 9000 rpm for 10 min in a Beckman JS 13.1 swing out rotor.

12) The supernatant was discarded and the pellet was now washed with 2 ml of 70% ethanol. The ethanol after centrifugation was removed and the excess ethanol was removed using a pipette.

13) The DNA was dissolved in 250 μl of 10 mM Tris-HCl pH 8 and allowed to dissolve overnight at 4°C. Genomic DNA extracts were stored at 4°C.

2.5.6.2 Analysis of replication intermediates by two-dimensional agarose gel electrophoresis (2D gel)

DNA replication can result in the formation of a variety of structures that differ each other by their size and shape. For example, passively replicated DNA result in the formation of Y-shaped DNA structures. 2D gel analysis allows you to separate DNA based on their size and shape complexity (L. Bell & Byers, 1983). This technique was further developed by Brewer and Fangman (Brewer & Fangman, 1987). This technique has been widely used to study replication and recombination related DNA structures at different origins of DNA replication in yeast chromosomes.

Principle:

The different shape of DNA structures results in different shift in the electrophoretic mobility of DNA molecules of equal mass. The region of interest is being digested with specific restriction enzymes and separated through a first-dimension gel, in conditions that emphasize the size differences and minimize the contribution of shape to the mobility (low agarose concentration, low voltage, no ethidium bromide). The region of specific size is then cut out from the first-dimension gel and placed and ran in a second-dimension gel where DNA runs orthogonally with respect to the first-dimension gel. The second-dimension gel is running under conditions that maximize the contribution of the shape to the mobility by means of a delay of complex structures during migration (high agarose concentration, high voltage and in the presence of ethidium bromide). The different replication structures that arises during replication origin firing, replication fork progression, pausing and recombination are depicted in the following schematic diagram (adapted from (Lucas, 2000)).

Here, 2d gel electrophoresis was performed according to protocol that was described in (Branzei et al., 2006, 2008).
Restriction digestion of DNA:

1. 10-20 μg of genomic DNA was digested following the reaction mixture (150 μl final reaction volume):
   - 10X Cut smart buffer – 15 μl
   - 100-120 units of each restriction enzyme (half of the amount is added after 30 min of incubation at 37°C)
   - 10-20 μg of DNA (concentration is based on the region of your interest)
   - Autoclaved water

2. DNA was digested overnight at 37°C.

3. 1/8V (19 μl) KAc 2.5 M pH6 (autoclaved) and 1V (169 μl) of 100% isopropanol was added the next day into this mixture, inverted delicately and kept at least for 1 hr at -20°C for better precipitation.

4. The samples were then centrifuged at 14000 rpm for 10 min at RT. The DNA was then washed in 70% ethanol and centrifuged again at 14000 rpm for 10 min at RT.

5. The supernatant was discarded and the remaining excess ethanol is removed using a pipette after a short spin.

6. The tubes were then kept at 37°C for about 10 min to dry the DNA and later, 20 μl 1X TE buffer was added to each tube. The tubes are then kept at 30°C, 300 rpm for dissolving at least for 3 hrs.

The following restriction enzymes are used for digesting the regions of interest. All the enzymes are purchased from New England Biolabs (NEB).
- Ncol: ARS 305
- EcoRI and HindIII: ARS305
- HindIII and PstI: TER302

First-dimension electrophoresis:

1. 0.35% agarose gel (Low EEO agarose without EtBr in 500 ml 1X TBE buffer) was prepared, poured and stored at 4°C for almost 1 hr.

2. 4 μl of 20X loading dye (Promega) was added to the digested DNA that was dissolved in 20 μl of TE buffer. This DNA was loaded in alternate wells of the first-dimension gel.

3. The gels were run at 50 V, 40 mA at room temperature in 1X TBE running buffer for about 18-20 hrs (time depends on the size of the fragment of interest).
Second-dimension electrophoresis:
1. After the running, the gel was stained with 10 mg/ml EtBr containing 1X TBE buffer in a plastic tray for 30 min.
2. Using the 1 kb marker as reference, the gel was cut in order to keep only the fragment(s) of interest. Individual slices are obtained by cutting in between the gel slices.
3. The slices were then arranged in a new tray allowing 12 cm of space for the second-dimension migration.
4. The second-dimension gel was poured above these slices at room temperature (0.9% low EEO agarose, 1 X TBE, 15 μl EtBr) and kept undisturbed for 30 min for complete solidification.
5. 2 L of cold 1X TBE buffer supplemented with 60 μl EtBr was prepared as running buffer for each second-dimension gel
6. The second-dimension gel runs under cold conditions with the following settings: 180 V, 140 mA, 7-9 hrs (time depends on the size of the fragment of interest).

Southern Blotting:
1. The second-dimension gels were then transferred to a UV stratalinker to de-crosslink the psoralen by irradiating them with 265 nm UV bulbs.
2. The gels were then treated with the following solutions with agitation:
   - HCl 0.25 N (1 x 8min)
   - Denaturing solution (0.5 M NaOH, 1.5 M NaCl) (1 x 20 min)
   - Blot # 2 (1 M ammonium acetate, 0.02 M NaOH; prepared fresh) (1 x 20min)
3. In the meantime, the genescreen membrane is equilibrated in 10 X SSC buffer.
4. Southern blotting was then set up in the following order: 3 M paper, gel, genescreen membrane, two wet 3 M paper, two dry 3 M paper, a pile of tissue and 1 kg weight on the top.
5. This set up was then kept intact overnight.
6. The next day, the above southern blotting apparatus was disassembled, the genescreen membrane was kept for drying at room temperature for around 30min. The DNA on the membrane was then auto crosslinked with UV irradiation (auto cross-linking program, with 265 nm UV lamps on Stratalinker).
Radioactive Hybridization:
1. 30 ml of pre-warmed Perfect Hyb plus solution (Sigma-Aldrich) was added to pre-warmed tubes.
2. The genescreen membrane was washed in water and positioned inside this pre-warmed tube containing Perfect Hyb plus solution with the DNA on the membrane facing inside.
3. The tubes were then incubated with rotation at 65°C for at least 1 hr, until probe is ready.
4. In the mean-time, the hybridization reaction using prime-a-gene labelling kit (Promega) was set up.
5. The template for desired area of interest was amplified using specific primers.

ARS305
ARS305F: CTCCGTTTTTAGCCCCCCGTG
ARS305R: GATTGAGGCCACAGCAAGACCG

TER302
TER302Fw: GAAGGTCAAACATCAATTTGATTGATTCTGCGCCCATGATC
TER302Rv: GCTTCCCTAGAACCCTTTATTATGTGGTACATGCGCTGGTGA

6. 50 ng of template DNA with water was boiled for 10 min before adding the rest of the reagents.
7. The hybridization reaction is set up as follows:
   - 50 ng of DNA
   - 30.4 ml H$_2$O
   - 10 ml 5X Buffer
   - 2 ml BSA
   - 0.7 ml of dATP, dTTP, dGTP solutions
   - 3-5 units of Klenow DNA polymerase
   - 50 μcurie of radioactive alpha-dCTP

8. The reaction was incubated at room temperature for about 60 min to allow incorporation of the radioactive nucleotides in the DNA fragments.

9. The radioactive DNA was recovered from this reaction mixture by using a G50 column (1st centrifuge 3000 rpm 1 min, change tube, add reaction, wait 1-2 min and spin again).

10. Both the labelled DNA and single-stranded carrier DNA was boiled for 10min and is added to the hybridization tube.

11. The tubes are then incubated at 65°C overnight in constant rotation prior to washing.

Washing of the Filters:

1. Prepare for each tube 500 ml Washing Solution I and 1000 ml Washing Solution II

<table>
<thead>
<tr>
<th>Washing Solution I (65°C)</th>
<th>Washing Solution II (42°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC 2x</td>
<td>20x SSC 0.1x</td>
</tr>
<tr>
<td>50 ml SSC</td>
<td>5ml SSC 20x</td>
</tr>
<tr>
<td>SDS 1%</td>
<td>SDS 0.1%</td>
</tr>
<tr>
<td>25 ml SDS 20%</td>
<td>5ml SDS 20%</td>
</tr>
<tr>
<td>Final volume 500ml H2O</td>
<td>Final volume 1000ml H2O</td>
</tr>
</tbody>
</table>

2. Wash the filters in the following order:

   50 ml Wash Sol. I at 65°C in tube 15 min, tube rotation
   450 ml Wash Sol. I at 65°C in a tray 20 min with agitation
   500 ml Wash Sol. II at 42°C in a tray 20 min with agitation
   500 ml Wash Sol. II a 42°C in a tray 20 min with agitation

3. The membrane was then dried using a 3 M paper cover with saran wrap and expose to a storage phosphor screen in an appropriate cassette.

4. The phospho-screen was then scanned using a Typhoon Scanner (GE healthcare)
Figure 2.2 Schematic representation of different replication fork signals obtained in the end of 2D gel electrophores (Lucas, 2000)

Using 2D gel electrophoresis we will able to visualize different replication as well as recombination intermediates. From left to right: single replication forks, replication bubbles, double replication forks, recombination intermediates (in the form of spikes).

Re-probing method:
- Boil a solution of 0.1 XSSC, 1% SDS
- Add it to the filter and agitate for 15-20 min at 65°C
- Wash filter with water

Quantification of replication intermediates
Quantification of X-shaped intermediate signals were performed using the Image Quant software (GE Healthcare) as described in (Branzei et al., 2008; Vanoli et al., 2010). For each time point, areas corresponding to the monomer spot (M), the X-spike signal and a region without any replication intermediates as background reference were selected and the signal intensities (SI) in percentage of each signal were obtained. The values for the X and monomer were corrected by subtracting from the SI value the background value after the latter was multiplied for the ratio between the dimension of the area for the intermediate of interest and for background. Thus, the values for X and M were calculated in the following way:
\[ X = SI(X) - \frac{SI(\text{background}) \cdot A(X)}{A(\text{background})} \]

\[ M = SI(M) - \frac{SI(\text{background}) \cdot A(M)}{A(\text{background})} \]

The relative signal intensity for the X was then determined by dividing the value for X with the sum of the total signals (the sum of the X and monomer values).

\[
\text{Spike} = \frac{X}{X + M}
\]

The resulting values for X signals were then normalized and converted to percentage by using the highest value number of X for each experiment as 100 and normalizing the other values to it.
3. AIM AND RATIONALE

DDK is an enzyme that is known to phosphorylate various factors and thereby regulate several important functions within the cell. For instance, DDK functions during replication initiation by phosphorylating different subunits of MCM helicase complex (Sheu & Stillman, 2006). During DNA damaging conditions, DDK is known to play roles in Translesion synthesis pathway by phosphorylating Rev7 subunit of the Translesion polymerase (Brandão et al., 2014; Pessoa-Brandão & Sclafani, 2004). DDK regulates recombination during meiosis by phosphorylating Mer2, a meiosis-specific double stranded break protein (Murakami & Keeney, 2014). DDK is also shown to promote Mus81-Mms4 resolvase activation during mitosis (Princz et al., 2017).

Here, in this project, we asked whether DDK has any roles in mitotic recombination during DNA damaging conditions. Several reports in literature have paved way to this hypothesis. For instance, in literature it was shown that the Rad 9 subunit of the 9-1-1 complex in fission yeast is a substrate for DDK which undergoes phosphorylation during DNA damaging conditions. 9-1-1 complex by itself was identified to promote the error-free DNA damage tolerance pathway (TS) in budding yeast (Furuya et al., 2010; Karras et al., 2013). It can be that Rad9 phosphorylation by DDK is required for its roles in the template switching pathway. Also, DDK is shown to interact with cohesin and is also required to load the cohesin loaders prior replication initiation. In addition to this, DDK was also shown to be involved in regulating the recruitment and retention of Tof1-Csm3 complex thereby promoting programmed fork arrest. Tof1-Csm3 is known to participate in many replication-associated functions, including sister chromatid cohesion (Murakami & Keeney, 2014; Natsume et al., 2013; T. S. Takahashi et al., 2008). Moreover, our lab has uncovered the importance of sister chromatid cohesion during Error-free recombination (Fumasoni et al., 2015). It is possible that DDK acts in one or many of the above-mentioned substrates to regulate recombination during stress conditions.

In other aspects, DDK is also known to phosphorylate the checkpoint protein claspin to support optimal replication fork speed. It was also reported that DDK is recruited to chromatin in a checkpoint dependent manner (Gold & Dunphy, 2010; Yanow et al., 2003). Our lab has previously showed the importance of replication checkpoint in template switching by preventing the premature action of Mus81-Mms4 nuclease on the recombination intermediates (Szakal & Branzei, 2013). It could be that DDK can indirectly
regulate template switching though its roles in checkpoint. All these possibilities has strengthened our hypothesis that DDK might regulate recombination in a direct and/or in an indirect manner.

In our experiments, we used methyl methane sulphonate (MMS) as the DNA damaging agent to induce genotoxic stress. MMS is an alkylating agent that modifies both guanine (to 7-methylguanine) and adenine (to 3-methyladenine) to cause base mismatches and thereby replication blocks.

Using *Saccharomyces cerevisiae* as a model system, here we tried to investigate the contribution of DDK towards replication associated recombination during MMS induced genotoxic stress conditions.
4. RESULTS

Part A: Characterization and validation of different DDK mutants and their functional significance in mitotic recombination

3.1 Experimental optimization of different ddk mutants for 2D analysis

3.1.1 Construction of various ddk mutants

In order to understand the roles of DDK kinase in mitotic recombination, we first decided to make use of existing ddk mutants and create new conditional mutants. The first type was the temperature sensitive alleles of DDK that were already available in the lab; cdc7-4 and dbf4-1 (Leland H. Hartwell, 1971). Mutations in Cdc7 or Dbf4 genes renders them inactive at high temperature (37°C) (Fig. 3.1). Therefore, we initiated the study by shifting these mutants at 37°C after initiation of DNA replication to completely inactivate them for the round of replication under investigation. Subsequently, we found that the mutant at semi-permissive temperature (28°C) is already defective in its function in recombination, but did not affected the origin firing function of DDK (as verified in our BrdU experiments shown in Figure 3.6). As DDK is an essential enzyme required for its function in initiating replication, we wanted to make sure that we are able to separate the origin firing function of DDK from its recombination function. For this reason, we performed the later experiments with the temperature sensitive mutant at 28°C.

Figure 3.1. Schematic representation of DDK subunits (Hartwell 1971)

DDK is composed of two subunits: The enzymatic subunit is called Cdc7(507aa) and the regulatory subunit is called Dbf4 (704aa). Cdc7 consists of 3 kinase domains while Dbf4 consists of N, M and C motifs which is required for its binding with Cdc7 and the DNA.
The other type of mutant system that we used was the AID conditional degron of DDK mutants. Auxin-inducible degradation (AID) system can induce rapid and reversible depletion of the desired protein. Here, auxin mediates the interaction of the AID degron domain that is inserted as a tag (here as a C-terminal tag to Cdc7) with the F-box protein TIR1. This leads to the ubiquitylation of the target protein by recruitment of an SCF-type ubiquitin ligase (Morawska & Ulrich, 2013). We established the cdc7-AID mutant where Cdc7 protein can be conditionally degraded by the addition of auxin. We verified that the cdc7-AID strain is lethal when grown in the presence of auxin as Cdc7 is essential (Fig. 3.2).

Figure 3.2. Construction of auxin inducible conditional DDK mutants
a) Schematic representation of auxin-induced degradation system (adapted from (Nishimura et al., 2009)). The protein of interest (here, Cdc7) is further fused to an AID tag in a yeast
strain that also expresses an F-box protein called TIR1. Auxin addition mediates the interaction of TIR1 with AID and this interaction recruits E2 and E3 enzymes that polyubiquitylate the AID tag, targeting it for proteasome degradation b) Schematic illustration of Cdc7 carrying a C-terminus AID tag. c) Spot assay analysis of cdc7-AID strains on YPD plates supplemented with 1mM of auxin. Plates were scanned after 3 days at 28°C.

Apart from the above mutants, we used a third type of DDK mutant, called ddk-KR which was reported by our lab to be SUMOylation defective but proficient in origin firing. It contains mutations within the DDK SUMOylation sites - cdc7-K16R, dbf4-K6R, K14R, K432R (Fig. 3.3) (Psakhye et al., 2019). This particular mutant is considered to be a "stronger DDK" with regard to its efficiency in origin firing as it is not targeted for degradation and shows higher levels of Mcm4 phosphorylation (Psakhye et al., 2019).

Figure 3.3. Schematic diagram of ddk-KR mutant with the respective mutations on DDK subunits (Psakhye et al., 2019)
Schematic drawing of Cdc7 and Dbf4 subunits of DDK with mutations in the major SUMOylation sites.

3.1.2 Cell cycle analysis of ddk mutants to optimize the experimental conditions for further experiments
As DDK is implicated in initiating DNA replication (Leland H. Hartwell, 1973), it was pivotal to verify that in our experiments, replication initiation is not affected because of DDK inactivation. Accordingly, for the temperature sensitive mutants we synchronized the cells in G1 phase by the addition of alpha factor and then decided to release them into S phase in 0.033% MMS containing medium at permissive temperature (25°C) for 20 min to allow for origin firing to occur. The cells were then shifted to pre-warmed medium at the restrictive temperature of 37°C to inactivate the Cdc7 protein. Cultures kept at 25°C during the whole experiment were used as control. Under these conditions, cdc7-4 and dbf4-1 mutants could
be released in S phase (Fig. 3.4). But after performing a few experiments at 37°C we also decided to try whether we can already see similar effects using semi-permissive temperatures for cdc7-4 and dbf4-1, that is, 28°C. To this purpose, we grew the cells overnight at 25°C and then synchronously released them from G1 phase into S phase at 28°C. We were able to obtain similar results for our experiments. We decided to proceed with the semi-permissive temperature for ddk temperature sensitive mutants for the future experiments.

Figure 3.4. ddk<sup>ts</sup> mutants progress well into S-phase with the optimized experimental condition

Experimental conditions for 2D gel electrophoresis using the cdc7-AID strain were standardized by analyzing the FACS data. Exponential cells were synchronized in G1 phase using alpha factor and released in permissive temperature (25°C) for the initial 20 min and then the cells were shifted to a non-permissive temperature (37°C) to inactivate Cdc7 or Dbf4 gene. Samples for FACS were collected every 30 min. Cultures kept at 25°C all throughout the experiment served as control. 1N and 2N below the graph represents G1 and G2/M phases of the cell cycle, respectively.

We followed the similar procedure also for the conditional AID mutant of DDK. G1 synchronized cells were released into S-phase containing 0.033% MMS for about 20 min for the replication initiation to occur prior to the addition of auxin. Remarkably, cells
progressed fine in S phase without any delay in G1 after the conditional depletion of Cdc7 by auxin (Fig. 3.5).

**Figure 3.5. Conditional AID ddk mutants progressed through S-phase with the optimized experimental conditions**

Experimental conditions for 2D gel electrophoresis using the *cdc7-AID* strain were standardized by analyzing the FACS data. Exponentially grown cells were synchronously released from G1 phase into 0.033% MMS containing YPD medium. Auxin was added after 20 min of its release from G1 phase to conditionally deplete Cdc7 and depletion of Cdc7 protein was verified using western blotting and the samples were collected for the indicated timepoints. Cdc7-protein levels were detected using anti-myc and Pgk1 is served as loading control which was immunodetected by anti-Pgk1. FACS samples were collected every 20 min from the G1 release. 1N and 2N below the graphs indicate G1 and G2/M phases respectively.

**3.1.3 DDK effects in replication-associated recombination are not due to its defective origin firing**

To further validate whether DNA replication initiation is not hampered in *ddk* mutants, we decided to measure the origin firing efficiency in these mutants with our standardized experimental condition (Heller et al., 2011). To estimate the origin firing efficiency, we performed a quantitative ChIP assay for measuring the amount of the thymidine analog
bromodeoxyuridine (BrdU) incorporated proximal to the early replication origin ARS305 where we observed recombination defects, as described below (Higuchi et al., 1993). Wildtype and cdc7-4 cells were released from G1 into 0.033% MMS containing medium for 90 min at 28°C and samples were collected at the indicated time-points for the BrdU-qPCR assay (Fig. 3.6). Since it is known that Cdc7 protein is inactivated at 37°C in cdc7-4 mutant, we used this non-permissive temperature as a control. We observe similar origin firing efficiency for wildtype and cdc7-4 at 28°C at which the 2D gel electrophoresis experiments were performed. Furthermore, at the non-permissive temperature (37°C) where Cdc7-4 becomes inactive, we observed a strong reduction in the firing of origins in cdc7-4 mutant as expected (Fig. 3.6). Therefore, we conclude that any defects we may observe in ddk mutants under this experimental condition, are not a consequence of defective origin firing.

Figure 3.6. DDK mutants have similar origin firing efficiency with wildtype cells
Exponentially grown culture of Wildtype and cdc7-4 cells were arrested in G1-phase and then released at a temperature of 28°C and 37°C in YPD medium containing 0.033% MMS and 200 μg/ml BrdU. Samples were collected at the indicated time points and BrdU incorporation was analyzed at these time points for ARS305 region by BrdU ChIP-qPCR assay. BrdU fold increase was calculated as a ratio: IP/Input. Each chip experiment was repeated three times and each real time PCR was performed in triplicates. Error bars represent the SEM (mean value +/- standard error of mean) of three independent experiments.
3.2 2D gel analysis to study DDK’s roles in recombination

Using 2D gel electrophoresis, we studied the recombination intermediates arising proximal to stalled replication forks. This was done by monitoring X-shaped structures composed of sister chromatid junctions (SCJs) forming at the vicinity of replication origins (Fig. 3.7). Here, we are studying the replication intermediates that are formed at an early origin of replication (ARS305) located in Chromosome III (Friedman & Brewer, 1995). Using 2D gel electrophoresis, we can not only study the recombination intermediates, but even termination intermediates. Termination intermediates will appear in a different shape in 2D, as these molecules migrate in a different pattern compared to the recombination intermediates. Hybridizing different probes for different regions along the length of the given restriction fragment provide information about the movement of replication fork progression and also facilitate the detection of replication termination points (Brewer & Fangman, 1991; Little et al., 1993). For this assay, G1-phase synchronized cells are released in YPD medium containing the alkylating reagent methyl methane sulfonate (MMS) and the pattern of different replication intermediates was analyzed at different time points during replication.

**Figure 3.7. Schematic illustration of final result of replication intermediates revealed by 2D gel electrophoresis and the 2D gel fragment analyzed**

Left: Schematic representation of replication intermediates detected in 2D gel electrophoresis. Here, monomer spot represents linear un-replicated molecules. Y arc indicates single replication forks and bubble arc represents bidirectional replication forks. The vertical spike (X-shaped molecules) corresponds to recombination intermediates.

Right: depicts the genomic region containing ARS305 spanned by Neol restriction sites, where the 2D gel signals are analyzed (Right).

Previous studies have reported the formation of recombination intermediates during replication of DNA damage. These structures accumulate when the Sgs1-Top3-Rmi1 complex is defective because of their compromised processing (Branzei et al., 2008;
Giannattasio et al., 2014; Liberi, 2005). As a consequence, sgs1Δ cells replicating in the presence of damaged templates accumulate recombination structures which can be visualized by 2D gel electrophoresis (Liberi, 2005) (Fig. 3.8). To gain insights into the contribution of DDK on recombination, we decided to work in an Sgs1 defective background as this increases our chances to detect contributions to this process.

Figure 3.8. Construction ddk sgs1 mutant
Left: schematic representing the role of Sgs1-Top3-Rmi1 complex in the initial processing of recombination intermediates that arises during DNA damage. Middle: Represents final 2D picture depicting the accumulation of recombination structures in sgs1Δ/Tc-sgs1 cells
Right: ddk mutants were combined with sgs1 mutants for 2D gel electrophoresis

3.2.1 Temperature inactivation of DDK causes reduction in recombination
As sgs1Δ cells have synthetic growth defects with several mutants affecting replication (Ooi et al., 2003), here we decided to alleviate this problem by using a tetracycline inducible conditional system of Sgs1. In Tc-sgs1 cells, Sgs1 protein translation is inhibited upon addition of Tetracycline (Gonzalez-Huici et al., 2014). We combined temperature sensitive mutants of DDK with Tc-sgs1 mutant for 2D experiments. For this assay, log-phase culture of Tc-sgs1, cdc7-4 Tc-sgs1 and dbf4-1 Tc-sgs1 that were grown at 25°C were synchronously released from G1 phase in YPD media containing 0.033% Methyl Methane Sulfonate (MMS) at 28°C. Samples were collected at 3 different timepoints (90 min, 120 min and 150 min respectively) and the pattern of recombination intermediates at the early replication origin ARS305 was then analyzed by 2D gel electrophoresis (Fig 3.7). Tc-sgs1 mutant was used as a control here which showed accumulation of recombination intermediates upon Tetracycline induced Sgs1 depletion (Liberi et al., 2005, Vanoli et al., 2010). Notably, we
observed a defect in the accumulation of X-shaped SCJs in *cdc7-4* and *dbf4-1* combined with *Tc-sgs1* when compared to *Tc-sgs1* alone, in conditions of similar depletion efficiency of Tc-HA-Sgs1 (Fig. 3.9).

**Figure 3.9. DDK promotes recombination**

*Tc-sgs1, cdc7-4 Tc-sgs1* and *dbf4-1 Tc-sgs1* cells were synchronously released from G1 arrest in medium containing 0.033% MMS and tetracycline (1mM) to induce Sgs1 depletion. Samples were collected at the indicated time points, followed by *in vivo* psoralen cross-linking. Genomic DNA was extracted from the samples and was subjected for digestion using NcoI restriction enzyme. 2D gels were analyzed using a probe for ARS305. Using western blotting, Sgs1 depletion was visualized using HA antibody while Pgk1 was served as loading control. FACS plots are also indicated for each strain. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.
3.2.2 Conditional depletion of DDK using auxin also caused reduction in recombination intermediate accumulation

Similar to temperature sensitive mutants, a 2D gel assay was also conducted in the conditional AID mutant of DDK, the cdc7-AID allele. Using sgs1Δ cells as control, sgs1Δ and cdc7-AID sgs1Δ cells grown at 28°C were synchronously released from G1 in 0.033% MMS containing media without the addition of auxin for 20min for origin firing to occur and then auxin was added to induce depletion of Cdc7. Samples were collected at three different time intervals. Markedly, we observed that the conditional depletion of Cdc7 also resulted in a decreased accumulation of X-shaped Sister Chromatid Junctions (SCJs) in the sgs1Δ background at ARS305, altogether indicating a role for DDK in recombination (Fig. 3.10).

![2D gel assay](image)

**Figure 3.10. DDK supports recombination**

Exponentially grown sgs1Δ and cdc7-AID sgs1Δ cells were released synchronously from G1 phase into 0.033% MMS containing media at 28°C. Auxin was added after 20 min of G1
release in cdc7-AID sgs1Δ to conditionally deplete Cdc7. Cells were collected at the indicated time points, psoralen crosslinked and the extracted DNA was further digested with NcoI restriction enzyme and was subjected for 2D gel analysis. Using western blotting, Cdc7 depletion was visualized using anti-myc antibody while Pgk1 was served as loading control. FACS plots are also indicated for each strain. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.

3.2.3 Enzymatic activity of DDK is required for its recombination function
As DDK is an important kinase that can phosphorylate various substrates, we asked whether the enzymatic activity of DDK accounts for its ability to support replication-associated recombination. In order to address this, we decided to work with a DDK mutant that is defective in its enzymatic activity, an analog sensitive cdc7-as3 allele. This is a conditional mutant of Cdc7 which will be inactivated based on a chemical genetic approach. In this allele, mutations were created in the ATP binding pocket of Cdc7 to enlarge the pocket to fit other purine analogs (Bishop et al., 2001). Addition of a purine analog, PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo[3,4-d]- pyrimidine) drug to a culture of cdc7-as3 inhibits the enzymatic activity of Cdc7. Using 2D gel electrophoresis, we examined the recombination intermediates formed in cdc7-as3 mutant, when it is inactivated. For this purpose, we constructed cdc7-as3 sgs1Δ mutant and used this mutant for 2D assay, keeping sgs1Δ as control. PP1 drug was added at a final concentration of 15 μM to the culture after 30 min of its release from G1 phase. Samples were collected for 2 time points, 90 min and 120 min and the recombination intermediates were analyzed for early replication origin, ARS305. We observed a reduction in the accumulation of recombination intermediates in cdc7-as3 mutant compared to control cells, highlighting the importance of the Cdc7 kinase activity in replication associated recombination (Fig. 3.11).
Figure 3.11. Kinase activity of DDK is required for recombination

Exponential cultures of sgs1Δ and cdc7-as3 sgs1Δ cells were arrested at 28°C in alpha factor and released in 0.033% MMS containing media. PP1 drug of 15 μM concentration was added after 20 min from G1 release to inhibit Cdc7. Samples were collected at 90 min and 120 min, psoralen crosslinked and extracted DNA was digested with NcoI and subjected for 2D gel analysis. Cell cycle progression of each strain was monitored by analyzing FACS plots. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.

3.2.4 SUMOylation defective ddk-KR mutant exhibits recombination defect

Recently, we have used a SUMOylation defective ddk-KR mutant where the major SUMOylation sites on both the subunits of DDK have been mutated. BrdU incorporation analysis revealed that the origin firing efficiency of this mutant is not affected and acts even slightly better than the wildtype (Psakhye et al., 2019). To assess the effect of this DDK
mutant on recombination during DNA damaging conditions, when DDK essential function in replication initiation remains undisturbed, we performed a 2D gel electrophoresis and monitored recombination intermediates at ARS305 region for ddk-KR allele. As performed for DDK temperature sensitive mutants and AID conditional alleles, ddk-KR was also combined with Tc-sgs1 allele for visualization and comparison of recombination intermediates. The experiment was conducted in exponentially grown cells, with cells arrested in G1 phase at 25°C and released at 28°C in 0.033% MMS containing medium. Samples for 2D were collected at 90 min, 120 min and 150 min. Remarkably, we obtained a reduction in the formation of recombination intermediates in ddk-KR Tc-sgs1 mutant compared to Tc-sgs1 alone at ARS305. Altogether, these results suggest that DDK supports replication-associated recombination and this function is uncoupled from its role in initiating genomic replication (Fig. 3.12).

Figure 3.12. ddk-KR shows replication-associated recombination defects
Exponentially grown Tc-sgs1 and ddk-KR Tc-sgs1 cells were synchronized in G1 phase at 25°C and released in YPD medium containing 0.033% MMS and tetracycline (1mM) to induce Sgs1 depletion at 28°C. Samples were collected at the indicated time points followed by psoralen crosslinking. Genomic DNA was extracted from the cells, digested with NcoI
and analyzed by 2D gel electrophoresis with a probe for ARS305. Using western blotting, Sgs1 depletion was visualized using HA antibody while Pgk1 was served as loading control. Cell cycle progression of each strain was monitored by analyzing FACS plots. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.

**Part B: Mechanisms, pathways and factors contributing to DDK recombination function**

3.3 DDK promotes the formation of inter-sister and inter-homologous chromosomes recombination intermediates

We found that DDK mutants, in haploid cells are defective in replication-associated mitotic recombination induced by MMS. In a diploid situation, it is possible that the observed inter-sister recombination defect in ddk mutants is compensated by an increase in recombination between homologous chromosomes, or that it represents an overall recombination defect. In order to address these possibilities, we proceeded to check the effect of DDK mutants on both sister chromatid junctions and inter-homologue junctions (IHJs) mediating damage-bypass in a modified diploid strain in which both events can be visualized. IHJs is the term used for the accumulation of recombination intermediates formed through the exchange between homologous chromosomes. To perform this, we used a previously established system where one of the chromosomes III carries a single polymorphic mutation at one of the EcoRV restriction site located near the early replication origin ARS305. This mutation allows us to discriminate, by size of the non-replicated and replicated fragments, the cruciform structures accumulating between sister chromatids (SCJs) or homologous chromosomes after restriction digestion using EcoRV and NcoI enzymes (Carotenuto & Liberi, 2010). In this assay, replication as well as recombination intermediates generated in MMS treated conditions of each homologous chromosomes give rise to SCJ signals of different size. Inter-homolog junctions appear in the form of a spike of intermediate size in the middle (Fig. 3.13a). According to the previous reports, recombination structures arising from sister chromatid junctions (SCJs) and homologous chromosomes (IHJs) share similar kinetics, while the inter homolog junctions are shown to be less abundant (Carotenuto & Liberi, 2010) in line with previously reported features of mitotic recombination.

For this experiment, we established Tc-sgs1/Tc-sgs1* homozygous diploid strains as well as the cdc7-4 Tc-sgs1/cdc7-4 Tc-sgs1* and ddk-KR Tc-sgs1/ddk-KR Tc-sgs1* strains, in which * accounts for the modified ARS305 locus on chromosome III. All these strains were only
heterozygous for the mutation at an EcoRV restriction site as described above. These cells were arrested in G2/M phase by nocodazole treatment at 25°C. The cells were then released into 0.033% MMS containing medium at 28°C. Samples were collected at 180 min and 240 min. Genomic DNA was extracted from this samples following in-vivo psoralen crosslinking and digested with EcoRV and NcoI. The digestion of the DNA for these strains gave 2 restriction fragments of different sizes, 3771 bp and 5106 (see Fig. 3.13). Those fragments were then subjected for 2D gel analysis and later probed for ARS305 region. Besides the expected defect in ddk mutants on sister chromatid junctions, we did not observe an increase in damage-bypass inter-homolog junctions, but rather a decrease (Fig. 3.13b). This highlights the fact that DDK affects recombination in general rather than causing a specific impairment on the inter-sister recombination.

Figure 3.13. Damage-bypass SCJs and IHJs were both decreased in ddk mutants
a) Schematic representation of the replication intermediates arising in the modified diploid system. IHJs are indicated by red arrows. b) Exponentially grown Tc-sgs1/Tc-sgs1*, cdc7-4

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Tc-sgs1/cdc7-4, ddk-KR Tc-sgs1/ddk-KR Tc-sgs1* (* indicates mutation at the EcoRV restriction site located near to the ARS305 region on the one of the chromosome III) cells were arrested in G2/M phase by nocodazole treatment at 25°C and released in YPD medium at 28°C in medium containing 0.033% MMS and tetracycline (1mM) to induce Sgs1 depletion. Samples were collected for the indicated timepoints, psoralen crosslinked and DNA was extracted for 2D gel analysis. Restriction digestion was performed with EcoRV and NcoI and the fragment undergoes 2d gel analysis and was later probed for ARS305 region. Samples for TCA protein extraction and western blotting were also collected. Using western blotting, Sgs1 depletion was visualized using HA antibody while Pgk1 was served as loading control. Cell cycle progression of each strain was monitored by analyzing FACS plots.

3.4 DDK promotes recombination jointly with the PCNA polyubiquitylation pathway
Rad18 and Rad5/Mms2/Ubc13-mediated polyubiquitylation of the PCNA clamp is known to facilitate error-free recombination related template switching (TS) where a newly synthesized strand serves as a template for the damaged nascent strand (Branzei, 2011; Branzei et al., 2008; Giannattasio et al., 2014). To examine possible effects of DDK on the Rad18 pathway, we combined cdc7-4 with rad18Δ in Wildtype and Tc-sgs1 backgrounds. 2D gel analysis reproduced the strong effect of rad18Δ (Branzei et al., 2008) and the intermediate effect of cdc7-4, and indicated similar reduction caused by the cdc7-4 rad18Δ combination with the one of rad18Δ (Fig. 3.14a). However, spot assay indicated additive MMS sensitivity between rad18Δ and cdc7-4 (Fig. 3.14b), likely due to TLS impairment caused by the cdc7-4 mutation (Brandão et al., 2014).
**Figure 3.14.** DDK acts jointly with PCNA ubiquitylation

*a*) Exponentially grown *Tc-sgs1*, *rad18A Tc-sgs1*, *cdc7-4 Tc-sgs1* and *cdc7-4 rad18A Tc-sgs1* cells were synchronized in G1, and released in medium containing 0.033% MMS and tetracycline (1mM) to induce Sgs1 depletion at 28°C. Samples were collected for the indicated timepoints, psoralen crosslinked and the extracted genomic DNA for the indicated time points were digested with NcoI and 2D gel signals were analyzed for ARS305. Samples
for western blotting and FACS analysis were collected. Using western blotting, Sgs1 depletion was visualized using HA antibody while Pgk1 was served as loading control. Cell cycle progression of each strain was monitored by analyzing FACS plots. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%. b) Wildtype, rad18Δ (#1 and # 2 represents 2 different clones), cdc7-4 (#1 and # 2 represents 2 different clones) and cdc7-4 rad18Δ (#1 and # 2 represents 2 different clones) cells were serially diluted and spotted on YPD and MMS containing YPD plates for the indicated MMS concentrations. Plates were incubated at 28°C and were scanned after 3 days.

Because of the strong effect of rad18Δ mutation on X-molecule formation that makes potential additivity effects difficult to observe in 2D gels, we further examined the combination of cdc7-4 with mms2Δ. Deletion of mms2 also resulted in reduced accumulation of recombination intermediates whose effects on X-molecules are more modest than the ones of rad18Δ (Branzei et al., 2008). In this case, quantification of the X-signals revealed a mild additivity between cdc7-4 and mms2Δ effects at the last time point, and epistasis for the other two (Fig. 3.15a), while spot assay again indicated additive MMS sensitivity between mms2Δ and cdc7-4 (Fig. 3.15b), likely due to TLS impairment function in cdc7-4. These results suggest joint functions of DDK and Rad18/Mms2 in template switching, but leave open the possibility that Cdc7 may affect replication-associated recombination by other means.
Figure 3.15. DDK shows mild additivity with PCNA ubiquitylation mutant, mms2

a) Exponentially grown Tc-sgs1, mms2Δ Tc-sgs1, cdc7-4 Tc-sgs1, cdc7-4 mms2Δ Tc-sgs1 cells were synchronized in G1 phase and released in YPD medium containing 0.033% MMS and tetracycline (1mM) to induce Sgs1 depletion at 28°C. Samples were collected for the indicated time points for 2D gel analysis, FACS, and for Western blotting. Collected samples were subjected to psoralen crosslinking, after which genomic DNA was extracted and digested using NcoI and the replication intermediates were analyzed for ARS305 region.
Using western blotting, Sgs1 depletion was visualized using HA antibody while Pgk1 was served as loading control. Cell cycle progression of each strain was monitored by analyzing FACS plots. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.

b) Wildtype, mms2Δ, cdc7-4 and mms2Δ cdc7-4 cells were serially diluted and spotted on YPD and MMS containing YPD plates for the indicated MMS concentrations. Plates were incubated at 28°C and were scanned after 3 days.

3.5 DDK functions beyond the ‘time-window’ of the PCNA polyubiquitylation pathway

Replication-associated recombination not only occurs through Rad18- and Rad5/Mms2/Ubc13-mediated polyubiquitylation of PCNA but also through a pathway independent of PCNA (Branzei et al., 2008). This latter pathway commonly called as salvage pathway gets activated in backgrounds defective in PCNA SUMOylation. While PCNA polyubiquitylation-dependent recombination primarily occurs in S-phase, the salvage pathway is shown to be preferentially deployed in the later stages of replication (Branzei & Szakal, 2016). We assessed the role of DDK in the salvage pathway by combining cdc7-4 with siz1Δ Tc-sgs1, where PCNA SUMOylation is largely abolished due to inactivation of the Siz1 SUMO ligase that is critical for PCNA SUMOylation at the K164 residue (Hoege et al., 2002; Stelter & Ulrich, 2003; Windecker & Ulrich, 2008). Here, siz1Δ Tc-sgs1 showed accumulation of recombination intermediates similar to that of Tc-sgs1, which occurs from the salvage pathway of recombination (Branzei et al., 2008). While, we observed a reduction in recombination intermediates caused by cdc7-4 in siz1Δ Tc-sgs1 background that is similar to the effect of cdc7-4 in Tc-sgs1 background (Fig. 3.16). Thus, DDK facilitates both template switching and the salvage pathway of recombination, similar in trend to what has been reported for Rad51 and other critical recombination factors or mediators.
Figure 3.16. DDK also plays a role in salvage pathway dependent recombination

Exponentially grown *Tc-sgs1*, *siz1Δ Tc-sgs1*, *cdc7-4 Tc-sgs1*, *cdc7-4 siz1Δ Tc-sgs1* cells were synchronized in G1 and released in YPD medium containing 0.033% MMS and tetracycline (1mM) to induce Sgs1 depletion at 28°C. Samples were taken at the indicated timepoints for FACS, Western blotting and 2D gel analysis. Collected samples were subjected to psoralen crosslinking, after which genomic DNA was extracted and digested using NcoI and the replication intermediates were analyzed for ARS305 region. Using western blotting, Sgs1 depletion was visualized using HA antibody while Pgk1 was served as loading control. Cell cycle progression of each strain was monitored by analyzing FACS plots. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the
monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.

We also examined the impact of SUMOylation defective ddk-KR mutant in the siz1Δ background. Keeping Tc-sgs1, ddk-KR Tc-sgs1 and siz1Δ Tc-sgs1 as controls, we analyzed the recombination intermediates formed in ddk-KR siz1Δ Tc-sgs1 mutant. We observed no difference in recombination intermediates in ddk-KR siz1Δ Tc-sgs1 mutant compared to siz1Δ Tc-sgs1 alone (Fig. 3.17). These results suggest that the recombination defect associated with the ddk-KR mutation is specific to TS and does not affect recombination in general.

Figure 3.17. ddk-KR mutant did not affect the salvage pathway
Exponentially grown Tc-sgs1, siz1Δ Tc-sgs1, ddk-KR Tc-sgs1, ddk-KR siz1Δ Tc-sgs1 cells were arrested in G1 phase and then released in YPD medium containing 0.033% MMS and tetracycline (1mM) to induce Sgs1 depletion at 28°C. Samples were taken at the indicated
timepoints for FACS, Western blotting and 2D gel analysis. Collected samples were subjected to psoralen crosslinking, after which genomic DNA was extracted and digested using NcoI and the replication intermediates were analyzed for ARS305 region. Using western blotting, Sgs1 depletion was visualized using HA antibody while Pgk1 was served as loading control. Cell cycle progression of each strain was monitored by analyzing FACS plots. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.

3.6 DDK Regulation of Recombinase and anti-recombinase activities

3.6.1 DDK facilitates Rad51 recruitment to damaged replication forks

As DDK seems to affect replication-associated recombination regardless of whether the Rad5 pathway is employed or not, we analyzed if recruitment of Rad51 to the damaged DNA is affected in ddk mutants by performing Chip-qPCR analysis. Wildtype and cdc7-4 cells were arrested in G1 at 25°C and released in MMS containing YPD medium at 28°C and the samples were collected at 20 min, 30 min and 40 min respectively. We chose earlier time points in comparison with the ones assessed by 2D gel, as Rad51 recruitment is one of the early process during DNA damage response. We observed a mild decrease in the recruitment of Rad51 to the damaged DNA in cdc7-4 mutants compared to wildtype (Fig. 3.18).
Figure 3.18. DDK promotes Rad51 recruitment

Exponentially grown wildtype and cdc7-4 cells were arrested in G1 phase at 25°C and released into 0.033% MMS containing YPD media at 28°C. Samples were collected for ChIP analysis at 20 min, 30 min and 40 min. Each chip experiment was repeated three times and each real time PCR was performed in triplicates. Error bars represent the SEM (mean value +/- standard error of mean) of three independent experiments.

We also checked the similar effect of Rad51 recruitment in ddk-KR mutant as it was also displaying a defect in recombination. For this purpose, Wildtype, ddk-KR and rad51Δ cells were exponentially grown and arrested in G1 phase. The cells were released into 0.033% MMS containing medium at 28°C and samples were collected for 20 min, 30 min and 40 min that were analyzed by Chip-qPCR. Compared to cdc7-4 mutant, ddk-KR showed a stronger decrease in the recruitment of Rad51 to the ARS305 region (Fig. 3.19). These results suggest an important role of DDK in the Rad51 recruitment/retention process.

Figure 3.19. DDK promotes Rad51 recruitment

Exponentially grown wildtype and ddk-KR cells were arrested in G1 phase at 25°C and released into 0.033% MMS containing YPD media at 28°C. Samples for ChIP-qPCR were collected at 20 min, 30 min and 40 min. Each chip experiment was repeated three times and each real time PCR was performed in triplicates. Error bars represent the SEM (mean value +/- standard error of mean) of three independent experiments.
3.6.2 Overexpression of Rad51 did not rescue the sensitivity of DDK mutants towards different DNA damaging agents

Spot assay analysis of cdc7-4 showed an increased sensitivity of this mutant to different DNA damaging agents like MMS at varying concentrations (Fig 3.14b & Fig 3.15b). As we see a decreased recruitment of Rad51 filaments to the damaged DNA for this mutant, we decided to investigate whether overexpression of Rad51 in this mutant can rescue the sensitivity of this mutant to different damaging agents. For this reason, extra copies of Rad51 were introduced to cdc7-4 along with wildtype using a plasmid Rad51YEplac195. Empty vector was also introduced to these strains which was kept as a control. Spot assay analysis revealed that overexpression of Rad51 in cdc7-4 mutant was not sufficient to rescue the sensitivity of the cdc7-4 mutant. On the other hand, when Rad51 was over-expressed in rad51Δ (used as control), there was a rescue in the sensitivity of rad51Δ towards different DNA damaging agents (Fig. 3.20). We did not perform similar rescue experiments in ddk-KR, due to their lack of MMS sensitivity (Psakhye et al., 2019). However, we plan to perform a 2D gel experiment in ddk-KR, specifically defect in TS, to assess if RAD51 overexpression rescues the associated recombination defect.

![Spot assay analysis](image)

**Figure 3.20.** Overexpression of RAD51 did not rescue the sensitivity of the cdc7-4 mutant towards DNA damaging agents

Wildtype, cdc7-4 and rad51Δ cells carrying empty vector or RAD51 overexpression vector were serially diluted and spotted on -URA, MMS and CPT containing -URA plates for the indicated MMS and CPT concentrations. Plates were incubated at 28°C and were scanned after 3 days.
3.6.4 RPA binding to DNA is dependent on DDK action

RPA or Replication protein A is a heterotrimeric complex that binds to the single stranded DNA and serves a protective function. This process is considered as one of the early steps in DNA damage response (DDR) (Wold, 1997). Rad51 filament formation in the vicinity of damaged replication forks is preceded by RPA (Replication protein A) nucleation on the ssDNA (Patrick Sung, 1997). As we observed a decreased recruitment of Rad51 to damaged replication forks in both ddk mutants, cdc7-4 and ddk-KR, we decided to look into the effect of RPA recruitment to the damaged DNA in these mutants. To assess the effect of RPA nucleation in ddk mutants, we measured by ChIP-qPCR the recruitment of Rfa1, a subunit of RPA, proximal to the early replication origin ARS305 for three different timepoints after MMS treatment. Rfa1-PK, cdc7-4 Rfa1-PK and ddk-KR Rfa1-PK cells were synchronized in G1 and released in the presence of 0.033% MMS. Samples for Chip analysis were collected for 20 min, 30 min, and 40 min respectively. Compared to PK-tagged Rfa1 cells, we observed an increase in the Rfa1 recruitment in cdc7-4 Rfa1-PK, but not in ddk-KR Rfa1-PK (Fig. 3.23). These results suggest a direct or indirect involvement of DDK in ssDNA formation and RPA recruitment during DNA damaging conditions.

Figure 3.21. ddk mutants exhibits increased RPA recruitment

Exponentially grown wildtype, cdc7-4 and ddk-KR cells were arrested in G1 phase at 25°C and released into 0.033% MMS containing YPD media at 28°C. Samples were collected for Chip-qPCR at 20 min, 30 min and 40 min. Chip experiment was repeated two times while
each real time PCR was performed in triplicates. Error bars represent the SEM (mean value +/- standard error of mean) of two independent experiments.

### 3.7 DDK mutants causes long ssDNA stretches at replication forks

To examine a possible impact of DDK mutants on ssDNA formed during replication, we examined the structure of replication forks by Transmission Electron microscopic analysis (TEM) coupled to in-vivo psoralen crosslinking. The experiment was conducted in Wildtype, cdc7-4 and ddk-KR cells released from G1 phase in the presence of 0.033% MMS. Cells were collected after 60 min upon release from G1 arrest, psoralen crosslinked, and then replication intermediates were extracted following the same procedure ad for 2D gels. We analyzed 144 molecules of Wildtype, 165 molecules of cdc7-4 and 173 molecules of ddk-KR respectively. In Wildtype and other mutants, more than 50% of the replication intermediates were composed of normal replication forks containing Y molecules and bubbles (Fig. 3.24). Interestingly, ddk mutants showed strongly elevated levels of gapped forks (35% and 36% of all replication intermediates in cdc7-4 and ddk-KR respectively compared to 9% in Wildtype) as well as broken forks (7% and 6% of all intermediates in cdc7-4 and ddk-KR respectively when compared to 4% in wildtype). Gapped forks were characterized by the presence of a long ssDNA continuity on one of the two replicated strands connected to the fork branching point, with cdc7-4 displaying a median value of 1502 nucleotides length of ssDNA and ddk-KR of 963 nucleotides, compared to a median of 704 nucleotides length of wildtype (Fig. 3.24). This is in accordance with the finding that cdc7-4 cells accumulate increased levels of RPA at damaged forks compared to wildtype and ddk-KR (Fig. 3.23). Altogether, these results indicate the importance of DDK in restraining abnormal formation of ssDNA at the fork junction in DNA damaging conditions (EM courtesy: Sabrina Dusi & Michele Giannattassio).
Figure 3.22. *ddk* mutants display increased gapped and broken forks

Transmission Electron microscopic analysis of replication intermediates in WT, *cdc7-4* and *ddk-KR* mutants. Left) The plot indicates the percentage of various types of DNA replication intermediates and the joint molecules divided in reversed forks and hemicatenanes, generated in *cdc7-4* and *ddk-KR* mutants in comparison with wildtype. The number (n) of DNA molecules analyzed for each genotype is indicated. Right) The graph represents the length of ssDNA gaps (in nucleotides) generated from *ddk* mutants in comparison with wildtype. Below) Typical examples of the visualized categories of normal forks, gapped forks, broken forks and reversed forks are indicated with the entire DNA structure (EM courtesy: Sabrina Dusi & Michele Giannattassio).

3.8 DDK impact on checkpoint activation

The replication checkpoint plays roles in maintaining genomic stability by protecting replication forks from unscheduled resection and formation of aberrant DNA structures. Previous reports show that checkpoint mutants show increased number of single stranded
molecules, such as gapped forks and hemi replicated bubbles, as well as reversed forks upon exposure to HU (Lopes et al., 2001; Sogo, 2002). In addition to this, our lab showed the importance of checkpoint activation in protecting error-free DNA damage tolerance intermediates from unscheduled nucleolytic processing (Szakal & Branzei, 2013). DDK is also known to play roles in regulating S-phase checkpoint signaling (Tsuji et al., 2008). As ddk mutants resembled in regard to fork resection checkpoint mutants exposed to HU, we wanted to investigate whether DDK functions in replication associated recombination may involve defects in checkpoint activation. We performed two different experiments to test this hypothesis:

a) Monitor the cell cycle progression and Rad53 phosphorylation status in DDK mutants during replication in the presence of MMS
b) Examine if DDK defect in recombination is due to unscheduled activity of Mus81-Mms4 nuclease

3.8.1 Temperature inactivation or conditional depletion of DDK did not affect cell cycle progression but reduced checkpoint activation during genotoxic stress

Previous reports revealed a role for yeast Cdc7-Dbf4 complex in the full activation of Rad53 in response to replication stress (Ogi et al., 2008). We first investigated whether there is any difference in the kinetics of cell cycle progression in ddk mutants. For this, Wildtype, cdc7-4 and rad53-K227A (kinase-defective mutant of Rad53) cells were synchronized in G1 phase and then released into S-phase in 0.033% MMS containing medium. Samples for FACS analysis was collected for every 10 min up to 180 min. We observed a faster cell cycle progression in the checkpoint defective mutant rad53-K227A as expected. While in cdc7-4 mutant, we did not observe any increase in the cell cycle progression compared to wildtype but rather a slow-down (Fig 3.25a). We also performed a similar experiment in conditional cdc7-AID mutants and obtained similar result to that of cdc7-4 where the cell-cycle progression remained slower than that of wildtype (Fig. 3.25b).
Figure 3.23. DDK mutants did not alter the cell-cycle progression in DNA-damaging conditions, differently from checkpoint defective mutants

a) Exponentially grown wildtype, *cdc7-4* and *rad54-K227A* cells were arrested in G1-phase using α-factor and released in medium containing 0.033% MMS. Samples were collected at the indicated time points to determine the DNA content by FACS analysis. b) *cdc7-AID* cells
were grown into exponential phase and arrested in G1-phase. Cells were released from G1 phase into 0.033% MMS containing medium both in the absence and presence of 1 mM auxin (auxin was added after 20 min of its release from G1 phase, see Figure 3.5). Samples for FACS were collected for the indicated time points.

In order to further validate whether DDK has any effect in activation of checkpoint kinases during genotoxic stress we decided to further monitor the Rad53 phosphorylation status of ddk temperature sensitive mutants as well as conditional AID mutant of DDK during genotoxic stress. For this purpose, G1 synchronized wildtype and cdc7-4 (25°C) cells were released in 0.033%MMS containing medium at 28°C. Samples for protein extraction were collected at 90 min, 120 min and 150 min as in 2D gel electrophoresis. We observed a reduction in the Rad53 phosphorylation in cdc7-4 mutant compared to wildtype (Fig. 3.26a). We performed a similar experiment also for cdc7-AID strain in the absence and presence of auxin. We also observed a reduction in the phosphorylation of Rad53 in these mutants in the presence of Auxin where Cdc7 is depleted by auxin after 20 min of its release from G1 phase into MMS containing S-phase (Fig. 3.26b).
Figure 3.24. DDK temperature sensitive as well as conditional *cdc7-AID* mutants show reduced Rad53 phosphorylation

a) Wildtype and *cdc7-4* cells were arrested in G1 using alpha factor and then released in 0.033% MMS containing medium at 28°C. Samples for western blotting were collected for the indicate timepoints. Rad53 phosphorylation levels were analyzed using anti-Rad53 antibody and Pgk1 served as loading control b) *cdc7-AID* cells were synchronously released from G1-phase into S-phase medium containing 0.033% MMS both in the absence and presence of 1 mM auxin (auxin was added after 20 min of its release from G1, see Figure 3.5). Samples for western blotting were collected for the indicated timepoints and Rad3 phosphorylation levels were analyzed using Rad53 specific antibody. Pgk1 is served as loading control.

We also analyzed the Rad53 phosphorylation status and cell cycle progression of *ddk-KR* mutant in a similar manner as in *cdc7-4* temperature sensitive and *cdc7-AID* conditional mutants. Rad53 phosphorylation status was monitored for wildtype and *ddk-KR* mutants that were arrested in G1-phase and released in medium containing 0.033%MMS. Samples for western blotting and FACS analysis was collected for 90 min, 120 min and 150 min time points. From the western blot analysis, it appears that there is not any difference in the Rad53 phosphorylation levels in *ddk-KR* mutant in comparison with the wildtype (Fig 3.27). Consistent with the Rad53 phosphorylation status, MMS treated *ddk-KR* cells completed DNA replication with slower kinetics compared to that of wildtype. These findings here indicate that *ddk-KR* mutant does not affect MMS-associated checkpoint activation.
Figure 3.25. *ddk-KR* mutants did not demonstrate any checkpoint defect

Wildtype, *ddk-KR* and *rad53-K227A* cells were exponentially grown in YPD medium, were synchronously in G1 phase and then released into 0.033% MMS containing YPD medium. Samples were collected for every 15 min until 120 min to determine the DNA content by FACS analysis. Kinetics of Rad53 phosphorylation in Wildtype and *ddk-KR* were analyzed by performing a western blotting with the samples collected for indicated timepoints using anti-Rad53 antibody. Immunodetection of Pgk1 is served as loading control.

3.8.2 DDK effects in replication-associated recombination is not due to premature Mus81-Mms4-mediated resolution

DDK is known to play roles in interacting and phosphorylating checkpoint factors (Gold & Dunphy, 2010; Yanow et al., 2003) and in checkpoint activation (Sasi et al., 2018; Tsuji et al., 2008). Since we observed reduced Rad53 phosphorylation levels in *ddk* temperature sensitive as well as conditional mutants, next we investigated whether the effect of DDK in recombination is related to its ability to prevent premature action of the Mus81-Mms4 nuclease, as previously reported to be the case in checkpoint mutants (Szakal & Branzei, 2013). To these ends, we examined whether the recombination defect associated with DDK
dysfunction can be rescued by concomitant inactivation of the Mus81-Mms4 nuclease. For this, we combined cdc7-4 with mms4Δ and examined whether there is any rescue in the recombination defect caused by the cdc7-4 mutation. As reported above, cdc7-4 Tc-sgs1 showed a decrease in the accumulation of recombination intermediates compared to Tc-sgs1 and mms4Δ Tc-sgs1, in line with previous reports that Mms4 does no contribute to the formation/resolution of these structures in checkpoint proficient cells (Szakal & Branzei, 2013). We observed a similar amount of X-structures in cdc7-4 Tc-sgs1 mms4Δ and cdc7-4 Tc-sgs1 (Fig. 3.28). Thus, DDK roles in replication-associated recombination are not due to defective stabilization of these structures against Mus81-Mms4-mediated resolution.

Figure 3.26. The recombination defect of cdc7-4 is not caused by unscheduled Mus81-Mms4 activity

Tc-sgs1, mms4Δ Tc-sgs1, cdc7-4 Tc-sgs1, cdc7-4 mms4Δ Tc-sgs1 cells were synchronized in G1 with α-factor and released in media containing 0.033% MMS and 1 mM Tetracycline

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to induce Sgs1 depletion. Samples were collected at indicated time points followed by genomic DNA extraction. DNA was digested with NcoI and the 2D gels were analyzed for ARS305 region. Western blotting for Sgs1 depletion, FACS and quantification plots are also included. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.

Previous reports have shown that DDK facilitates activation of the Mus81-Mms4 nuclease in mitosis (Princz et al., 2017). SUMOylation defective ddk-KR mutant is a a stabilized and stronger version of DDK in regard to MCM activation. Because it also shows a defect in recombination, we decided to check if this is due to unscheduled activation of the Mus81-Mms4 nuclease, potentially due to extensive phosphorylation by ddk-KR mutant. For this purpose, we combined ddk-KR Tc-sgs1 with mms4Δ and checked the status of recombination intermediate accumulation in this mutants. 2d gel analysis revealed a decrease in the accumulation of recombination intermediates in ddk-KR Tc-sgs1 mutant when compared to Tc-sgs1 alone, as previously reported. While the triple mutant of ddk-KR mms4Δ Tc-sgs1 did not showed any rescue in the recombination defect associated with ddk-KR mutant (Fig 3.29). In conclusion, this data suggests that activation of the Mus81-Mms4 nuclease due to the prolonged binding of DDK on DNA is not the cause for the observed recombination defect.
Figure 3.27. Recombination defect of ddk-KR mutant does not rely on its dysfunction in regulating Mus81-Mms4 activity

Tc-sgs1, mms4Δ Tc-sgs1, ddk-KR Tc-sgs1, ddk-KR mms4Δ Tc-sgs1 cells were synchronized in G1 with α-factor and released in media containing 0.033% MMS and 1 mM tetracycline to induce Sgs1 depletion. Samples were collected at indicated time points for FACS, protein and 2D gel analysis. Genomic DNA was extracted from this samples and were digested with NcoI. 2D gel signals were visualized using a probe specific for ARS305. The relative enrichment of X-shaped replication intermediates is represented in the quantified plots. In the graph, signal intensities were normalized with respect to the monomer spot and the highest value obtained during quantification of the X molecule was assigned as 100%.
3.9 Artificial restoration of sister chromatid cohesion does not rescue the recombination defect of DDK temperature sensitive mutant cdc7-4

DDK is reported to interact with cohesin and is shown to load the cohesin loader complex prior to replication initiation (Natsume et al., 2013; T. S. Takahashi et al., 2008). As it is known that cohesin supports replication-associated recombination (Fumasoni et al., 2015), we investigated if artificial restoration of sister chromatid proximity by sister chromatid tethering bypasses the local template switch defect observed in the cdc7-4 mutant. For this purpose, we used a system that artificially re-establishes sister chromatid cohesion in a locus-specific manner and relies on the ability of Lactose inhibitor (Lacl) to bind to the Lactose operator sequence (LacO). In wildtype (tetramer) form, Lacl is able to bind the LacO sequences present in both chromatids bringing them together, while the truncated version of Lacl forms a dimer that can only bind the LacO sequences present on one chromatid (Fig. 3.30a) (Straight et al., 1996). In cohesion-defective cohesin mutants, only the tetramer version can restore the sister chromatid cohesion but not the dimer (Straight et al., 1996). Likewise, the replication-associated recombination defect associated with cohesin mutants can be locally bypassed by artificial sister chromatid tethering induced by the Lacl tetramer, but not the dimer, whereas the recombination defect of other replication fork-associated mutants is not (Fumasoni et al., 2015).

By utilizing the above assay, we examined whether the recombination defect in DDK mutants can be rescued by artificially restoring local cohesion. For this, Tc-sgs1 and cdc7-4 Tc-sgs1 cells carrying the dimer and tetramer system were released from G1 after the activation of Lacl expression, into media containing 0.033% MMS at 28°C. Samples were collected at the indicated time points and proceeded with 2D protocol to be analysed for a LEU2 locus situated at chromosome III, where the LacO sequence was integrated. By performing 2D gel electrophoresis, we compared the pattern of recombination intermediates in the dimer versus the tetramer system. The reduction in recombination intermediates was observed in cdc7-4 in both systems (Fig. 3.30b). This indicates that artificial sister chromatid tethering does not restore the formation of X-molecules in the sgs1 cdc7-4 strain. However, this result will need to be validated to include additional controls of cohesin mutants and on the functionality of the tetramer system. Altogether, these data support the notion that DDK facilitates recombination independently of its roles in facilitating cohesin loading and sister chromatid cohesion.
Figure 3.28. Artificial sister chromatid tethering did not restore the recombination defect in DDK mutants

a) Schematic representations of LacI dimer and tetramer systems for artificial sister chromatid tethering as mentioned in (Fumasoni et al., 2015). b) Tc-sgs1 and cdc7-4 Tc-sgs1 cells carrying dimeric LacI or tetrameric LacI were grown at 25°C and arrested in G1 phase using α-factor. Cultures were then transferred to a synthetic complete media lacking histidine
that is further supplemented with 10 mM of 3-aminotrizole and α-factor for the final 30 min of G1 arrest to induce LacI expression. Cells were released from this medium to 0.033% MMS containing YPD media at 28°C and samples were collected for the indicated time points. Genomic DNA was extracted and digested with EcoRV and XhoI and analyzed using a probe flanking Leu2 locus. FACS, Western blotting and X molecule quantifications are indicated.

3.10 DDK and Natural Pausing sites

DDK was reported to mediate robust pausing at Fob1-mediated replication fork barriers (RFBs) in rDNA (Bastia et al., 2016). This finding was of interest as it might be relevant for identifying potential roles of DDK in mediating recombination at forks generally stalled at NPSs genome-wide, where our lab identified that activities required for fork pausing (i.e. Tof1 and Csm3) mediate formation of recombination structures when the Smc5/6 complex functionality is impaired (Menolfi et al., 2015). We began by reproducing the report on Cdc7 role in fork pausing at rDNA (Bastia et al., 2016), using tof1 as control mutant defective in pausing. Cells were synchronously released from G1 phase into 25°C YPD medium for 20 min and then shifted to 37°C pre-warmed medium. Samples were collected after three hours before proceeding with DNA extraction and 2D analysis. In line with previous reports, we were able to reproduce that cdc7-4 and tof1 are defective in strong pausing at RFBs (Fig. 3.31).
Figure 3.29. Inactivation of DDK caused significant reduction of PFA

a) Schematic representation of rDNA repeats indicating the location of the RFBs. Blue arrows show the direction of rDNA transcripts (Adapted from Bastia et al., 2016). b) (Above) Schematic workflow of the experiment. (Below) Log phase cells of the indicated genotype were synchronized in G1, released in YPD media for 20 minutes at 25°C and then shifted to 37°C. The samples were collected after 3hr and analyzed by 2D gel electrophoresis. The scheme represents replication fragments arising from 2D gel electrophoresis. Cell cycle progression was monitored by performing FACS which is also displayed.

As cdc7 mutant alleles is reported to have an effect similar to tof1 mutant on pausing at rDNA region (Bastia et al., 2016), we asked whether cdc7-4 also behaves analogously with tof1 mutation in exhibiting an altered pattern of replication/recombination DNA structures at NPSs when Smc5/6 is dysfunctional (Menolfi et al., 2015). Prior to this, we initiated the
study by checking the direct effect of *cdc7-4* temperature sensitive mutant at NPSs. In order to address this, we performed a 2D gel electrophoresis and chose a well characterized termination region to visualize the DNA structures, TER302. This is a 5 kb region located at chromosome III, characterized by the presence of a tRNA and an LTR containing-Ty element which is known to associate with replication fork pausing (Fig. 3.32). Wildtype, *smc6-P4* and *cdc7-4* mutants were synchronously released from G1 phase into 200 mM HU containing medium at 28°C. Samples for 2D were collected at 60 min, 180 min and 300 min. Interestingly, we observed a mild accumulation of X-shaped recombination structures in *cdc7-4* temperature sensitive mutant, similar but more modest than in *smc6-P4* (Fig. 3.32). Based on this preliminary data, we assume that DDK might also play roles at NPS, a hypothesis that will need to be reevaluated later.

![Diagram](image)

**Figure 3.30 DDK mutants accumulate recombination structures at NPSs**

a) Schematic representation of the 2D gel fragment analyzed that spans the TER302 region.

b) Schematic representation of type of 2D gel signals arising at the TER302 region revealed
by 2D gel electrophoresis. c) Wildtype, smc6-P4, and cdc7-4 cells were synchronously released from G1 arrest into S-phase in the presence of 200 mM HU at 28°C for 2D gel analysis. Samples were collected for the indicated timepoints and the extracted DNA from this samples were further digested with HindIII and PstI enzymes. The restriction fragment is further subjected 2D gel analysis and the 2D gel signals were analyzed with a probe for TER302. Samples were also collected for FACS and are displayed. Orange arrow indicates accumulation of recombination intermediates.
5. DISCUSSION

Cellular proliferation attained through proper DNA replication is an evolutionary conserved process among all eukaryotes. Maintenance of genome integrity by accurate duplication of genetic information and its propagation to coming generations is essential. However, the genetic material is constantly exposed to a plethora of threats of either exogenous or endogenous nature. Due to this exposure, the genetic material is often damaged through several types of DNA lesions. In eukaryotic cells, these lesions are constantly tracked and cleared by a repair machinery within the cell, specifically designed to ensure that the lesions are either repaired or tolerated. Pathways that get activated to deal with these lesions are collectively referred to as DNA Damage Responses or DDR (S. P. Jackson & Bartek, 2009). DDR responses include the involvement of various cellular processes like checkpoint activation, activation of DNA damage tolerance pathways and so on. This response plays a crucial part in preserving the genome integrity as perturbances in these processes are often implicated in several developmental and neurological disorders. This finally drives genomic instability, a hallmark of cancer (O’Driscoll & Jeggo, 2008). Therefore, it is important to understand the workings of various DNA repair pathways, which can lead to the development of therapeutics to various disorders including cancer.

Till date, extensive studies have been carried out on several factors that regulate the DDR pathway. We put forth one such player, Dbf4-dependent kinase or DDK, which we show to have specific roles in recombination-mediated DDT. S-phase kinase DDK (Cdc7-Dbf4) has been shown to phosphorylate several substrates and is known to regulate a wide range of cellular functions. In recent years, it drew a lot of attention as target for chemotherapy (Hisao Masai, 2008; Alessia Montagnoli et al., 2004). One essential role of DDK is its involvement in DNA replication initiation by phosphorylating various factors associated with origin firing (Larasati & Duncker, 2016). Overexpression of DDK subunits (Cdc7 and Dbf4) is correlated with poor prognosis resulting in advanced tumor grade in many cancers (Bonte et al., 2008; A. N. Cheng et al., 2013; Ghatalia et al., 2016; Melling et al., 2015). Even though several potent Cdc7 inhibitors are available, a better insight into the identification of different substrates of Cdc7 could help in the development of better cancer therapeutics. It may also resolve the chemoresistance cases occurring in many cancers by developing combinatorial therapies (Iwai et al., 2019; Alessia Montagnoli et al., 2008).

As mentioned above, DDK is shown to take part in different cellular processes including DNA replication. Although some important findings have been made in the recent years
regarding its importance during replication stress, the significance of DDK during replication stress remains enigmatic. DDK was also indicated to have roles in replication stress response by allowing full checkpoint activation and mutagenesis (Pessoa-Brandão & Sclafani, 2004; Tsuji et al., 2008). However, its roles in DDT remain poorly understood.

Using *S. cerevisiae* as a model system, here we investigated and uncovered roles for DDK in replication associated recombination during genotoxic stress in a manner uncoupled from replication initiation. Genotoxicity induced here is by the DNA damage alkylating agent methyl methane sulfonate or MMS, which methylates nitrogen bases on the DNA. Effects on recombination were thoroughly investigated by assessing the levels of recombination structures associated with DNA replication via 2D gel electrophoresis.

4.1 DDK is important for mitotic recombination

First, we investigated the levels of recombination structures formed in various *ddk* mutants by performing 2D gel electrophoresis. We used two kinds of DDK mutants: kinase deficient or unstable mutants (temperature sensitive, conditional AID *ddk* mutants) and kinase proficient and stable mutants of DDK (SUMOylation defective *ddk*-KR mutants) described in (Psakhye et al., 2019). We created *ddk* mutants in the *sgs1* mutant background where there is compromised resolution of the transiently forming recombination structures arising during chromosome replication (Branzei et al., 2008; Giannatasio et al., 2014; Liberi, 2005). We found that DDK contributes to replication associated recombination as we observed a recombination defect in all tested *ddk* mutants (*cdc7-4, cdc7-AID, ddk-KR*), with differences that will be summarized below.

DDK is known to phosphorylate numerous factors in the cell. As per the literature, the factors undergoing DDK-mediated phosphorylation participate in many essential cellular processes, including initiation of DNA replication (L. H. Hartwell, 1974; L. H. Hartwell et al., 1974; Leland H. Hartwell, 1976). This emphasizes the importance of this enzyme in cellular homeostasis as the absence of this kinase in budding yeast can result in cell lethality. Our findings also demonstrate the importance of enzymatic activity of DDK in recombination function. However, as the SUMOylation defective DDK mutant that we find defective in recombination function is active in origin firing (Psakhye et al., 2019), our results suggest that these two functions are genetically separable.
The fact that ddk<sup>ts</sup> (temperature sensitive) mutants show recombination defects in both sgs1Δ and sgs1Δ siz1Δ backgrounds explains the importance of DDK in both classical template switching involving PCNA polyubiquitylation as well as in the salvage pathway independent of PCNA polyubiquitylation. The salvage pathway is active in backgrounds in which PCNA SUMOylation is impaired. In those backgrounds, recombination structures arises from a Rad51-dependent recombination pathway that is independent of Rad18 and PCNA polyubiquitylation activities and is not further inhibited by the Srs2 anti-recombinase (Branzei & Szakal, 2016). The migration pattern of the recombination intermediates is similar to the one mediated by the interplay between homologous recombination activities and PCNA polyubiquitylation, and therefore it is currently unknown whether the underlying DNA structures mediating TS and the salvage pathway are identical or not. Based on the cycles of PCNA SUMOylation and polyubiquitylation, it is believed that the salvage pathway is restricted to later stages of the cell cycle whereas TS is activated early during replication. Importantly, both recombination pathways involve formation of SCJs, whose dissolution is primarily mediated by Sgs1 (Branzei & Szakal, 2016). In what respects the salvage pathway is different from template switching is not well understood. What is known from the literature is both this pathways are dependent on Rad51 dependent recombination (Pfander et al., 2005; Vanoli et al., 2010). We found that cdc7-4 was defective in both recombination pathways, highlighting a role for DDK on Rad51 and its regulators. This experiment was done in a Siz1 deleted background where PCNA SUMOylation is impaired. Siz1 is an E3 SUMO ligase enzyme that can not only SUMOylate PCNA but also other recombination and repair proteins. In order to rule out the possibility that recombination structures arising in Siz1 deleted background is not due to the functional defect of some other proteins, in future, it will be also interesting to repeat the same experiment in a pol30-RR background instead of siz1Δ background, where PCNA lacks two major SUMOylation sites (Branzei et al., 2006; Pfander et al., 2005).

Unlike cdc7-4, SUMOylation defective ddk-KR mutant showed recombination defect only in sgs1Δ background but not in sgs1Δ siz1Δ background indicating that the SUMOylation of DDK is acting in TS and the same window as PCNA polyubiquitylation, but is not required for the salvage pathway of recombination. It was also previously reported that cells that are defective in the TS pathway often have increased usage of TLS (Translesion synthesis) and can generate increased mutagenesis rates (Fumasoni et al., 2015; Storchova, 2001). It will be of interest to examine whether the role of SUMOylated DDK in facilitating TS is coupled with a defect in mutagenesis as reported for cdc7-4 (Brandão et al., 2014;
Pessoa-Brandão & Sclafani, 2004, or rather compensated by increased mutagenesis. This is a relevant piece of information as DDK is known to target Rad18 E3 ubiquitin ligase (Day et al., 2010). It is also known from the literature that PCNA can interact with Rad18 through its SUMO moiety as Rad18 contains SUMO interaction motifs (SIMs) and that this interaction is required for its roles in the regulation of PCNA ubiquitylation (Parker & Ulrich, 2012). Based on our current findings, what we would like to speculate here is that, like PCNA, DDK might also interact with Rad18 through its SUMO motif and that this interaction together with PCNA-SUMO is required for the regulation of Rad18 function in PCNA ubiquitylation (Model A).

**Model A: Hypothetical model representing the role of ddk-KR in regulating template switching:** SUMOylation defective ddk-KR mutant exhibited recombination defects only in the Template switching pathway (Figure 3.12) but not in the salvage pathway of recombination (Figure 3.17). SUMO modified PCNA is shown to interact Rad18 E3 Ubiquitin ligase via its SUMO moiety as Rad18 contains SIM motifs. Moreover, in human cells DDK is known to target Rad18. Here, we hypothesize that together with PCNA, DDK can also interact with Rad18 via its SUMO moiety that is required for the full activation of Rad18 and thereby its regulation of Template switching pathway.
4.2 DDK regulates recombination efficiency

We further investigated whether this recombination defect associated with ddk mutants is probably because of the lack of function of one of its known substrates. DDK is known to play roles in checkpoint regulation by phosphorylating and activating several checkpoint-associated factors. For instance, DDK is shown to be recruited to the the fork by the checkpoint protein Mrc1/Claspin and was also reported to interact and phosphorylate Mrc1/Claspin (Yang et al., 2016; Chen et al., 2013; Sasi et al., 2018). From our data it is clear that there is a checkpoint defect for temperature sensitive mutants of DDK but not for the SUMOylation defective ddk mutants. Additionally, DDK was also reported to activate Mus81-Mms4 complex for its process of dissolution of joint molecules thereby acting as a novel regulator of homologous recombination (Princz et al., 2017). Moreover, it was previously shown from our lab that checkpoint in itself can prevent the premature activation of this complex (Szakal & Branzei, 2013). However, the replication-associated recombination defect of ddk mutants was not identical to the one of replication checkpoint deficient mutants at least in regard to the fact that low levels of replication-associated recombination intermediates associated with ddk mutants were not rescued by concomitant depletion of the Mus81-Mms4 nuclease (Szakal & Branzei, 2013).

DDK was also shown to interact with cohesin, which is required to load cohesin-loaders prior to replication initiation. Moreover, cohesin was also shown to support replication-associated recombination (Branzei et al., 2008; Fumasoni et al., 2015). Although no reports so far indicate a role of DDK in replication-associated cohesion, this is an interesting possibility, especially considering DDK’s interaction with the replication fork protection complex, Tof1-Csm3 (Murakami & Keeney, 2014). Tof1-Csm3 is part of the replication fork machinery and has multiple replication-associated functions that maintain genome integrity, including its roles in sister chromatid cohesion (Mayer et al., 2004). To the contrary, from our 2D experiments (Fig 3.30) we found that the recombination defect associated with ddkts mutant was different from what was reported for cohesin mutants, whose role in replication-associated recombination is rescued by artificial sister chromatid tethering (Fumasoni et al., 2015).

One of the initial events induced by DNA damage is the recruitment of RPA followed by Rad51 filament nucleation on the exposed ssDNA that is involved in DNA recombination-mediated repair. Here, we found a modest reduction in Rad51 recruitment to the damaged DNA in ddk mutants which may explain reduced formation of replication associated
recombination factors by 2D gel electrophoresis. A role for DDK in modulating Rad51 availability may explain why DDK does not behave as a typical template switch mutant of the Rad5 pathway at least in ddk<sup>ts</sup> mutants, but as a homologous recombination mutant with roles in both the template switch and the salvage pathway, yet with more modest effects. This may suggest that DDK plays a more direct role in recombination by acting on factors that participate in recombination.

Rad51 assembly to the damaged DNA is counteracted by the anti-recombinase activity of Srs2, and the cell cycle dependent kinase, CDK which also shares consensus sites with DDK, can phosphorylate Srs2 (Saponaro et al., 2010) to facilitate Srs2 turnover. Turnover of Srs2 protein levels have already been identified as a mechanism that is proposed to regulate recombination at sites of replication stress (Urulangodi et al., 2015). Therefore, it would be further interesting to check whether DDK can potentially target Srs2 like CDK and whether this phosphorylation is required for its turnover and thereby promoting mitotic recombination. In addition to this, by using a Group based prediction system (GPS) 3.0 algorithm that has successfully adopted to predict phosphorylation sites in proteins, we identified few sites in Srs2 that can be potential targets of DDK (Table 4.1) (Lim et al., 2020; Xue et al., 2008). Whether these sites are getting phosphorylated by DDK resulting in its turnover, is coupled with its observed recombination defect is something which needs further investigation. Alternatively, or in addition to this, 9-1-1 complex that is known to target DDK in S. pombe and itself promotes replication-associated recombination (Karras et al., 2013), could also be an effector of DDK in this process (Furuya et al., 2010).

By Chip-qPCR, we observed that in cdc7-4 mutants there is an increased RPA recruitment but not in ddk-KR, which behaves like wildtype. It may be that the recombination defect common to the two analyzed ddk mutants is a consequence of an impairment at two different steps or that the penetrance of the defect is different. We observe that Rad51 recruitment is reduced in both ddk mutants. Importantly, ddk-KR is likely fully proficient in DDK kinase activity, but loss of SUMOylation may affect the formation of critical protein complexes required for TS. We also envisage that DDK might be involved in the regulation of exchange between Rad51 with RPA. Rad52 is another recombination protein that is shown in literature to be involved in displacement of RPA from ssDNA (Gasior et al., 1998; Plate et al., 2008). Alternatively, it is also possible that DDK is involved in the phosphorylation of other proteins that catalyze exchange of RPA with Rad51, such as Rad52, or of Rad51 paralogs, such as Rad55 and Rad57 proteins, which stabilize the Rad51 filament (Table 4.1).
As we observed a decrease in the Rad51 recruitment and a reduction in recombination intermediates, it is worth examining if there are predicted DDK phosphorylation sites in Rad51. For this, we analyzed the amino acid sequences of Rad51 using the group based prediction software (GPS) 3.0 algorithm (Lim et al., 2020), as mentioned above. This software identified two major phosphorylation sites on Rad51, S12 and S272, as putative Cdc7-specific phosphorylation sites (Table 4.1). We also found that both these positions are well conserved among different eukaryotic species. Another interesting observation is that both these positions belong to an ‘SES’ sequence motif which is also well conserved (data not shown). The significance of this motif is not well understood. However, whether these sites of Rad51 are phosphorylated by DDK and how much this phosphorylation influences the recombination process under investigation are experiments that deserve further future consideration. Alternatively, in cdc7-4 mutants, RPA may fail to be exchanged with Rad51 because of a phosphorylation defect due to which there is an increased RPA recruitment associated with reduction in Rad51.

Importantly, our results reveal that yeast cells with impaired DDK SUMOylation and DDK function accumulate increased ssDNA gaps with very long ssDNA length compared to wildtype during replication in the presence of DNA damage. Although both ddk mutants (cdc7-4 and ddk-KR) displayed higher number of ssDNA gaps, the major difference between cdc7-4 and ddk-KR is the ssDNA nucleotide length, whereby cdc7-4 showed longer ssDNA gaps than that of ddk-KR. It is unknown about the location on the whole genomic DNA where these gaps are arising. It can be that in cdc7-4 mutants, significant proportion of these defective forks arises at rDNA because of its importance indicated at replication fork pausing acting through Tof1 (Bastia et al., 2016).

There are several studies related to the relation between DDK and various nucleases, such as Exo1 and Mre11. In vitro studies reported that DDK can phosphorylate Exo1 upon replication fork stalling and in this way is involved in the regulation of Exo1 nuclease activity and/or stability, thus regulating resection (Sasi et al., 2018). A very recent paper also uncovered a role for DDK in promoting Mre11 fork processing, which is a function independent from DNA replication initiation (Rainey et al., 2020). The authors have speculated on Mre11 being a direct target of DDK phosphorylation where this phosphorylation may be required to increase its nuclease activity in cells. In ddk mutants, the observed accumulation of single stranded DNA gaps can be attributed to the fact that there is increased DDK activity at damaged replication forks followed by extensive nuclease’s activity leading to increased ssDNA gaps. This speculation can be probed by
examining the status of ssDNA gaps in yeast cells upon Mre11, Exo1 and/or Dna2 deletion in the ddk-KR background. This will not answer our question on why there is not any increased RPA recruitment in ddk-KR mutant compared to that of the wildtype while we still observe an increased number of ssDNA gaps. But what we observe from the electron microscopy experiments is that apart from the increased ssDNA gaps, the length of the gaps in ddk-KR mutants compared to wildtype is not very high, which is consistent with the results of RPA recruitment in this mutant. The threshold of the difference of RPA recruitment is still unknown. But taking together all the observed differences between cdc7-4 and ddk-KR especially in terms of recombination, and the differences on the DDK kinase activity, it will be of interest to examine how these effects are reflected in the regulation of fork resection or fork uncoupling, as both these processes can account for the observed EM phenotype.

What happens in cdc7-4 in terms of increased RPA recruitment as well as increased ssDNA gap length requires further experimentation. cdc7-4 acts as a functionally defective DDK mutant, especially in terms of its kinase function. It might be that in this mutant, there is downregulation of Mre11 which leads to the expression of other nucleases causing extensive resection. These possibilities can be tested by checking by EM what happens in ddkts mutant when the activities of other nucleases are inhibited. Importantly, similar analysis needs to take place in the kinase proficient ddk-KR mutant. The connection between the observed accumulation of ssDNA gaps in ddk mutants and the reduced recombination intermediate accumulation has still to be studied.

Additionally, our experiments unveiled a checkpoint defect for ddkts mutants but not for ddk-KR mutants. Literature studies show that the checkpoint kinases are involved in preventing the action of nucleases during replication stress conditions (Cotta-Ramusino et al., 2005; Segurado & Diffley, 2008). One speculation is that there might be unscheduled nucleolytic events that arise at the replication fork because of an impaired checkpoint activation which then leads to increased ssDNA gaps that we observe in ddkts mutants. It might be that the recombination defect that we observe in ddkts mutants is compounded by reduced checkpoint activation.

Another interesting possibility is that DDK restricts fork uncoupling. This is because of the finding that there are several replication forks associated factors that are targets or can be possible targets of DDK kinase. For instance, as DDK interacts with the replication fork protection complex Tof1-Csm3 and is recruited to the fork by Mrc1/Claspin (Murakami & Keeney, 2014; Yang et al., 2016), its role in recombination may be manifested upon
recruitment of Tof1/Csm3 to the replication fork. We posit that this increased single stranded DNA gaps we are observing in the $\text{cdc7-4}$ temperature sensitive mutant is probably because of dysregulation of factors associated with replication fork uncoupling which ultimately leads to exposure of more single stranded gaps probably because phosphorylation by DDK might be regulating stable association of some of these replisome components. This could also explain why we are not seeing any increased RPA recruitment or increased ssDNA gap length in $\text{ddk-KR}$ mutant but only in $\text{cdc7-4}$. In the $\text{ddk-KR}$ mutant there is stable DDK associated with the replication fork, which may not lead to fork uncoupling. As mentioned previously, what is happening in the $\text{ddk-KR}$ mutant is probably because of an excessive nuclease activity, but this still needs further investigation.

Additionally, Bastia et al., proposed a role for DDK in replication fork pausing under non-damaging conditions (Bastia et al., 2016). They have showed the importance of Tof1 phosphorylation in programmed fork arrest (PFA). They have proposed Tof1 as possible target of DDK as they observe a similar replication fork pausing defect in both mutants. We also confirmed these results. It is known that Tof1-Csm3-Mrc1 associates with replication forks and makes a stable complex (Bando et al., 2009; Katou et al., 2003). These components may facilitate pausing to prevent the uncoupling of the replisome from the template (Katou et al., 2003). In $\text{S. pombe}$, it was shown that Dbf4 homolog interacts with components of the fork protection complex (Matsumoto et al., 2005). Also we know that in meiosis ,DDK is involved in physical association with Tof1/Csm3 and is involved in the recruitment of DDK to the replication fork (Murakami & Keeney, 2014). Whether recruitment of DDK to the replication fork is required for the maintenance of replication fork complex, is currently unknown. A rational explanation is that Tof1 phosphorylation by DDK is required for its recruitment and maintenance at the replisome to form a stable replication fork complex. This raises the possibility that when DDK is dysfunctional, Tof1 retention is affected resulting in replication fork uncoupling that further results in the exposure of ssDNA which we visualize through our electron microscopy experiments.

Alternatively, it was shown that limited re-priming can lead to fork uncoupling and exposure of long stretches of ssDNA at the fork (Fumasoni et al., 2015). Instead of physiologically affecting the fork uncoupling on the leading strand, DDK may affect fork coupling by modulating re-priming. It was reported that error-free recombination during replication relies on the $\text{Pol\alpha/Primase/Ctf4}$ complex (Fumasoni et al., 2015). One possibility is that DDK might be involved in the regulation of factors like Ctf4 that connect different replisome components and act as hubs for other replisome associated factors (Villa et al.,
From our group-based prediction software analysis, we have also identified some DDK phosphorylation sites also for Ctf4 (Table 4.1). As per our notion that DDK might regulate nuclease activities, DDK mode of action may be manifested through Ctf4, also because of its known interaction with Dna2 nuclease (Formosa & Nittis, 1999; Villa et al., 2016).

<table>
<thead>
<tr>
<th>Suspected substrates of DDK</th>
<th>Predicted phosphorylation sites</th>
<th>High threshold score (cut off = 11.93)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad51</td>
<td>S12 S272</td>
<td>12.66 12.66</td>
</tr>
<tr>
<td>Ctf4</td>
<td>T212 S872</td>
<td>12.33 15.33</td>
</tr>
<tr>
<td>Srs2</td>
<td>S399 S628 S682 T980</td>
<td>15.33 12.66 12.66 12.33</td>
</tr>
<tr>
<td>Tof1</td>
<td>T29 S796 S1150 S1208</td>
<td>12.33 12.66 12.66 15.33</td>
</tr>
<tr>
<td>Rad52</td>
<td>T419</td>
<td>12.93</td>
</tr>
</tbody>
</table>

Table 4.1 Possible substrates of DDK and its phosphorylation sites as predicted by GPS (Group-based prediction software 3.0)
As per our preliminary findings, we found our predicted phosphorylation sites for DDK in Rad51. Rad51 can be potentially phosphorylated at two major sites: S12 and S272. Interestingly, out of these two sites, 272\textsuperscript{nd} Serine was found to be well conserved among several eukaryotic species. A structure prediction analysis also indicate that this position is situated on the outer interface of Rad51 when bound to the DNA, making it exposed for a possible modification. As a recent advancement of the study, we have created a point mutation at this 272\textsuperscript{nd} position of serine, converting serine to alanine and found that this mutant is sensitive to different damaging drugs like MMS, HU and CPT. Interestingly, this mutant also displayed recombination defect similar to that of \textit{ddk} mutants. We further plan to investigate whether this is a direct phosphorylation site for DDK by developing and using phospho-specific antibodies for this particular site of DDK. In addition to this, we also plan to perform a phospho-proteomics screening, to identify whether Rad51 can a potential substrate for DDK. Based on the results obtained from the above experiments, we might also proceed to perform invitro kinase assays using purified \textit{ddk} mutants and Rad51 variant.

It was proposed that the binding of Rad51 to ssDNA plays a role in protecting the DNA from excessive uncoupling of the replication fork during DNA synthesis. In this way, Rad51 functions in preventing excessive ssDNA accumulation (Hashimoto et al., 2010; Mason et al., 2019). We also speculate that Rad51 phosphorylation by DDK might be important for its protective role of replication forks from the action of various nucleases, a hypothesis that we also plan to test in the near future.

As SUMOylation defective \textit{ddk-KR} mutant also exhibited recombination defects, using co-immunoprecipitation studies, we will be also checking whether ddk-KR interacts with Rad18 via its SUMO interacting motif.

Based on the current findings, what we speculate here is, DDK can phosphorylate Rad51 during genotoxic stress conditions and that this phosphorylation is required for either its recruitment or stabilization on the single stranded DNA that is further required for specific activation of homologous recombination. In the absence of DDK, Rad51 becomes defective in phosphorylation thereby affecting the recombination (Model B).
Model B: DDK phosphoregulates Rad51 that is required for the activation of Homologous recombination. Upon induction of genotoxic stress during replication, fork stalling occurs that results in the exposure of single stranded DNA. RPA gets recruited to the ssDNA which will be later replaced by Rad51. DDK can phosphorylate Rad51 and that this phosphorylation is required for either its recruitment to the ssDNA and/or its stabilization. This promotes further recombination and repair. In the absence of DDK, Rad51 phosphorylation is impaired resulting in recombination defects and thereby genomic instability.

As DDK is a kinase involved in the phosphorylation of different substrates, knowledge of the identity of DDK substrates is important to understand its cellular functions. In this regard, it will also be worth performing a SILAC screen to compare and identify different phosphoproteins in ddk mutants versus wildtype in MMS conditions. Identifying different substrates and functions of DDK can unravel more possibilities in cancer chemotherapy. Overall, our study provides novel insights on DDK roles in DDT and relevant substrates, and may inform potentially new means to block replication of dividing cancerous cells relying on DDT.
6. APPENDIX 1

During my PhD term, I had also contributed to another work in collaboration with Prof. José Antonio Tercero from the Department of Centro de Biología Molecular Severo Ochoa (CSIC/UAM), Spanish National research council, Madrid, Spain. The manuscript related to this work is published in the following journal.

Journal: Science advances
Date: 08 April 2020
DOI: 10.1126/sciadv.aaz3327

Manuscript is attached to the thesis.

Title:
The Mgs1/WRNIP1 ATPase is required to prevent a recombination salvage pathway at damaged replication forks

Abstract
DNA damage tolerance (DDT) is crucial for genome integrity maintenance. DDT is mainly carried out by template switch recombination, an error-free mode of overcoming DNA lesions, or translesion DNA synthesis, which is error-prone. Here, we investigated the role of Mgs1/WRNIP1 in modulating DDT. Using budding yeast, we found that elimination of Mgs1 in cells lacking Rad5, an essential protein for DDT, activates an alternative mode of DNA damage bypass, driven by recombination, which allows chromosome replication and cell viability under stress conditions that block DNA replication forks. This salvage pathway is RAD52 and RAD59 dependent, requires the DNA polymerase δ and PCNA modification at K164, and is enabled by Esc2 and the PCNA unloader Elg1, being inhibited when Mgs1 is present. We propose that Mgs1 is necessary to prevent a potentially toxic recombination salvage pathway at sites of perturbed replication, which, in turn, favors Rad5-dependent template switching, thus helping to preserve genome stability.
4. APPENDIX 2

During my PhD, I had also worked on another project in collaboration with Dr. Peter de Wulf from the Department of Experimental Oncology, European Institute of Oncology, Italy. This work is published in the following journal.

Journal: Nucleic Acids Research
Date: 06 September 2018
DOI: 10.1093/nar/gky618

Manuscript is in attachment to the thesis.

Title:

Integrating Rio1 activities discloses its nutrient-activated network in Saccharomyces cerevisiae

Abstract

The Saccharomyces cerevisiae kinase/adenosine triphosphatase Rio1 regulates rDNA transcription and segregation, pre-rRNA processing and small ribosomal subunit maturation. Other roles are unknown. When overexpressed, human ortholog RIOK1 drives tumor growth and metastasis. Likewise, RIOK1 promotes 40S ribosomal subunit biogenesis and has not been characterized globally. We show that Rio1 manages directly and via a series of regulators, an essential signaling network at the protein, chromatin and RNA levels. Rio1 orchestrates growth and division depending on resource availability, in parallel to the nutrient-activated Tor1 kinase. To define the Rio1 network, we identified its physical interactors, profiled its target genes/transcripts, mapped its chromatin-binding sites and integrated our data with yeast's protein-protein and protein-DNA interaction catalogs using network computation. We experimentally confirmed network components and localized Rio1 also to mitochondria and vacuoles. Via its network, Rio1 commands protein synthesis (ribosomal gene expression, assembly and activity) and turnover (26S proteasome expression), and impinges on metabolic, energy-production and cell-cycle programs. We find that Rio1 activity is conserved to humans and propose that pathological RIOK1 may fuel promiscuous transcription, ribosome production, chromosomal instability, unrestrained metabolism and proliferation; established contributors to cancer. Our study will advance the understanding of numerous processes, here revealed to depend on Rio1 activity.

5. APPENDIX 3

I have also internally collaborated with one of my colleague’s project and the resulting paper is currently under peer-review.
6. REFERENCE


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The Mgs1/WRNIP1 ATPase is required to prevent a recombination salvage pathway at damaged replication forks

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DNA damage tolerance (DDT) is crucial for genome integrity maintenance. DDT is mainly carried out by template switch recombination, an error-free mode of overcoming DNA lesions, or translesion DNA synthesis, which is error-prone. Here, we investigated the role of Mgs1/WRNIP1 in modulating DDT. Using budding yeast, we found that elimination of Mgs1 in cells lacking Rad5, an essential protein for DDT, activates an alternative mode of DNA damage bypass, driven by recombination, which allows chromosome replication and cell viability under stress conditions that block DNA replication forks. This salvage pathway is RAD52 and RAD59 dependent, requires the DNA polymerase δ and PCNA modification at K164, and is enabled by Esc2 and the PCNA unloader Elg1, being inhibited when Mgs1 is present. We propose that Mgs1 is necessary to prevent a potentially toxic recombination salvage pathway at sites of perturbed replication, which, in turn, favors Rad5-dependent template switching, thus helping to preserve genome stability.

INTRODUCTION

The presence of DNA damage is largely inevitable and a main source of genomic instability (1). DNA lesions can cause pathological conditions that may lead to disease or cell death, and in consequence, cells require efficient mechanisms that first detect and then either repair or tolerate DNA insults (1, 2). Cells are especially vulnerable to DNA damage during chromosome replication, as unrepaired lesions at the time of replication may hamper the progression of replication forks. These lesions need to be tolerated, leaving their repair for a later time, to avoid permanent fork stalling or fork breakdown that would result in incomplete genome replication (2, 3).

In eukaryotes, the DNA damage tolerance (DDT) is mainly carried out by the RAD6/RAD18 pathway (2, 3). When DNA replication forks stall due to DNA lesions or replicative stress, the DNA polymerases and the replicative helicase can partially uncouple, leading to long stretches of single-stranded DNA (ssDNA) that are coated by the replication protein A. This coated ssDNA is the signal for the activation of DDT, triggering the recruitment to chromatin of the E3-ubiquitin ligase Rad18 (4). Both proteins form a heterodimer that mono-ubiquitylates the proliferating cell nuclear antigen (PCNA) sliding clamp protein at K164 (5). This PCNA modification activates translesion DNA synthesis (TLS) by favoring its interaction with bypass (TLS) polymerases (6). TLS polymerases have low fidelity and are able to replicate across the DNA lesions, a mode of DNA damage bypass that is frequently error-prone. The monoubiquitin modification of PCNA can be further extended to K63-linked polyubiquitin chains, a process that is carried out by the E3-ubiquitin ligase Rad5 in budding yeast (5) (HLTF and SHPRH in mammals) together with the E2 complex Ubc13–Mms2 (UBC13–UEV1 in mammals). PCNA polyubiquitylation mediates a second mode of DNA damage bypass that requires the DNA-dependent adenosine triphosphatase (ATPase)/helicase activity of Rad5 and is driven by transient template switch recombination (7, 8). In this type of bypass, the blocked DNA nascent strand uses the recently synthesized undamaged strand of the sister chromatid as a template for replication over the lesion, and the process is error-free. Both modes of DDT are interconnected, as Rad5 is also required for the recruitment of TLS polymerases to stressed replication forks and for TLS activity (9).

In addition to ubiquitylation, PCNA is also modified during chromosome replication by SUMOylation at K164 and, to a minor extent, at K127 (5). PCNA SUMOylation promotes the recruitment of Srs2, an antirecombinogenic helicase that prevents unscheduled recombination at replication forks (10, 11) by dismantling Rad51 filaments (12, 13). In higher eukaryotes, a similar antirecombinogenic role is carried out by PARI (14, 15). Both modifications of PCNA, polyubiquitylation and SUMOylation, cooperate to facilitate template switching (16, 17), and, at least in budding yeast, SUMO-PCNA is the physiological substrate of Rad18 (17).

The fact that homologous recombination is inhibited by Srs2 after its recruitment by SUMO-PCNA, while template switch recombination works as an efficient error-free DNA damage bypass mechanism, raised an apparent paradox and an interesting biological problem that was recently deciphered (18). This work showed that whereas homologous recombination is inhibited globally during chromosome replication by the Srs2 helicase at ongoing forks, the template switching mode of recombination is allowed locally via the action of the SUMO-like domain protein Esc2, which counteracts Srs2 at damaged or stalled replication forks (18). Esc2 binds to sites of stalled replication and promotes Elg1-dependent local unloading of SUMO-PCNA, together with bound Srs2, and Sbx5–Sbx8–mediated proteasome degradation of the antirecombinase, which notably reduces the levels of Srs2 at the stalled forks. These low Srs2 levels allow local Rad51 filament formation and recombination-mediated damage bypass via template switching at sites of perturbed replication (18). A question derived from these findings, however, is how the template switch mode of recombination is favored, whereas, despite the local

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counteraction of Srs2 at damaged forks, a salvage pathway of recombination is still inhibited or postponed for later in the cell cycle. This action is important because, unlike template switching, a salvage pathway is potentially toxic as it can lead to deleterious genomic rearrangements during replication or the accumulation of DNA intermediates that are not properly resolved.

Posttranslational modifications of PCNA are central for DDT, and therefore, the study of the proteins that interact with modified PCNA may provide a better understanding of how the mechanisms of DDT are modulated. Among them, budding yeast maintenance of genome stability 1 [Mgs1; MgsA/RarA in bacteria and WRNIP1 (Werner helicase interacting protein 1) in humans] is an evolutionarily conserved DNA-dependent AAA+ ATPase with ssDNA annealing activity (19), whose function is poorly understood. Mgs1 interacts with PCNA in vivo and in vitro (19, 20) and shows preference for the association with polyubiquitylated PCNA via its ubiquitin-binding zinc finger (UBZ) domain, which allows its recruitment to sites of replication stress (20). Mgs1 also interacts genetically and physically with the polymerase δ (Pol δ) (21–23). By interfering with the Pol δ–PCNA interaction, Mgs1 might facilitate the release of this polymerase during different processes (20). Mgs1 levels are important for its function because overexpression affects mutation rates and recombination and makes cells sensitive to genotoxic stress (19, 21). However, MGS1 deletion does not confer sensitivity to DNA-damaging agents, but it causes an increase in the frequency of mitotic recombination (19, 21). Although the function of Mgs1 is unclear, this protein has been linked to DDT due to the interaction with PCNA described above and because Mgs1 becomes essential in the absence of Rad6/Rad18 (22, 23). In addition, consistent with the requirement of Mgs1 and its homologs for genome stability during chromosome replication, it was recently shown that human WRNIP1, with proposed roles in DNA transactions (24), is important for the maintenance of the integrity of stalled forks and for replication resumption (25). Likewise, it has been proposed that Bacillus subtilis RarA assembles at blocked forks and plays a role in preventing pathological replication fork restart (26).

In this work, we have investigated the contribution of Mgs1 to the DDT and have found that, in the absence of Rad5, this protein is required to prevent a recombination salvage pathway at damaged and stalled replication forks. We propose that Mgs1 contributes to channeling DDT to error-free template switch recombination by helping to block other potentially detrimental recombination processes at the replication fork, an action that is fundamental for genome stability.

RESULTS
The MMS and HU sensitivity of cells lacking the DDT protein Rad5 is suppressed by deletion of MGS1 or elimination of Mgs1-ATPase activity

To start investigating the contribution of Mgs1 to DDT, we first studied its functional relevance in the absence of Rad5. We analyzed Saccharomyces cerevisiae the sensitivity of a double mutant mgs1Δrad5Δ to methyl methanesulfonate (MMS) or hydroxyurea (HU) (Fig. 1). As described (22, 23), mgs1Δrad5Δ cells showed a growth defect with respect to the parental strains when spores germinate after tetrad dissection (Fig. S1A), but this defect was not apparent when cells growing exponentially were spotted onto rich-medium plates (Fig. 1A). As previously reported (19, 21), cells lacking MGS1 did not present differences in the sensitivity to MMS or HU with respect to wild-type cells, whereas rad5Δ mutant cells were highly sensitive to the treatment with both compounds (Fig. 1A). Previous reports had indicated a similar sensitivity of rad5Δ and mgs1Δrad5Δ cells to MMS and a higher sensitivity of the mgs1Δrad5Δ double mutant to HU than the single rad5Δ (22). Unexpectedly, however, and probably as a result of the different experimental approach used, our drop dilution assays showed that mgs1Δrad5Δ cells were significantly more resistant to chronic exposure to MMS or HU than rad5Δ cells (Fig. 1A), indicating that deletion of MGS1 causes a significant suppression of the sensitivity of cells lacking Rad5 to those agents. To rule out that this result was due to the genetic background used (W303), we carried out the same kind of experiments using DF5 S. cerevisiae cells (fig. S1B). The recovery of the viability of the rad5Δ mutant after MMS or HU treatment when MGS1 was deleted was similar to that obtained with W303 (Fig. 1A and fig. S1B), which eliminated a potential influence of the background on our data.

In the drop dilution assays described above, cells were treated with MMS or HU for several generations. As Rad5 is required for the completion of chromosome replication and the maintenance of viability during S phase in the presence of MMS-damaged DNA (27), we also examined the sensitivity to MMS of mgs1Δrad5Δ cells and the corresponding individual mutants during a single S phase (Fig. 1B). Cells were first synchronized in G1 phase with the α factor pheromone and then released into S phase in fresh medium containing different concentrations of MMS. The analysis of the viability along the experiment indicated that wild-type control and mgs1Δ cells were not sensitive to the treatment with MMS during S phase, unlike rad5Δ cells, which were highly sensitive to all MMS doses used (Fig. 1B). Similar and consistent to the results obtained with the drop dilution assays (Fig. 1A), elimination of MGS1 allowed cells lacking Rad5 to significantly reduce their sensitivity to MMS at all the concentrations used, even at the highest doses of MMS and at the longest exposure times during S phase (Fig. 1B). Thus, deletion of MGS1 notably reduces the sensitivity of rad5Δ mutant cells to the treatment with MMS, not only after chronic exposure to this DNA-damaging agent but also during a single S phase.

Mgs1 contains an ATPase domain in its central region (19) and a zinc finger domain (UBZ) at its C terminus that is necessary for the interaction of this protein with PCNA (20). To differentiate whether the effect of MGS1 deletion on rad5Δ cells was due to the absence of the whole Mgs1 protein or can just be explained by the elimination or some of its properties, we examined the consequences of mutating the ATPase or the UBZ domains of Mgs1 on the sensitivity of rad5Δ to MMS or HU. We constructed strains combining previously characterized mutants of these domains [mgs1-K183A for ATPase (19) and mgs1-D31A for UBZ (20)] with rad5Δ deletion and analyzed their sensitivity to MMS and HU after treatment for several generations (Fig. 1C). Drop dilution assays (Fig. 1C) showed that cells lacking Rad5 were highly sensitive to MMS or HU and that this sensitivity was significantly suppressed after MGS1 deletion, in agreement with the data in Fig. 1A. The mutation of the UBZ domain of Mgs1 did not have any effect on the sensitivity to MMS or HU of rad5Δ cells (mgs1-D31A rad5Δ strain; Fig. 1C), but the mutation of the ATPase activity of Mgs1 suppressed, to a large extent, the sensitivity of the rad5Δ mutant to the same drugs (mgs1-K183A rad5Δ strain; Fig. 1C). Likewise, the inactivation of the ATPase activity of Mgs1 allowed a significant suppression of the sensitivity of rad5Δ cells to MMS during a single S phase (Fig. 1D), at all MMS concentrations.
used, to a similar extent as the deletion of \textit{MGS1} (Fig. 1, B and D). Thus, elimination of the ATPase activity of Mgs1 is sufficient to significantly suppress the high sensitivity to treatment with MMS or HU of cells lacking the DDT protein Rad5.

\textbf{Mgs1 elimination in rad5\textDelta cells facilitates a pol \textdelta – and PCNA-K164 modification–dependent tolerance pathway}

Rad5 is required for the completion of DNA replication in the presence of DNA-damaging agents such as MMS or adozelesin (27–29), making possible the progression of replication forks through damaged DNA and thus contributing to the maintenance of cell viability (27). As chromosome replication in the presence of MMS-induced DNA damage is halted in \textit{rad5\textDelta} cells (27), we investigated whether the absence of Mgs1 could revert that situation, which would help to explain the \textit{mgs1\textDelta rad5\textDelta} results in Fig. 1. With this purpose, we analyzed the dynamics of chromosomal replication by pulsed-field gel electrophoresis (PFGE) after treating cells with MMS (Fig. 2A). \textit{mgs1\textDelta rad5\textDelta} and wild-type, \textit{mgs1\textDelta}, \textit{rad5\textDelta}, \textit{mgs1-D31A rad5\textDelta}, \textit{mgs1-K183A rad5\textDelta}, \textit{mgs1-D31A}, \textit{mgs1-K183A}, \textit{rad5\textDelta}, \textit{mgs1-D31A rad5\textDelta}, \textit{mgs1-K183A rad5\textDelta}, \textit{rad5\textDelta}, and \textit{mgs1-D31A rad5\textDelta} control cells were synchronized in G\textsubscript{1} phase with \textalpha factor and then released into S phase in medium containing different MMS concentrations. Strains are as in (A). The plots represent the means ± SD from three independent experiments.
right (PFGE) and left bottom (quantification), consistent with flow cytometry (Fig. 2A, left top). In wild-type control and mgs1Δ cells, full-length chromosomes reentered the gel after 120-min recovery in medium without MMS, as shown by a clear signal from discrete bands that increased after 240 min to nearly 2× with respect to that in G1 [Fig. 2A, right and left (bottom)]. These data indicated that, in most cells, the chromosomes recovered from the DNA lesions induced by MMS and completed replication. In contrast, in rad5Δ cells, and in agreement with the requirement of Rad5 for replication of damaged DNA (27–29), most of the DNA was retained in the wells even 240 min after recovery from MMS treatment, indicating that chromosomes were not replicated [Fig. 2A, right and left (bottom)]. However, the elimination of Mgs1 suppressed to a high extent the replication problems of cells lacking Rad5. Thus, unlike rad5Δ cells and similar to wild-type and mgs1Δ controls, in mgs1Δrad5Δ cells, there were discrete bands corresponding to intact chromosomes 120 min after recovery from MMS exposure, with band signals increasing to similar levels to those of wild type and mgs1Δ after 240 min [Fig. 2A, right and left (bottom)], indicating that, in most cells, chromosomal replication had been completed. Therefore, the absence of Mgs1 in rad5Δ cells allows DNA damage bypass and chromosome replication, which may explain why cells lacking Rad5 reduce their sensitivity to agents causing DNA damage or replication stress when MGS1 is deleted.

To understand how DNA damage bypass takes place in mgs1Δrad5Δ cells, we first asked whether the replicative Pol δ, whose interaction with PCNA is modulated by Mgs1 (20), is necessary for the suppression described so far. Although the gene encoding the catalytic subunit of Pol δ, Pol3, is essential, the removal of the last four amino acids of Pol3 (pol3Δct mutant) allows cell survival while conferring sensitivity to certain agents (30). We made a triple mutant pol3Δct mgs1Δrad5Δ, which was viable, and carried out sensitivity assays to MMS or HU, as before (Fig. 2B). Drop dilution assays showed that the double mutants mgs1Δpol3Δct and rad5Δpol3Δct behaved as their corresponding individual parental mutants, whereas mgs1Δrad5Δ cells were more resistant to MMS or HU than the rad5Δ mutant. However, the suppression of the sensitivity to MMS or HU of rad5Δ by MGS1 deletion was not possible when the last amino acids of Pol3 were deleted (pol3Δct mgs1Δrad5Δ strain). This result indicates that Pol δ is necessary for the process that allows DNA replication and viability of mgs1Δrad5Δ cells after MMS or HU treatment.

The absence of the E3-ubiquitin ligase Rad5 impedes PCNA polyubiquitylation, but this protein can be still modified by mono-ubiquitylation at K164 and by SUMOylation at the same residue and,
to a minor extent, at K127 (5, 6). To analyze whether PCNA modification was relevant for the replication through damaged DNA in mgs1Δrad5Δ cells, we used a PCNA mutant not modifiable at K164, pol30K164R, and performed drug sensitivity assays as before (Fig. 2C). Drop dilution assays showed that pol30K164R cells were as sensitive to MMS as rad5Δ cells. Moreover, deletion of MGS1 in a pol30K164R rad5Δ mutant did not suppress the sensitivity of these cells to MMS treatment (Fig. 2C). Therefore, together with the requirement for Pol δ, PCNA modification at K164 is necessary for DNA replication and cell viability under stress conditions in rad5Δ cells when Mgs1 is eliminated.

Translesion synthesis polymerases make only a minor contribution to replication stress tolerance in mgs1Δrad5Δ cells

As template switching is not present in the mgs1Δrad5Δ mutant due to the lack of Rad5, it could be expected that DNA damage bypass could rely solely on translesion DNA synthesis. In MGS1+ rad5Δ cells, TLS activity is not enough to allow the completion of chromosome replication under conditions of MMS-damaged DNA (27), but spontaneous mutagenesis is increased in mgs1Δrad5Δ cells (22), which could be the result of a higher activity of TLS polymerases in this mutant. Taking these data and the results above into account, we analyzed whether TLS activity is responsible for allowing DNA damage bypass and thus for the completion of chromosome replication and cell viability in mgs1Δrad5Δ mutant, at least in response to MMS treatment. According to this hypothesis, TLS polymerases would bypass the DNA lesions in mgs1Δrad5Δ cells for which the modification of PCNA at K164 is necessary, and then Pol δ would continue DNA synthesis.

rev1Δmgs1Δrad5Δ and rev3Δmgs1Δrad5Δ mutants exhibit severe growth defects [Fig. S2 and (23)], but the use of the mgs1-K183A allele, which in combination with rad5Δ behaves similarly to mgs1Δ (Fig. 1, C and D), allowed the construction of viable strains (mgs1-K183A rev1Δrad5Δ, mgs1-K183A rev3Δrad5Δ, and mgs1-K183A rev1Δrev3Δrad5Δ—30Δ) where the last one is referred to as mgs1-K183A tilsΔrad5Δ; Fig. 3, A and B) that were useful to test the mentioned hypothesis. Drop dilution assays (Fig. 3B) indicated that cells lacking Rad5 were highly sensitive to MMS or HU and that elimination of MGS1 or its ATPase activity (mgs1-K183A rad5Δ strain) significantly suppressed this sensitivity, in agreement with the data in Fig. 1. However, in the absence of any of the TLS polymerases or even all of them (Fig. 3B, last four lanes), there was only a small reduction in the suppression of the sensitivity of rad5Δ cells to MMS when Mgs1-ATPase activity was eliminated, and there was no effect on the sensitivity to HU. This result indicates that TLS polymerases have only a minor role in the process that allows DNA replication and cell viability in the absence of Rad5 when MGS1 is deleted or Mgs1-ATPase activity is eliminated, and therefore, other mechanisms that explain the mgs1Δrad5Δ phenotype must be involved.

Elimination of Mgs1 in cells lacking Rad5 promotes a Rad52- and Rad59-dependent recombination-driven tolerance pathway

Previous studies had shown that the elimination of the Srs2 helicase, which binds SUMO-PCNA causing inhibition of homologous recombination (10, 11), suppresses the sensitivity of rad5Δ cells to ultraviolet (UV) light or γ-irradiation (31, 32). Considering these data, we sought to analyze whether homologous recombination was involved in the mechanism that allows chromosome replication in mgs1Δrad5Δ in the presence of DNA damage or replicative stress, which, as shown above, cannot be explained (only) by the action of TLS polymerases. We first tested in our genetic background the consequences of eliminating Srs2 in combination with mgs1Δ or rad5Δ and found that, similar to what happens in mgs1Δrad5Δ cells and consistent with the aforementioned reports (31, 32), SRS2 deletion reduced the sensitivity of rad5Δ cells to MMS or HU (Fig. S3). Moreover, in the absence of Srs2, Mgs1 elimination did not have apparent consequences on rad5Δ cells and vice versa.

The similarity of the phenotype of the mgs1Δrad5Δ mutant to that of the strains harboring SRS2 deletion (fig. S3) could support the hypothesis that, as in srs2Δ cells, homologous recombination was facilitated in rad5Δ cells after Mgs1 deletion. To directly test this idea, we analyzed recombination molecularly, studying the formation/disappearance of X-shaped structures in the proximity of DNA replication forks by two-dimensional (2D) gel electrophoresis, in cells treated with MMS (Fig. 4A and fig. S4). The presence of these types of structures following DNA damage indicates recombination events, which are largely due to Rad5-dependent template switching mechanisms (16, 33). Cells lacking Rad5 show a reduction of these X-DNA intermediates under DNA-damaging conditions (29). We synchronized cells in G1 phase with α factor and then released them into


Fig. 3. TLS polymerases have a minor role in the suppression of the sensitivity of rad5Δ cells to MMS or HU when the Mgs1-ATPase activity is eliminated. (A) The elimination of the ATPase activity of Mgs1 is compatible with the deletion of RAD5 and the genes encoding TLS polymerases. Examples of tetrad dissection after combining RAD5 deletion in the absence of Mgs1-ATPase activity (mgs1-K183A) with deletions of REV1 (YAJ117 × YAJ96 strains; left), REV3 (YAJ117 × YAJ76; center), or all TLS (REV1, REV3, and RAD30; YAJ183 × YAJ206; right). Spores were grown at 30°C for 48 hours. (B) Drop dilution assays. Serial dilutions (10-fold) of normalized exponentially growing cultures were spotted onto YPD plates containing different amounts of MMS or HU and incubated for 48 hours at 30°C. Strains: Wild-type (YAJ111), mgs1Δ (YAJ110), mgs1-K183A (YAJ113), rad5Δ (YAJ115), mgs1Δrad5Δ (YAJ114), mgs1-K183A rad5Δ (YAJ117), rev1Δrev3Δrad5Δ (tlsΔ) (YAJ231), mgs1ΔtlsΔ (YAJ233), mgs1-K183A tlsΔ (YAJ235), tlsΔrad5Δ (YAJ237), mgs1-K183A tlsΔrad5Δ (YAJ230), mgs1-K183A rev3Δrad5Δ (YAJ165), mgs1-K183A rev1Δrad5Δ (YAJ196), and mgs1-K183A rev30Δrad5Δ (YAJ205).

Fig. 4. Mgs1-ATPase activity plays a role in the Rad52-dependent recombination pathway. (A) The elimination of the ATPase activity of Mgs1 is compatible with the deletion of RAD5 and the genes encoding TLS polymerases. Examples of tetrad dissection after combining RAD5 deletion in the absence of Mgs1-ATPase activity (mgs1-K183A) with deletions of REV1 (YAJ117 × YAJ96 strains; left), REV3 (YAJ117 × YAJ76; center), or all TLS (REV1, REV3, and RAD30; YAJ183 × YAJ206; right). Spores were grown at 30°C for 48 hours. (B) Drop dilution assays. Serial dilutions (10-fold) of normalized exponentially growing cultures were spotted onto YPD plates containing different amounts of MMS or HU and incubated for 48 hours at 30°C. Strains: Wild-type (YAJ111), mgs1Δ (YAJ110), mgs1-K183A (YAJ113), rad5Δ (YAJ115), mgs1Δrad5Δ (YAJ114), mgs1-K183A rad5Δ (YAJ117), rev1Δrev3Δrad5Δ (tlsΔ) (YAJ231), mgs1ΔtlsΔ (YAJ233), mgs1-K183A tlsΔ (YAJ235), tlsΔrad5Δ (YAJ237), mgs1-K183A tlsΔrad5Δ (YAJ230), mgs1-K183A rev3Δrad5Δ (YAJ165), mgs1-K183A rev1Δrad5Δ (YAJ196), and mgs1-K183A rev30Δrad5Δ (YAJ205).
S phase in medium containing MMS. The pattern of replication intermediates at the ARS305 early origin of replication was analyzed at different times during chromosome replication (Fig. 4A and fig. S4). In agreement with previous studies (29), rad5Δ cells showed an important reduction in the percentage of X molecules with respect to wild-type and mgs1Δ cells. Notably, this situation was significantly reverted when MGS1 was eliminated in the rad5Δ mutant. Thus, in mgs1Δrad5Δ cells, there was a notable increase in the percentage of X-shaped intermediates with respect to rad5Δ at 45 and 90 min after release from the G1 block when, according to flow cytometry, cells were in S phase (Fig. 4A). This result indicates that, in cells lacking Rad5 and under conditions of MMS-induced DNA damage, the absence of Mgs1 allows the formation of recombination structures at damaged replication forks that very likely facilitate replication. These structures are not originated by template switching, as this mechanism is absent in this strain due to the lack of Rad5.

To genetically support the 2D gel data in Fig. 4A, we next studied the involvement of the recombination proteins Rad51 and Rad52 in the phenotype of mgs1Δrad5Δ cells. The triple mutant rad51Δmgs1Δrad5Δ was inviable, and unlike the case of the strategy used to study the role of TLS polymerases (Fig. 3), the combination of rad51Δ with the mgs1-ATPase mutant and rad5Δ (rad51-1K183A rad5Δ) did not yield a viable strain either (fig. S5). On the contrary, although they exhibited a growth defect, rad52Δmgs1Δrad5Δ cells were viable (Fig. 4B). Drop dilution assays showed that RAD52 deletion increased the sensitivity of cells lacking Rad5 to MMS or HU. Moreover, even considering the growth defect of the triple mutant, these drop dilution assays indicated that, in the absence of Rad52, elimination of Mgs1 could not rescue the viability of rad5Δ cells after treatment with the drugs (Fig. 4B). Together, and in agreement with the data obtained by 2D gel electrophoresis (Fig. 4A), the results suggest that homologous recombination is crucial to allow replication and viability of mgs1Δrad5Δ cells when treated with MMS or HU.

To further understand the requirement of recombination for replication and viability in the mgs1Δrad5Δ mutant after MMS or HU treatment, we analyzed the potential implication of Rad59 in the studied process. RAD59 is a paralog of RAD52, and although the Rad59 protein contributes to a subset of homologous recombination events, it is not required for template switching (34). We combined the deletion of RAD59 with those of MGS1 and RAD5 and analyzed the sensitivity of this strain and the corresponding controls to MMS or HU by drop dilution assays (Fig. 4C). The data obtained indicated that Rad59 is necessary for the mechanism that allows viability of mgs1Δrad5Δ cells after exposure to MMS or HU, thus reinforcing the importance of homologous recombination for this process and showing that the mode of recombination in these cells is genetically different from template switching.

Survival of mgs1Δrad5Δ cells under genotoxic stress conditions depends on the SUMO-like domain protein Esc2 and the PCNA unloader Elg1

The requirement of homologous recombination for the replication and survival of mgs1Δrad5Δ cells following genotoxic replicative stress raises the question of how this mechanism is allowed at damaged or stalled forks, as SUMOylation of PCNA recruits the antirecombinase Srs2 helicase and prevents potentially toxic recombination (10, 11). As explained previously, recombination is globally inhibited at forks during chromosome replication in “wild-type” (MGS1’ RAD5’) cells,
but the SUMO-like domain protein Esc2 counteracts Srs2 locally at perturbed forks, facilitating recombination-mediated DNA damage bypass by error-free template switching (18). This is achieved by Elg1-dependent local unloading of SUMOylated PCNA and increased turnover of Srs2, which reduces Srs2 levels at sites of perturbed replication (18). The question is how a recombination mode alternative to template switching is allowed in a mgs1 mutant, but not in MGS1’rad5Δ cells, in which forks stall in the presence of DNA damage causing cell death (27). We thought that a possibility was that this mode of recombination, which could be considered a potentially mutagenic “salvage pathway,” was facilitated in the mgs1 mutant by the same factors that promote template switching at stalled forks in wild-type cells but inhibited when Mgs1 is present.

To analyze whether similar requirements to those that facilitate template switching in MGS1’RAD5Δ cells make possible a recombination salvage pathway in the mgs1’rad5Δ mutant, we examined the potential involvement of Esc2 (Fig. 5A) and Elg1 (Fig. 5B) in the phenotype of these latter cells. Drop dilution assays (Fig. 5A) showed that, like wild-type and mgs1Δ cells, esc2Δ and esc2Δmgs1Δ cells had little sensitivity to MMS or HU treatment similar to mgs1’rad5Δ and that esc2Δmgs1Δ cells were as sensitive to these drugs as the rad5Δ mutant. Notably, deletion of ESC2 in cells lacking Rad5 impeded the suppression of their sensitivity to MMS or HU when Mgs1 is eliminated (esc2Δmgs1Δrad5Δ strain). Drop dilution assays (Fig. 5B) also showed that elg1Δ and elg1Δmgs1Δ cells had similar sensitivity to MMS or HU as wild-type or mgs1Δ cells. In agreement with previous data (35), ELG1 deletion allowed a modest recovery of the viability of rad5Δ cells after MMS treatment. However, ELG1 elimination prevented the suppression of the sensitivity of the rad5Δ mutant to MMS or HU when MGS1 was deleted (elg1Δmgs1Δrad5Δ strain) (Fig. 5B). Moreover, both interacting peptide and SUMO-interacting motifs of Elg1 (35) were necessary for the suppression of the sensitivity of rad5Δ cells by MGS1 deletion (fig. S6), consistent with the requirement of PCNA modification at K164 for this process (Fig. 5C). These results indicate that both Esc2 and Elg1 are required to allow recombination-driven replication and cell survival in the presence of genotoxic replication stress in mgs1’rad5Δ cells, which strongly suggests that the mechanism allowing for this process is similar to that facilitating template switching at stalled forks in wild-type cells (18). Notably, although Esc2 and Elg1 are present in cells lacking Rad5, this mechanism does not work if they have Mgs1.

To further study how Mgs1 influences this alternative recombination mechanism providing tolerance in mgs1’rad5Δ cells, we asked whether this protein affects recruitment of Elg1 to damaged replication forks. To test this possibility, we used chromatin immunoprecipitation (ChIP)–on-chip to analyze the binding of Elg1 along the entire genome.
in rad5Δ, mgs1Δrad5Δ cells, and the corresponding wild-type and mgs1Δ controls (Fig. 5C). Cells were first synchronized in G1 with α factor and then released into S phase in fresh medium containing MMS. The genome-wide clusters of Elg1 were examined after 30-min treatment with this drug. ChIP-on-chip analysis showed that the overall genomic coverage of Elg1 was significantly higher in rad5Δ cells than in the rest of the strains. The genomic coverage of this protein in cells lacking Rad5 was reduced to levels close to those of wild-type cells when MGS1 was eliminated (Fig. 5C). We also studied the genomic coverage of the Pol δ catalytic subunit Pol3 in the same strains (Fig. 5D). The ChIP-on-chip analysis showed that the main peaks that indicate Pol3 binding significantly coincide in their location with those of Elg1 under the same experimental conditions (Fig. 5, C and D). This similar ChIP-on-chip pattern shown by both proteins indicates that Elg1 is enriched at regions containing replication forks, which are marked by the polymerase. Concerning the binding of Pol3, the fact that the genomic coverage of this protein is higher in mgs1Δ cells than in MGS1Δ cells is consistent with the proposed role for Mgs1 in the modulation of Pol δ interaction with PCNA (20). These data indicate that in the absence of Rad5 and under DNA-damaging conditions, there is a direct correlation between the presence of Mgs1 and the accumulation of Elg1 at damaged stalled forks. In this situation, a recombination-driven tolerance pathway that allows replication does not take place, all of which is reverted when Mgs1 is eliminated.

Together, our results strongly suggest that Mgs1 is required to prevent unscheduled and potentially mutagenic recombination at damaged forks in the absence of Rad5. Likewise, from these data, it is possible to deduce that in wild-type cells, in which recombination by template switching, but not other modes of recombination, is facilitated locally at damaged forks (18), Mgs1 contributes to avoid a Rad52/Rad59-dependent recombination salvage pathway, thus helping to channel DNA damage bypass to the error-free mode of DDT.

**DISCUSSION**

The evolutionarily conserved AAA+ ATPase Mgs1 is involved in the maintenance of genome stability (19–23), but its precise function has remained enigmatic in part due to the absence of a clear phenotype of Mgs1-deficient cells. In this work, we addressed the relevance of this ATPase in cells lacking Rad5, a protein that is central for DDT by template switching and TLS (9). This approach revealed a role for Mgs1 in preventing a recombination salvage pathway at damaged or stalled replication forks.

We found that the elimination of Mgs1 or its ATPase activity significantly suppresses the sensitivity of rad5Δ cells to agents that cause DNA damage, such as MMS, or replicative stress, such as HU. This suppression can be explained by the activation of an alternative pathway in mgs1Δrad5Δ cells, but not in rad5Δ cells, where forks stall and cells die under genotoxic stress conditions (27–29). We showed that the mechanism that allows cell viability by overcoming DNA obstacles during replication in mgs1Δrad5Δ cells is dependent on Pol δ and requires modification of PCNA at K164. Notably, translesion synthesis polymerases have only a minor role in this process, despite being the main known DDT branch in the absence of template switching, perhaps because TLS is not fully functional in the absence of Rad5 (9). Instead, we uncovered that replication in mgs1Δrad5Δ cells in the presence of DNA damage is mainly driven by homologous recombination. This conclusion is based on (i) physical evidence provided by the increase in the percentage of X molecules at forks under DNA damage conditions in mgs1Δrad5Δ cells with respect to rad5Δ and (ii) genetic data such as the dependency of mgs1Δrad5Δ resistance to MMS and HU on RAD52 and RAD59, the latter, in turn, indicates that this type of recombination is genetically distinguishable from template switching (34). Notably, Rad59 is not required for the alternative recombination pathway that is facilitated in rad18Δels1 cells treated with UV light (10, 11), suggesting distinct mechanisms for the bypass of different DNA insults. As recombination-mediated template switching is absent in mgs1Δrad5Δ cells, and the observed mode of recombination is independent of PCNA poly-ubiquitylation, it is possible to conclude that elimination of Mgs1 or its ATPase activity in cells lacking Rad5 allows a recombination salvage pathway that facilitates chromosome replication in the presence of genotoxic stress. The extrapolation of these results to wild-type (RAD5Δ) cells might help to understand why the mgs1Δ mutant shows an increase in the frequency of mitotic recombination (19, 21).

The salvage pathway of recombination described before is normally restricted to late S phase or G2-M (7, 36) and inhibited in principle by the antirecombinase Srs2 (10, 11). However, this or a related salvage pathway is permitted at perturbed replication forks in mgs1Δrad5Δ cells. Urunagodi et al. (18) revealed that although recombination is globally inhibited during replication by Srs2, the SUMO-like domain protein Esc2, together with Elg1, can counteract Srs2 locally, allowing recombination-mediated DNA damage bypass by template switching at damaged stalled forks (Fig. 6A). We found that Esc2 and Elg1 are also required for replication stress tolerance in mgs1Δrad5Δ cells (Fig. 6B). These dependencies strongly suggest that template switching at damaged forks in wild-type cells and the salvage recombination pathway operating in mgs1Δrad5Δ cells are driven in a similar fashion by a mechanism facilitated by Esc2 and Elg1. Thus, in cells lacking both Rad5 and Mgs1, as in wild-type cells (18), Esc2 might bind to sites of stalled replication promoting Elg1 association to damaged replication forks, which, in turn, would lead to unloading of Srs2 bound to SUMO-PCNA followed by its degradation. Reduced local levels of Srs2 would allow binding of Rad51 and subsequently a recombination-driven replication pathway that is Rad52 and Rad59 dependent and requires DNA synthesis by Pol δ (Fig. 6B). Our data showed that this recombination-driven replication process is inhibited when Mgs1 is present (Fig. 6C) and that Mgs1 manifests its inhibitory function in a manner driven by its ATPase activity. In MGS1Δrad5Δ cells, we observed an accumulation of Elg1 at forks that is reverted by Mgs1 elimination, which indicates a correlation between Elg1 accumulation and the presence of Mgs1. Elg1 accumulation may lead to PCNA stabilization or, conversely, may be a consequence of it. Either way, the result could be the retention of Srs2 at damaged stalled forks, which would prevent recombination in a Mgs1-dependent way, impeding the completion of chromosome replication in MGS1Δrad5Δ cells (Fig. 6C) (27–29).

Our results suggest an important role for Mgs1 in preventing unscheduled recombination at damaged replication forks. They offer a rationale to explain why in wild-type cells a recombination-mediated error-free template switching, which depends on Rad5, is facilitated at damaged stalled forks, whereas a potentially toxic recombination salvage pathway that could lead to genomic rearrangements or faulty replication is inhibited. The discrimination between these two modes of recombination exists, although both might be in principle enabled locally at sites of stalled replication by the same Esc2- and Elg1-dependent mechanism that results in low
levels of the Srs2 antirecombinase (18). We propose that Mgs1 is a key factor required to prevent a salvage pathway of recombination at damaged or stalled forks. This, in turn, would help to channel DNA damage bypass to template switching, thus importantly contributing to the maintenance of genome stability during chromosome replication.

MATERIALS AND METHODS

Strains, media, and cell cycle experiments
The budding yeast strains used in this work are derivatives of W303 or DF5. Their relevant genotypes are indicated in table S1. All the strains were constructed by standard techniques or genetic crosses. The pML (37) and pYM (38) plasmid series were used as templates.
for polymerase chain reaction. Yeasts were routinely grown at 30°C in YP medium (1% yeast extract and 2% Bacto Peptone) containing 2% glucose. Bacto agar (2%) was added for solid medium. Cells were synchronized in G1 with the α factor pheromone (5 to 10 μg/ml). Nocodazole was used at 5 μg/ml. Samples for flow cytometry were collected and processed as described (39) and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Drug sensitivity assays
Cell viability after MMS treatment during a single S phase was determined by plating cells in triplicate onto YP-glucose plates containing different concentrations of MMS or HU. The plates were incubated at 30°C for 48 to 72 hours.

Pulse-field gel electrophoresis
Genomic DNA was obtained from 10^8 cells and prepared in plugs of low melting agarose, as previously described (39). The chromosomes were separated in a 1% agarose–tris-borate EDTA (TBE) gel by PFGE at 14°C using a CHEF-DR II system (Bio-Rad). The electrophoresis were carried out at 200 V (6 V/cm) for 24 hours, with 60- and 90-s pulses for 15 and 9 hours, respectively. The gels were stained with ethidium bromide and scanned after UV exposure. Quantification of the chromosome bands was performed using the ImageJ program (National Institutes of Health).

2D gel analysis
Purification of DNA intermediates, 2D gel analysis, and X-molecules quantification were performed as previously described (40).

ChIP-on-chip
The ChIP-on-chip experiments and the analysis of genome-wide clusters were carried out as previously described (18).

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/15/eaaz3327/DC1

View request a protocol for this paper from bio.protocol.

REFERENCES AND NOTES

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Integrating Rio1 activities discloses its nutrient-activated network in \textit{Saccharomyces cerevisiae}

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ABSTRACT

The \textit{Saccharomyces cerevisiae} kinase/adenosine triphosphatase Rio1 regulates rDNA transcription and segregation, pre-rRNA processing and small ribosomal subunit maturation. Other roles are unknown. When overexpressed, human ortholog RIOK1 drives tumor growth and metastasis. Likewise, RIOK1 promotes 40S ribosomal subunit biogenesis and has not been characterized globally. We show that Rio1 manages directly and via a series of regulators, an essential signaling network at the protein, chromatin and RNA levels. Rio1 orchestrates growth and division depending on resource availability, in parallel to the nutrient-activated Tor1 kinase. To define the Rio1 network, we identified its physical interactors, profiled its target genes/transcripts, mapped its chromatin-binding sites and integrated our data with yeast’s protein–protein and protein–DNA interaction catalogs using network computation. We experimentally confirmed network components and localized Rio1 also to mitochondria and vacuoles. Via its network, Rio1 commands protein synthesis (ribosomal gene expression, assembly and activity) and turnover (26S proteasome expression), and impinges on metabolic, energy-production and cell-cycle programs. We find that Rio1 activity is conserved to humans and propose that pathological RIO1 may fuel promiscuous transcription, ribosome production, chromosomal instability, unrestrained metabolism and proliferation; established contributors to cancer. Our study will advance the understanding of numerous processes, here revealed to depend on Rio1 activity.

INTRODUCTION

The RIO family of atypical protein kinases is found in most archaea, bacteria and eukaryotes (1–5). Lower eukaryotes including the budding yeast \textit{Saccharomyces cerevisiae} comprise two subfamilies: Rio1/RIO1K1 and Rio2/RIOK2, while higher eukaryotes, including humans, contain an additional subfamily: RIOK3. From archaea to higher eukaryotes, Rio1 promotes the 3′-end processing of the small ribosomal subunit pre-rRNA and mediates the release of late biogenesis factors during small ribosomal particle maturation. Importantly, as far as the process of ribosome biogenesis is concerned, Rio1 can act as a kinase as well as an adenosine triphosphatase (ATPase) (6–15). In budding...
yeast, Rio1 kinase activity also regulates 35S rDNA transcription by RNA polymerase I to ensure a timely onset of the cell cycle, to safeguard rDNA copy-number homeostasis, and to promote rDNA condensation and segregation (16). Its involvement in rDNA and rRNA biology, and its promotion of 40S ribosomal subunit maturation are consistent with RIO1 being a member of the ribosome biogenesis (Ribi) regulon (17–19). The latter comprises 236 genes whose proteins participate in the synthesis, assembly and functioning of the ribosome. Importantly, they do not encode the structural components of the ribosome as these are encoded by the ribosomal protein (RP) regulon. Ribi and RP expression are co-regulated in response to environmental conditions and the ability of a cell to grow and proliferate therein (e.g. nutrient availability): activated when resources are abundant, repressed when they are scarce (17–19).

Heterozygous rio1Δ yeast is sensitive to drugs perturbing Golgi activity, DNA replication, sphingolipid and ergosterol biosynthesis (20). These phenotypes suggest an involvement of Rio1 also in these processes, beyond ribosome biology.

In human cells, next to its role as a ribosome biogenesis factor (21–23), RIOK1 has also been implicated in the regulation of intracellular signaling, gene expression, cancer initiation, development and metastasis. Indeed; RIOK1 phosphorylates the TORC2 kinase complex to activate the kinase AKT and its downstream signaling network (24). RIOK1 is also part of the PRMT5 arginine N-methyltransferase complex, which methylates, among others, histone H4R3 to silence globin expression (25) and the ribonucleoprotein hnRNP A1 to control the translation of the cyclin D1 and c-Myc transcripts (26). A genetic lethality screen in colon cancer cells expressing the oncogenic KRASG13D mutation identified RIOK1 (27), suggesting involvement of RIOK1 in the activity of the GTPase KRAS. The latter functions as a molecular switch for signaling involvement of Rio1 also in these processes, beyond ribosome biology.

Although RIOK1 overexpression promotes cancer growth and invasion (30) its contributions to both events remain unclear because the protein -as is true also for Rio1- has not yet been mapped functionally at the whole-cell level. To advance our understanding of Rio1 and RIOK1 biology, we charted Rio1 activity in S. cerevisiae. Next, we revealed its functional conservation from yeast to human cells. Specifically, we identified physical and functional interactors of Rio1 by yeast two-hybrid and genetic synthetic screens, the genes and/or transcripts it regulates by RNA-sequencing, we localized Rio1 across the genome via ChIP-sequencing analysis and throughout the cell by indirect immunofluorescence (IF) and cryo-immunogold electron microscopy (EM). Using network computation we integrated our biological datasets with empirically verified and curated yeast protein–protein and protein–DNA interaction catalogs. This effort produced Rio1’s multi-layered network, which functions at the protein, gene/chromatin and RNA levels. Rio1 controls its target open reading frames (ORFs) both directly and indirectly using a host of transcription factors and regulators. Combined, the identities of its gene targets, protein interactors and intracellular localization patterns suggest that Rio1 regulates ribosome production, protein synthesis and turnover, metabolism, energy production and cell division (rDNA replication and chromosome transmission). RIO1 transcription, which we find is auto-regulated, is high under nutritionally rich conditions. Upon nutritional deprivation (glucose, amino acids), RIO1 expression becomes auto-repressed and the translation of its few transcripts increases. This pattern of regulation correlates with the expression status of its network regulon members, as indicated by a host of reporter genes involved in the most divergent intracellular processes. Noteworthy, Rio1 and nitrogen stress-response transcription factor Gcn4 cross-regulate each other and their gene networks in adaptation to growth conditions. Our singular datasets and tractable activity map represent valuable resources revealing how Rio1 and its network manage various essential biological processes, now associated with Rio1 activity.

MATERIALS AND METHODS
Yeast strains and culture conditions

The S. cerevisiae strains used in this study (Supplementary Table S14) have a W303-1A genetic background and were created by mating, tetrad dissection followed by spore selection, or by transformation and homologous recombination of polymerase chain reaction (PCR)-generated deletion or epitope cassettes. The RIO1-AID, PADH1-OsTIR1-9Myc degron strain (next named the RIO1-AID strain) was engineered as described (34). Strains were grown exponentially (OD600 = 0.8–1.0) at 24°C (200 rpm) in YPD (1% yeast extract, 2% peptone, 2% glucose) or YPRG medium (1% yeast extract, 2% peptone, 2% raffinose, 2% galactose). To deplete Rio1 from yeast, exponentially growing RIO1-AID cells were treated for 1 h with 500 μM of auxin (indole-3-acetic acid; Sigma-Aldrich, Cat # 13750). RIO1-AID depletion was confirmed by anti-AID western blot hybridization and by RT-qPCR analysis of 35S pre-rRNA transcript levels. For the starvation experiments; RIO1-AID cells were grown in synthetic complete medium (0.67% yeast nitrogen base without amino acids (Difco, Cat # 291940), 2% glucose, 0.002% amino acid drop-out mix), collected, washed with water, and re-suspended in synthetic drop-out medium (0.67% yeast nitrogen base without amino acids) provided with 2% glucose or with 0.002% amino acid drop-out mix, and supplemented with 500 μM of auxin or mock solution. One hour after being shifted to the new growth medium, the cells were collected, all RNAs extracted, and converted into cDNA for quantitation of certain cDNAs using a set of TaqMan RT-qPCR probes.

Yeast two-hybrid interaction screens, and confirmation by co-immunoprecipitation and western blot hybridization analysis

Yeast two-hybrid (Y2H) screens were performed by Hybrigenics Services (www.hybrigenics-services.com; Paris, France). The RIO1 coding sequence (YOR119C) was fused in frame at its N- or C-terminus to the LexA or the Gal4
DNA-binding domain. The four bait constructs were then screened three times each against all *S. cerevisiae* genes, fused N- or C-terminally to the Gal4 activation domain. In the screens, the complexity of the *S. cerevisiae* genomic library was covered 5- to 14-fold. Prey fragments of positive clones were amplified by PCR, sequenced and identified in the GenBank database (NCBI). Next, a predicted biological score (e-value) was calculated for each identified interaction. First, a local score took into account the redundancy and independency of the prey fragments, as well as the distribution of reading frames and stop codons in overlapping fragments. Second, a global score considered the interactions found in all the screens performed previously with the same genomic library. The combined calculated score (e-value ≤ 1.0E-02) positively correlates with the biological significance of the identified interaction (35–38).

To corroborate biochemically that Rio1 physically interacts with Y2H hits Rvb2, Sky1 and Sam37, we performed co-immunoprecipitation (co-IP) experiments. Since the endogenous expression level of *RIO1* is very low, and given that the efficacy of co-IP and elution of the interacting proteins is very low as well, we decided to perform the co-IP analysis using strains in which the expression of *RIO1*, *RVB2*, *SKY1* and *SAM37* were slightly elevated (details below) allowing for the identification of the Rio1-protein interaction by western blot hybridization analysis. Specifically, we created four strains in which the *RIO1* coding sequence was N-terminally labeled with a tandem 1FLAG-PrA (protein A) tag. Next, in three strains, the *RVB2*, *SKY1* or *SAM37* coding sequence was marked with an N-terminal 3HA epitope (in the fourth, negative control strain, no protein was labeled with a 3HA epitope). The expression of the proteins was placed under control of the *P_gal1* promoter and the strains then grown in 2% raffinose YP medium (YPR) to slightly enhance protein levels. Cell extracts were made with acid washed glass beads (FastPrep FP120 homogenizer, MP Biomedicals) and incubated for 4h (at 4°C) with anti-1FLAG M2 affinity agarose (GE Healthcare) and radiography (GE Healthcare).

**Western blot hybridization analysis of Rio1-AID protein levels**

Cells were collected from a 5 ml culture sample (14 000 rpm; 1 min, 4°C), treated with 5% trichloroacetic acid (Sigma-Aldrich) and processed as described (16). Membranes (Immobilon-P PVDF; Millipore) were incubated with mouse monoclonal anti-AID antibody (1:1000; Cosmo Bio Co., Cat # BRS-APC004AM) or with mouse monoclonal anti-Pgk1 antibody (1:5000; Life Technologies, Cat # 459250). Following incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10 000; BioRad, Cat # 170-6516) the proteins were visualized using ECL chemiluminescence solution (GE Healthcare) and radiography (GE Healthcare).

**Serial dilution growth analysis**

Yeast strains were grown overnight at 24°C (200 rpm) in YPD or YPR medium, back-diluted to an OD600 = 1.0, and serially diluted (1:4.5 dilution steps) in 96-well plates. The cells were transferred with a multi-pin replicator from the multi-well plates onto YPD or YPRG agar plates and incubated for 2–3 days at the indicated temperatures (Supplementary Figures S2B-C and 3A).

**Indirect immunofluorescence widefield imaging and cryo-immunogold electron microscopy**

Indirect IF widefield deconvolution imaging of yeast cells endogenously expressing 6Myc-Rio1, Rio1-GFP, Ndc80-3GFP or Nop1, or of nuclei isolated from the cells, was performed as described (16). In short, a 1 ml sample of yeast cells grown exponentially in YPD medium (24°C, 200 rpm) was centrifuged and cross-linked overnight in 1 ml of 3.7% formaldehyde. The cell walls were then digested (30 min) with zymolyase (100 μg/ml, Amsbio Cat # 100T), washed with 1.2 M sorbitol plus 100 mM phospho-citrate pH 5.9, and bound to a multiwall poly-L-lysine coated glass slide (Sigma-Aldrich). The slide was treated with DAPI, and hybridized with a rabbit anti-GFP primary antibody (Living Colours Full-Length GFP Polyclonal Antibody, Clontech, Cat # 632593), a mouse monoclonal anti-Myc primary antibody (9E10, Covance, Cat # MMS-150R) or a mouse monoclonal anti-Nop1 primary antibody (ThermoFisher, Cat # 28F2). Cy3 or fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Jackson ImmunoResearch Laboratories) were used to visualize the proteins. Images were cap-
tered with a DeltaVision ELITE microscope (Applied Precision) carrying an Olympus IX71 UPlanSapo objective lens (100×, NA 1.40) and a CoolSnap HQ2 CCD camera (Photometrics). Fifteen Z-stacks were acquired every 0.4 μm, deconvoluted (SoftWoRx) and virtually projected with maximum intensity.

Cryo-immunogold EM was performed as described (39). In short, S. cerevisiae cells endogenously expressing Rio1-GFP were grown in YPD medium (200 rpm, 24°C) till an OD₆₀₀ = 0.8. Next, the cells were pelleted by centrifugation, embedded in 12% gelatin, cooled on ice and cut into 1 mm³ cubes at 4°C. The cubes were immersed in 2.3 M sucrose (4°C, overnight), fast-frozen in liquid nitrogen, and cut with a Leica EM FC7 ultramicrotome. Thin sections (50–60 nm thickness) were picked up in a 1:1 mix of 2% methylcellulose and 2.3 M sucrose, and incubated with the rabbit anti-GFP antibody (1:10 dilution). Next, the sections were incubated with gold (10 nm)-labeled protein A (Cell Sciences) and treated with 1% glutaraldehyde and embedded in methylcellulose uranyl acetate. Images were acquired with a Tecnai-20 electron microscope (FEI, Eindhoven, The Netherlands), treated with 1% glutaraldehyde and embedded in methylcellulose uranyl acetate. Images were acquired with a Tecnai-20 electron microscope (FEI, Eindhoven, The Netherlands). To calculate the volumes of cells and organelles, we used the discretized version of the vertical rotator procedure. Specifically, we drew the central vertical axis through the center of the maximal diameter of the cell and then placed the stereological grids, as published (40). The gold labeling density was quantitated as described (41). For the nucleus, mitochondria and vacuoles, the numeric volume density (gold/μm²) was quantitated, whereas for the plasma and Golgi membranes, we measured the numeric surface density (gold/μm).

**Fluorescence-activated cell sorter (FACS) analysis**

Per time point, 1 × 10⁷ cells were collected by centrifugation and resuspended in 70% ethanol for 16 h. The cells were then washed in 250 mM Tris–HCl (pH 7.5), resuspended in this buffer containing 2 mg/ml of RNase A (37°C, 4 h) and treated with proteinase K (1 mg/ml) (37°C, 30 min). Next, the cells were resuspended in 200 mM Tris–HCl (pH 7.5), 200 mM NaCl and 80 mM MgCl₂ and their DNA then stained with 1 μM Sytox-green (Invitrogen). Samples were diluted 10-fold in 50 mM Tris–HCl (pH 7.8) and analyzed with a Becton Dickinson FACScan instrument.

**Extraction of replication intermediates and analysis by 2D agarose gel electrophoresis**

Purification of DNA intermediates and 2D agarose gel electrophoretic analysis were performed as described (42). In short, following the synchronous release of cells enriched in late G1 (START) using α-factor, a 200 ml culture of RIO1-AID cells containing or depleted of Rio1-AID and arrested with 200 mM of hydroxyurea (HU), were sampled (2–4 × 10⁶ cells), treated with 0.1% Na₂S, spheroplasted and submitted to DNA extraction with chloroform/isomylalcohol (24:1). Next, the purified DNA was treated with restriction enzymes EcoRV and HindIII to analyze ARS305, or with BgIII to analyze 5S rDNA, separated using 2D agarose gel electrophoresis, and submitted to Southern blot hybridization with radiolabeled probes targeting ARS305 and 5S rDNA (43). Recombination intermediate signals were quantitated with ImageQuant software (GE Healthcare).

For each time point, areas corresponding to the monomer spot (M), the Y arc and a region without replication intermediates (background reference) were selected and the signal intensities in % of each signal obtained. The values for the Y arc and monomer were corrected by subtracting from the signal intensity value the background value after the latter was multiplied for the ratio between the dimension of the area for the intermediate of interest and for the background. The relative signal intensity for the Y arc was determined by dividing the value for Y arc/termination with the monomer values.

**RNA isolation**

Total RNA was isolated from 5 ml of an exponential culture. The cells were harvested (14 000 rpm, 1 min, 4°C), washed with ice-cold buffer (50 mM sodium acetate pH 5.2, 10 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0) and stored at −80°C. RNA was then extracted using phenol and chloroform, as described (16). Next, residual DNA was removed by incubation (30 min, 37°C) with DNase I (New England Biolabs, Cat # M0303S) and the RNA further purified with the RNeasy Kit (Qiagen, Cat # 74104). RNA concentrations were measured with a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific) and its quality and integrity examined with a 2100 Bioanalyzer (Agilent Technologies).

**Real-time qPCR (RT-qPCR)-based quantitation of RNA transcripts**

A total of 1 μg of RNA, isolated as indicated above, was reverse transcribed into cDNA with ImProm-II reverse transcriptase (Promega, Cat # A3801) and random primers (Life Technologies, Cat # 48190-011). A total of 5 ng of the cDNA were then submitted to RT-qPCR analysis (7500 Fast Real-Time PCR, Life Technologies) using either custom-made or commercially available TaqMan probes (Thermo Fisher Scientific). The custom-made ETS1-1 probe (named probe nr. 4) measures the cDNAs derived from the primary 5S pre-rRNA transcripts at the 5′ETS sequence:

- forward primer: 5′-GATTTGTTGGATTACAGCTAATAGCAATCT-3′,
- reverse primer: 5′-GGAGGTACACTGGAAGAATCAGCT-3′,
- reporter sequence: 5′-CAACAAGGCGTTCCTCC-3′,
- 5′-fluorophore: 6-carboxyfluorescein (FAM), 3′-quencher: non-fluorescent quencher (NFQ).

The custom-made RPL7B probe measures the cDNAs derived from the RPL7B transcripts:

- forward primer: 5′-GAAGAAACATCATTCAAGCTAAGCGT-3′,
- reverse primer: 5′-GTTGAGCTCCAGGAGT-3′,
- reporter sequence: 5′-CAGCAGCCTTGGCATC-3′,
- 5′-fluorophore: FAM, 3′-quencher: NFQ.
The commercially available TaqMan RT-qPCR probes used in this study (ThermoFisher Scientific) are listed in Supplementary Table S15.

Ribosome profiling, pulse labeling of neo-synthesized RNAs and proteins

Ribosome profiles (40S and 60S subunits, 80S monosomes and polysomes) were produced by growing the RIO1 wild-type and RIO1-AID strains to an OD$_{600}$ = 0.5 in YPD medium (24°C). The cells were then treated for 1 h with 500 μM of auxin, isolated, treated on ice (5 min) with 100 μg ml$^{-1}$ cycloheximide (to stabilize the polysomes), and washed with extraction buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 10 mM MgCl$_2$, 100 μg ml$^{-1}$ cycloheximide). The cells were broken with glass beads and the cleared extract loaded on a 10.5 ml 5–45% sucrose gradient in extraction buffer lacking cycloheximide. Following centrifugation (16 h; 21 000 rpm; SW41 Beckman rotor), gradients were collected at 1 ml min$^{-1}$ flow rate and the UV profile recorded at 254 nm with a UV detector (linked to BioLogic LB fractionators), visualized with LP Data View software (Bio-Rad) and exported to Excel (Microsoft Office).

To study whether a 1 h depletion of Rio1 affected (r)RNA production we grew the RIO1 wild-type and RIO1-AID strains overnight in 2% glucose synthetic medium containing 10% (w/v) sucrose. The cells were then back-diluted in 2% glucose synthetic medium comprising 2.5 mM uracil, grown 10 mM uracil. The cells were then back-diluted in 2% glucose synthetic medium containing 10 mM uracil. The cells were then harvested after 20 min and total RNA extracted (16). The newly synthesized, 4TU-incorporated RNAs were biotinylated in the dark using 5 μg MTSEA Biotin-XX (Biotium, Cat # 440736) was added to the culture. The cells were then treated for 1 h with 500 μM of auxin or with a mock solution. A total of 5 μg of total RNA were isolated as described above, and ribosomal RNAs (rRNAs) removed with the Ribo-Zero Gold rRNA Removal Kit Yeast (Illumina, Cat # MRZY1324). rRNA depletion was confirmed by 2100 Bioanalyzer RNA6000 Pico Chip analysis (Agilent Technologies). A total of 2 μl of the rRNA-depleted RNAs were then reverse transcribed into a cDNA library with the ScriptSeq v2 RNA-Seq Library Preparation Kit (Illumina, Cat # SSV21124). cDNA library concentrations were quantified with a Qubit Fluorometer (Invitrogen) and its quality examined on a 2100 Bioanalyzer using the High Sensitivity DNA assay (Agilent Technologies). The cDNA library was then deep sequenced in multiplex fashion (6 samples per run) for 75 bases in the paired-end mode on a MiSeq Sequencing System (Illumina).

The library reads were aligned on the reference genome sacCer3 (S. cerevisiae S288c assembly from the Saccharomyces Genome Database (GCA_000146055.2)) (46) using TopHat software (47). The mapped sequences were processed with HTSeq software (48) using parameters: -m intersection strict, -a skip quality reads less than 1. Differential gene expression analyses including size-factor normalization, shrinkage estimation for the distribution's variance and negative binomial distributions were performed using the R package DESeq (49).

Chromatin immunoprecipitation (ChIP), ChIP~RT-qPCR and ChIP-chip analysis

RIO1-AID cells were grown exponentially till an OD$_{600}$ = 0.8 in YPD medium (24°C, 200 rpm). Culture samples (50 ml) were then cross-linked with 1% formaldehyde (24°C, 1 h) and treated as described (16). Mouse monoclonal anti-AID antibody (10 μg (Cosmo Bio Co., Cat # BRS-APC004AM) conjugated to protein A-agarose beads (ThermoFisher Scientific, Cat # 15918014) was then used to isolate Rio1-AID from the cell extracts. After washing and resuspending the beads, the crosslinks were reversed overnight (1% SDS, 65°C). All proteins were removed with Proteinase K (Roche, Cat # 03115828001) and contaminant RNA degraded with RNase A (Sigma-Aldrich, Cat # 10109169001). The chromatin that co-immunoprecipitated with Rio1-AID was extracted with phenol and chloroform, resuspended in 40 μl of double-distilled water and quantified with a NanoDrop 2000c UV-Vis Spectrophotometer (ThermoFisher Scientific). In a first set of parallel negative
control ChIP experiments; RIO1-AID cells were submitted to the ChIP protocol in which only protein A beads were used (no anti-AID antibody). In a second set, the untagged RIO1 wild-type yeast was subjected to the ChIP protocol in the presence or absence of anti-AID antibody (protein A beads used). The amount of rDNA chromatin that co-immunoprecipitated with Rio1-AID was quantitated by TaqMan RT-qPCR using five probes whose binding sites are indicated in Supplementary Figure S6. The primer sequences of the probes are described in (16).

To localize endogenous Rio1 to centromeres, the 6Myc-RIO1 strain and its untagged RIO1 parent (acting as the negative control) were grown at 24°C and enriched in late G1 with 5 μg/ml α-factor. Cross-linking was done as described above and 6Myc-Rio1 immunoprecipitated with an anti-Myc monoclonal antibody (Covance, Cat # 9E11). The co-immunoprecipitated chromatin was then isolated and amplified with the GenomePlex Complete Whole Genome Amplification Kit (Sigma-Aldrich, Cat # WGA2-10RXN), labeled with biotin-N11-ddATP (1 nM/μL) (NEB) using terminal transferase (Roche, Cat # 0328969103) and hybridized to Affymetrix S. cerevisiae Genome Tiling Array 1.0R. The data were analyzed with GeneChip Command Console software (Affymetrix).

ChIP-Sequencing (ChIP-Seq) and data analysis

One to five nanograms of input and chromatin immunoprecipitated DNA were blunt-ended with T4 DNA polymerase (New England Biolabs, Cat # M0203L) and phosphorylated with T4 polynucleotide kinase (New England Biolabs, Cat # M0201L). A single adenine was then added to the 3'-ends using DNA polymerase I Klenow Fragment (3′→5′ exo-) (New England Biolabs, Cat # M0212L) allowing for ligation to an adapter containing a single tyrosine overhang. The ligation products were purified by Agencourt AMPure XP beads (Beckman Coulter, Cat # A63880) and PCR-amplified with PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, Cat # 600674) to enrich fragments with adapters on both ends (Biomek FX, Beckman Coulter). cDNA library concentrations were measured with a Qubit dsDNA HS Assay kit (ThermoFisher Scientific, Cat # 32851) and its size distribution and quality evaluated on a Bioanalyzer 2100 (Agilent Technologies) before cluster generation (FlowCell, Illumina). The libraries were deep sequenced for 50 bases in single-read mode on a HiSeq 2000 sequencing system (Illumina).

Reads were initially filtered (for identification of genomic clusters) or not (for global genomic and mitochondrial clusters) for sacCer3 reference abundant sequences obtained from the Illumina iGenomes support web page (using bowtie version 1.1.1 with default parameters). Next, artefacts were eliminated with Cutadapt v.1.8.3 (50) and reads aligned on the reference genome sacCer3 (46) with Bowtie2 v.2.2.1 software (51) using pre-set end-to-end parameters, while discarding reads with more than one mapping. Duplicate reads were removed with SAMtools rmdup (v.0.1.19) obtaining uniquely mapped reads. After random down-sampling of ChIP-Seq1 aligned reads to the number of ChIP-Seq2 and Input samples using SAMtools, the enriched ChIP-Seq signals were identified using MACS2 v.2.0.10 (52) with the option shift size set to 73 and disabling model and dynamic lambda. The q-value threshold was set to 0.005. Data visualizations and gene annotations were carried out using the R package ChIPseeker, the web applications ChIPseek (53) and CEAS (54). The significance of the ChIP-Seq signal cluster distribution across genomic areas and the significance of overlap between clusters of different biological replicates were calculated using the Genomic Association Test (55) python script gat-run, using mappability_36bp.bed as work space file and num-samples = 10000 parameter. The ChIP-Seq maps are available at https://genome.ifom.eu/cgi-bin/hgTracks?db=sacCer3&position=chrXII%3A1-1078177&hgsid=569991_PXM70Mv3Pnyg0VIUClaQxpdP7CuY.

Bioinformatic protein-interaction and gene-expression analyses

Interactions were obtained using the web-based version of STRING DB (v.10) (http://string-db.org) (56), setting the ‘Active Prediction Methods’ to ‘experiments’ and ‘databases’ only. Physical or genetic interactions were weighted based on the Y2H e-values, the number of times a co-purifying protein was identified and the strength of a genetic growth effect.

The confidence (score) was left at the default setting of medium confidence (0.4) as the extracted combined score was used to weight edge thicknesses. The resulting network was imported into Cytoscape (v. 3.2.1.) (57). Clusters were formed as follows: (i) the MCODE algorithm (58) was used, via the ClusterViz plugin, to determine clusters based on network connectivity (degree threshold:2, k-core threshold:2, Maxdepth:100). (ii) A Gene Ontology (GO) enrichment analysis was then performed in DAVID (59) to establish the dominant functional nature of each network cluster. (iii) A pre-performed global GO analysis (biological processes, cellular components), based on the entire list of 818 significantly regulated genes/transcripts, was then interrogated to extract additional genes related to the function of the cluster. (iv) Clusters were then spatially re-organized using the Allegro spring-electric layout from Allegrolayout (Allegroviva, USA) followed by manual refinement. Nodes were colored by their Log2 Fold Change value, with values of −4.5 set to red, 0 set to white and +4.5 set to blue. For ease of visualization, the node sizes were also set in proportion to the magnitude of their Log2 Fold Change values. Edge thicknesses were weighted corresponding to their STRING combined scores. Genes derived from GO terms related to the general description of each cluster were combined to assess enrichment, and related significance of displayed clusters in the networks, with respect to a theoretical human/yeast genome composition.

Creation of Rio1’s functional network

data, curated by BIOGRID (60–61), IntAct (62), MINT (63) and TRANSFAC (64). Data integration and significance evaluation were performed with the ResponseNet algorithm, biased toward signaling pathways, as described (65–67). Network layouts were generated with Cytoscape 3.0.1.

Statistical data analysis

Fisher’s exact test (hypergeometric data distribution analysis), used to determine non-random associations between two categorical variables, was computed using the Fisher_exact method from SciPy.stats, version 0.19.0. Data averages, standard deviation and standard errors, were calculated with the Excel spreadsheet (Microsoft Office). Statistical significance of independence between datasets ($P < 0.05, P < 0.01$ and $P < 0.001$, graphically represented by *, ** and ***, respectively) was calculated using the unpaired, two-tailed student $t$-test (Prism 6.01; GraphPad).

RESULTS

The physical and functional interactome map of Rio1

To determine whether Rio1 is involved in activities beyond regulating rDNA transcription, pre-rRNA processing and 40S small ribosomal subunit maturation, we searched for Rio1 interactors/substrates via yeast two-hybrid (Y2H) interaction screens. To date, only two direct substrates of Rio1 in yeast are known: Rio1 itself (16,68,69) and Rpa43; a conserved subunit of RNA polymerase I (16). To identify protein interactors, we tagged Rio1 N- or C-terminally with either the LexA- or the Gal4 DNA-binding domain. These four bait constructs were then screened three times against all $S. cerevisiae$ genes, fused N- or C-terminally to the Gal4 activation domain. Only interactors identified in all three independent screens were retained and provided with a statistical score ($e$-value, explained in the ‘Materials and Methods’ section), which correlates with the biological significance of the interaction (35–38). The final list of high-confidence hits ($e$-values from 1.0E-02 to 1.0E-24) comprised 97 interactors, including Rio1 itself (15 hits, 7 unique prey fragments, $e$-values = 1.0E-02) (Supplementary Table S1). We previously confirmed that the rDNA helicase Sgs1 identified in the above Y2H screens (3 hits, 1 unique prey fragment, $e$-values = 1.0E-02), co-immunoprecipitates (co-IPs) with and recruits Rio1 to the rDNA (16). To further validate our Y2H dataset we probed an additional three interactors: Rvb2 (28 hits, 2 unique fragments, $e$-values = 1.0E-02), Sky1 (26 hits, 5 unique fragments, $e$-values from 1.0E-16 to 1.0E-24), Sam37 (11 hits, 5 unique fragments, $e$-values = 1.0E-02) by co-IP analysis. These proteins were selected firstly because they localize to different organelles and contribute to dissimilar activities: Rvb2 is a nuclear ATPase with helicase activity; Sky1 is a nuclear and cytoplasmic protein kinase, while Sam37 is mitochondrial membrane component (46). Second, because some of these proteins perform multiple roles: Rvb2 contributes to the transcriptional regulation of ribosome biogenesis and ribonucleoprotein complex genes, to the assembly of box C/D snoRNP complexes (70–72) but also promotes RNA polymerase II assembly, DNA damage repair, telomerase complex assembly, mitotic spindle formation, the heat response and phosphatidylinositol three kinase related signaling (71–74), Sky1 regulates mRNA export, cation homeostasis, sulfur metabolism, mitophagy and apoptosis (74–77), while Sam37 is required for mitochondrial protein import (78,79). Third, because the $e$-values of these three interactors cover the entire range of our hit list (1.0E-02 to 1.0E-24; Supplementary Table S1). As shown in Supplementary Figure S1, all three proteins co-IPed with Rio1. Together with the Sgs1-Rio1 co-IP (16), they further validate our Y2H dataset.

Next, our list of 97 proteins was enlarged with 61 Rio1 interactors, reported in the literature (Supplementary Table S2). These included nine proteins identified in Y2H screens (80), Yeast Resource Center Informatics Platform (52) and 52 proteins derived from 16 proteomic and biochemical studies (8,9,16,68,81–92). Of the latter, Zuo1; the ribosome-associated chaperone for nascent polypeptide chains, was also identified in our Y2H screens. Next, our 156 unique Rio1 interactors were weighted (see Methods) and functionally clustered (GO term: Biological Process), yielding Rio1’s protein–protein interaction map (Figure 1A, enrichment $P$-values are listed in the figure legend). The map exposed Rio1 as a node that integrates eight key activities: ribosome biogenesis and function; cell-cycle regulation; chromosome segregation; physiology; the stress response; transcriptional, chromatin organization and modification; DNA replication, damage and repair; and metabolism. Using Fisher’s exact test (computes the probability that a sub-population in one sample is over- or under-represented in a second sample beyond what is expected by chance) we compared our 156 interactors against those of 179 other yeast kinases (60). We found that Rio1 integrates sizable subgroups (sharing >10 proteins) of 16 kinase networks (Figure 1B, $P$-values are listed in the figure legend), each of which contributes to a certain cell activity (color-coded as in Figure 1A) (the kinases and their targets are listed in Supplementary Table S3).

Since our list of Rio1 interactors comprised interphase and mitotic proteins, we performed a small-scale genetic study to corroborate its involvement in the cell cycle. By mere luck, while epitope tagging Rio1 (6Myc-Rio1 (16), Rio1-3HA and Rio1-GFP (Supplementary Figure S2B and C)) we found that only the C-terminal 3HA tag negatively affected Rio1 activity, resulting in $RIO1$-3HA behaving like a ‘reduced-function’ allele. As compared to the $RIO1$ control strain, the curtailed activity of Rio1-3HA was evidenced by slow growth (smaller-sized colonies, Supplementary Figure S2B) and by elevated levels of $35S$ pre-rRNA transcripts (Supplementary Figure S2A), likely caused by de-repressed transcription and/or reduced processing. In addition, we made a Rio1-overproduction strain by placing endogenous $RIO1$ under control of the $P_{GAL1}$ promoter (induced by galactose, repressed by glucose). As expected, $35S$ rDNA transcription and/or processing of the $35S$ pre-rRNA transcripts were reduced in galactose-containing medium and elevated in the presence of glucose (Supplementary Figure S2A). Next, $RIO1$-3HA and $P_{GAL1}$-$RIO1$ were combined with 20 mutant alleles of proteins mediating key cell-cycle activities (DNA replication, DNA repair, cohesion, condensation, segregation, spindle assembly
Figure 1. The physical and genetic interaction map of Rio1. (A) The Rio1 interactome comprises 184 proteins (156 physical + 28 genetic interactors). Ninety-seven physical interactors were identified in our Y2H screens, nine were retrieved from published (80) and deposited (Yeast Resource Center Informatics Platform, www.yeastrc.org) Y2H screens (black-edged circles). Fifty-two physical interactors were retrieved from published affinity purifications and biochemical studies ((8,9,16,68,81–92), white-edged circles). Rio1 and Zuo1 were identified twice (in our Y2H screens and biochemically (88,91)). Twenty genetic interactors were identified in this study (black-edged diamonds), eight were retrieved from the literature ((12,93–95), white-edged diamonds) (Supplementary Figure S2B and Tables S1, 2 and 4). Each interactor was functionally clustered based on the Gene Ontology term ‘Biological Process’ associated with it and then color-coded. Proteins belonging to multiple clusters are colored representing each cluster. The P-values for functional enrichment were: ribosome biogenesis and activity: 2.1E-10, cell cycle: 1.6E-08; chromosome segregation: 3.7E-08, stress response: 1.8E-10, chromatoin organization and modification: 1.2E-01, transcription: 2.4E-03, DNA replication, damage and repair: 5.7E-07. (B) Overlap between the Rio1-protein interaction network (includes only its physical interactors) and those of 179 other kinases (60). The 16 top-scoring kinases sharing >10 interacting proteins with Rio1 are shown. Proteins are connected to their master kinase, placed in the outer ring. Shared proteins were functionally clustered and color-coded as in (A). Proteins shared by multiple kinases are shown with a white core. The P-values of the overlaps between the Rio1-protein interaction list and those of the other kinases were calculated with Fisher’s exact test. They are: Yck2: 4.9E-03, Fus3: 3.5E-04, Cka2: 2.4E-07, Ksp1: 2.9E-03, Snf1: 9.8E-08, Pho85: 6.1E-03, Hrr25: 1.5E-04, Tpk3: 5.3E-04, Tpk1: 3.2E-04, Atg1: 5.5E-04, Yck1: 1.6E-03, Cdc28: 2.5E-02, Dbf2: 4.6E-04, Hsl1: 3.49E-05, Vhs1: 7.3E-06, Rio2: 2.8E-10 (Supplementary Table S3).
checkpoint activity and exit from mitosis). The growth defects of the mutants were either rescued (positive genetic interaction) or exacerbated (negative interaction), indicating that Rio1 antagonizes or supports, respectively, the activity of the investigated protein (Supplementary Figure S2B and Table S4). These data substantiate a functional (and likely also physical) involvement of Rio1 in the activity of the above cell-cycle proteins and processes. Next, four published synthetic genetic screens performed with a heterozygous rio1Δ strain extended our map with another eight interaction partners (involved in ribosome biogenesis and translation, metabolism, transcription and cell-cycle regulation) (12,93–95) (Supplementary Table S2). These 28 genetic interactors were added to Rio1’s protein-interaction diagram, creating its physical and functional interactome net (Figure 1A).

During our study, Costanzo and colleagues performed a global pair-wise genetic interaction screen to produce the wired diagram of yeast cell function (96). The sub-screen with a temperature-sensitive rio1 allele identified 142 negative and 125 positive interactors (P-values < 0.05, Supplementary Figure S2D). The 267 gene products act across the cell in the processes we had already associated with Rio1 function (Figure 1); including RNA processing (23% of the annotated interactors), cell-cycle activity (21%), ribosome biogenesis (20%), protein folding and turnover (14%), and transcription (13%).

**Rio1 localizes to kinetochores**

Notwithstanding our positive co-IPs and the full overlap between our physical–genetic interaction map of Rio1, and Rio1’s genetic diagram (96), we validated further the involvement of Rio1 in processes and events identified above. Both Costanzo et al. and us recognized that Rio1 interacts with the kinetochore, the protein complex that assembles on centromeres to orchestrate chromosome segregation (kinetochore subunit Mcm21 as a Y2H partner, 12 alleles encoding kinetochore components that strongly genetically interacted with Rio1). To examine whether Rio1 actually localizes to kinetochores, we immunoprecipitated 6Myc-Rio1 from crosslinked G1 cells (enriched at START with the α-factor pheromone) using a monoclonal anti-Myc antibody (the untagged RIO1 strain acted as the negative control) and submitted the chromatin co-immunoprecipitating with 6Myc-Rio1 to genome hybridization analysis (ChIP-chip). We localized 6Myc-Rio1 at kinetochores (representative signals at CEN5 and CEN10 are shown in Figure 2A) while the protein was not identified at kinetochores in the untagged strain. To further corroborate its presence at kinetochores, we localized 6Myc-Rio1 by IF microscopy of spread nuclei isolated from cells enriched in G1 (cell cycle stage in which all kinetochores are clustered near the spindle pole). Next to localizing 6Myc-Rio1 to the nucleolus (rDNA, positive control; Nop1 as the nucleolar marker) we also identified the protein at kinetochores (kinetochore subunit Ndc80 as the marker) (Figure 2B). Combined, its presence at kinetochores underscores a contribution of Rio1 to kinetochore activity and consequently to chromosome transmission. This observation helps to explain why a reduced-activity rio1 mutant suffers from elevated chromosome loss and metaphase delay (88).

**Rio1 promotes rDNA replication**

Heterozygous rio1Δ mutants are sensitive to drugs obstructing DNA replication (20). This phenotype indicates an involvement of Rio1 in the process, a hypothesis that is supported by both its physical and genetic interactions with proteins mediating DNA replication (Figure 1A; Supplementary Figure S2B and Table S1, (96)). To verify its contribution, we wished to probe replication fork activity at the rDNA (at which Rio1 accumulates (16)), in wild-type yeast and in yeast lacking Rio1. However, since Rio1 activity is essential for viability, we had to conditionally eliminate the protein and chose the auxin-inducible degron (AID) system for that (34). After tagging Rio1 at its C-terminus with the AID degron cassette (hence producing the RIO1-AID, PADH1-OctTIRI-9Myc strain; hereafter named RIO1-AID), we probed Rio1-AID protein activity and confirmed it was identical to that exhibited by Rio1 in the untagged parent strain. Indeed, growth assays did not reveal any negative effect on fitness or growth rate (colony size, Supplementary Figure S3A) and 3S5 pre-rRNA transcript levels were also identical to those measured in the RIO1 stain (Supplementary Figure S3B). Next, we assayed the Rio1-AID degradation kinetics. Within 30 min of adding 500 μM of auxin to the RIO1-AID culture, Rio1-AID became undetectable by western blot hybridization analysis (Supplementary Figure S3C). Its depletion was supported by an increase in 3S5 pre-rRNA transcript levels (Supplementary Figure S3B). Because of its key roles in 3S5 rDNA transcription, pre-rRNA processing and 40S small ribosomal particle maturation, we examined whether depleting Rio1-AID for 1 h affected ribosome biogenesis and translation activity. Specifically, the wild-type and RIO1-AID strains were treated for 1 h with 500 μM of auxin. Whole-cell extracts were then made and run through a sucrose gradient to profile the 40S and 60S ribosomal subunits, the mature 80S monosomes and the translationally active polyosomes. A minor reduction in 40S and a slight increase in 60S ribosomal subunits was observed (Supplementary Figure S3D).

When we treated the cells with 4TU to label the newly synthesized (r)RNAs, 40 min into auxin treatment, we detected an increase in 20S pre-rRNA levels, reflecting reduced Rio1 activity in the cell (Supplementary Figure S3E). However, despite the mild 40S biogenesis defect, the ratio between the concentration of 80S monosomes and polyosomes was similar in the wild-type and Rio1-AID-depleted strains (5.2 versus 5.6), respectively, suggesting that cellular translation activity was not significantly affected. Indeed, a short methyl-3H-methionine pulse revealed no differences in the levels of radiolabeled, neo-synthesized proteins that were produced in both strains (Supplementary Figure S3F). This finding implies that any phenotype observed in yeast depleted of Rio1 for only 1 h is not caused by pleiotropic effects due to reduced intracellular protein synthesis but rather by the absence of Rio1 activity in the process being studied.

To finally assay DNA replication at the rDNA and control ARS305 sequence in the presence and absence of Rio1 activity, we synchronized the RIO1-AID cells in late G1
Figure 2. Rio1 localizes to kinetochores and promotes replication fork velocity/stability at the rDNA. (A) To probe the localization of Rio1 to kinetochores, we submitted a strain endogenously expressing 6Myc-RIO1 and its untagged parent to anti-Myc based chromatin immunoprecipitation, followed by microarray hybridization (ChIP-chip). The hybridization profiles (orange) at CEN5 and CEN10 are shown. (B) To corroborate the localization of Rio1 to kinetochores by microscopy, we isolated the nuclei from G1 cells (all 16 kinetochores are concentrated near the spindle pole) and then spread, washed and submitted them to indirect IF analysis. We identified Rio1 at both the rDNA (Nop1 as the rDNA/nucleolar marker) and at kinetochores (Ndc80 as the marker). DAPI strongly dyes the chromosomes but not the nucleolus as the latter is highly enriched with proteins, preventing DAPI from staining the rDNA. Scale bar = 5 μm. (C) To evaluate the contribution of Rio1 activity to chromosome replication we analyzed replication intermediates at the rDNA array and at negative control ARS305 in HU-treated RIO1-AID, P_400H_400-OsTIR1-9Myc cells (next named RIO1-AID) that contained or were depleted of Rio1 activity. The RIO1-AID strain was grown exponentially (expon), then synchronized in G1 with α-factor (αF) and finally released into the cell cycle using 200 mM HU-containing YPD medium supplemented with 500 μM of auxin or mock solution. Samples were collected at the indicated time points. Rio1-AID depletion was evidenced by anti-AID western blot hybridization (3-phosphoglycerate kinase Pgk1 acted as the loading control) (upper left blots). Cell-cycle progression was tracked by FACS-based analysis of DNA content (upper right plots). After releasing the cells from G1, genomic DNA was extracted from culture samples taken at the indicated time point (60, 120, 180 and 240 min) and treated with the restriction endonucleases EcoRV and HindIII to analyze ARS305, or with BglII to analyze 5S rDNA. Following 2D agarose gel electrophoresis, replication intermediates accumulating at both sites were visualized by Southern blot hybridization. The topologies of replication intermediates potentially accumulating in the presence of HU are drawn. The bottom left radiographs show the Southern hybridization images depicting replication fork intermediates at ARS305 and 5S rDNA in RIO1-AID cells exposed to HU and treated with 500 μM of auxin or mock solution. Results of a representative experiment are shown. The bottom right graphs show the relative values of the Y-arc signals quantitated against those of the monomer spots. The number of unidirectional Y-arc replication intermediates was consistently reduced at the rDNA locus in the Rio1-depleted cells. Error bars = standard deviation. The data were calculated from four (rDNA) or five (ARS305) independent experiments. Statistical (in)significance of independence between the mock- and auxin-treated datasets (P < 0.05 (*) and P < 0.01 (**), N.S. = insignificant data independence) was calculated using the unpaired, two-tailed student t-test.
with α-factor. The cells were then released into the cell cycle using YPD medium, supplemented with 500 μM of auxin or a mock solution and with 200 mM of HU to halt DNA replication. After confirming Rio1-AID depletion (western blot hybridization) and interphase arrest by the S-phase checkpoint (FACS analysis) (Figure 2C), we performed 2D agarose gel electrophoresis followed by Southern blot hybridization analysis using anti-rDNA and anti-ARS305 probes. Four (rDNA) or five (ARS305) independent experiments were performed for each condition (auxin and mock treatment). Examination of the replication fork intermediates revealed a significant reduction in unidirectional replication forks (Y arcs) at the rDNA, as compared to the ARS305 sequence, in the Rio1-depleted cells challenged with HU (Figure 2C). This observation underscores a supportive role for Rio1 in rDNA replication by regulating local replication fork velocity and stability. This contribution of Rio1 complements its interaction with the rDNA helicase Sgs1 and its repression of rDNA transcription during S-phase (16) in order to avoid clashes between the replicosome and RNA polymerase I, which trigger the genetic instability of this essential repeat region.

### Establishing Rio1’s transcriptome map

Our Rio1-protein interaction list contained histone H3 proteins Hht1 and Hht2, transcription factors, and proteins implicated in chromatin assembly, modification and remodeling. In addition, Costanzo et al. identified 29 rio1-interacting genes (14 negative, 15 positive) whose proteins regulate gene transcription. These findings and the fact that Rio1 regulates rDNA transcription by RNA polymerase I (16) convinced us to probe whether the enzyme controls gene expression beyond the rDNA as well. As such, six Rio1-AID cultures were grown exponentially in rich medium (YPD). Three of them were then treated for 1 h with 500 μM of auxin, the other three with a mock solution. A 1-h depletion was chosen because it allows for the removal of Rio1 without affecting global translation activity (see above). This window also permits for a solid response at the transcriptional level. Next, total RNA was isolated from the cells. The nuclear and mitochondrial rRNAs were removed (representing >95% of the yeast transcripts) to expose the less abundant RNAs, which were reverse transcribed and deep sequenced (RNA-Seq). After averaging and filtering the triple datasets (RPKM [reads per kilobase of transcript per million reads mapped] >1), transcript levels of 818 genes (nuclear + mitochondrial) emerged as being differentially changed in yeast depleted of Rio1 (P-value ≤ 0.05) (Supplementary Figures S4 and 5A–C; Supplementary Table S5). While its impact on gene expression/transcript levels ranged from 11-fold increase (RPL7B) to 18-fold reduction (YDR215C), Rio1 upregulated most (66%) of its target genes (Supplementary Figure S5C). We underline that the measured changes in transcript levels produced from the 818 identified ORFs can be the consequence not only of changes in gene expression but also of altered transcript decay and stability due to the lack of Rio1 activity.

Next, we assessed the functionality of the 818 genes via GO enrichment analysis (P-values are tabulated in Supplementary Table S6). We then color- and size-weighted the impact that Rio1 has on the transcript levels of each gene. The 548 genes/transcripts that are upregulated by Rio1 activity are colored red, while the 270 genes/transcripts that are downregulated by Rio1 activity are depicted in blue (Figure 3).

Our study revealed, first and foremost, that Rio1 upregulates the transcript levels of all RP genes (RP regulon) and of the RP transcriptional activator Rap1 (Supplementary Table S5). Since Rio1 is a Ribi member, we did not anticipate it to regulate the RP regulon, possibly by controlling Rap1 expression (Supplementary Figure S11B). Also, Rio1 modulates the transcriptome state of 20% of the Ribi regulon (18, 19) hence additionally regulating ribosome biogenesis (e.g. subunits of the rRNA-processing SSU processome, ribosome chaperones, transporters, etc.) and activity (translation initiation factors, aminoacyl-tRNA synthetases, etc.) through Ribi gene expression. We underscore that the significant changes observed in RP and Ribi transcript levels measured after 1 h of Rio1-AID depletion are fully compatible with the cells being translationally competent since the ribosomes that were synthesized before and hence were active during the 1h-depletion window, ensured bulk protein synthesis at normal capacity (Supplementary Figure S3F).

Second, Rio1 reduces the levels of transcripts derived from the genes encoding all 26S proteasome components (including its ATPases and the ubiquitin-specific protease Ubp6) and of the gene coding for proteasomal transcription factor Rpn4. As such, Rio1 can control protein degradation/inactivation in the cell. Noteworthy, proteasome expression opposes that of the RPs, indicating that when protein synthesis is required, Rio1 may downregulate proteasome expression (and vice versa). Also, Rio1 affects the transcript levels of an assortment of chaperones, emphasizing its protagonist role in managing protein levels, quality and activity.

Third; carbon and amino acid biosynthesis, purine, pyrimidine, phosphorous and lipid biosynthetic pathways, as well as ATP synthesis and/or hydrolysis are subject to regulation by Rio1.

Fourth, Rio1 regulates the mRNA levels of chromatin assembly and remodeling factors, cytoskeleton components and regulators, cell wall biogenesis enzymes, cell cycle determinants and proteins mediating exo- and endocytosis.

Our transcriptome data further show that Rio1 not only physically interacts with a large number of proteins but also regulates the expression of 29 of them (listed in Supplementary Table S7), including regulators such as the kinase Sky1, vacuolar proteinase Prb1, various protein chaperones, histone H3 proteins Hht1 and Hht2, translational initiation factor eIF4A -via which the 40S ribosomal subunit scans for the start codon-, RPs and metabolic enzymes. As such, Rio1 seems to orchestrate intracellular activities both at the protein and chromatin/RNA levels.

### Establishing Rio1’s chromatin interaction map

After disclosing its transcriptome, we examined to which extent Rio1 regulates its target genes by localizing to promoters and/or coding sequences, by acting in off-DNA fashion on transcription factors, RNA polymerases or chro-
Figure 3. The Rio1 transcriptome map. The 818 genes whose transcript levels were found to have differentially changed in yeast depleted of Rio1 (as compared to the RIO1-AID strain not depleted of Rio1), were functionally clustered based on the Gene Ontology term 'Biological Process' associated with each gene (enrichment P-values are listed in Supplementary Table S6). The differential state of each transcript is indicated by symbol size and color intensity (see gradient on the left). Genes whose transcript levels increased due to Rio1 activity are shown in red whereas those whose levels decreased due to Rio1 activity are indicated in blue. The gray lines in the background represent all known interactions between the 818 gene products, as cataloged in protein interaction databases.

matin remodelers/modifiers, or by affecting RNA turnover or stability (Figure 1A, Supplementary Tables S1 and 2). As such, three RIO1-AID cultures were exponentially grown in rich medium (YPD, same conditions as for the RNA-Seq experiments, allowing us to correlate both datasets). The cells were then subjected to ChIP analysis with a monoclonal anti-AID antibody, and the generated DNA libraries deep-sequenced (ChIP-Seq). In three parallel negative control experiments, RIO1-AID cultures were subjected to the ChIP protocol that did not employ the anti-AID antibody, while untagged RIO1 cultures were submitted to ChIP experiments that did and did not use the anti-AID antibody. RT-qPCR analysis of the rDNA sequence, to which Rio1 localizes, indicated the absence of background noise in our Rio1-AID ChIP experiments (Supplementary Figure S6). Examination of the anti-AID ChIP-sequencing data derived from the three RIO1-AID cultures revealed a preponderance of reads originating from the mitochondrial chromosome (present in 20–50 copies per haploid cell), obscur- ing the signals derived from the nuclear chromatin. After separating the mitochondrial chromatin reads, we distinguished 297, 397 and 481 Rio1-binding sites across the nuclear genome, respectively, of which 125 sites were consistently identified in all three experiments (Supplementary Figure S7A-B and Table S8). Along the nuclear DNA, Rio1 was enriched at promoter regions (10.5 ± 0.5%), coding sequences (69 ± 2%) and near transcription termination sites (10 ± 2.0%) (Supplementary Figure S7C). As for the Rio1-controlled genes identified by RNA-Seq: in at least 2 of the 3 ChIP experiments we found that 10 harbored a Rio1-binding signal in their promoters, 12 in their coding sequences, while 1 gene (SSA1) had Rio1 bound to its promoter and coding sequence (these 23 genes are indicated in bold italic font in Figure 4A, examples are shown in Figure 4B). In one of the three ChIP-Seq experiments, we captured Rio1 at the promoter or coding sequence of an additional 29 Rio1-controlled genes (italic font in Figure 4A), suggesting short-lived interactions between Rio1 and these genes. With the exception of these 52 transcriptome members and the rDNA (Figure 4C and Supplementary Fig-
Figure 4. The Rio1-chromatin binding map. (A) Fifty-two nuclear genes whose expressions are under the direct regulation of Rio1 and which harbor its footprint in their promoter regions (red segment), coding sequences (orange segment) or both (purple segment). The genes indicated in bold italic font contained a high-scoring Rio1 signal in at least two independent ChIP-Seq experiments. The genes indicated in italic font contained a high-scoring Rio1 signal in singular ChIP-Seq experiments. (B) Visual representations of Rio1 localizing to target genes at their promoter sequence (CDC19, FES1), coding sequence (RPS8B, TDH2) or both (SSA1). The LogLR (likelihood ratio) values for the local enrichment of Rio1 relative to the input are shown. The black marks underneath each Rio1 signal indicate the part of the footprint that is significant (q-value ≤ 0.005). (C) Enrichment of Rio1 at specific nuclear regions and loci. The data represent the average values measured in three independent ChIP-Seq experiments. Error bars = standard errors. (D) Visual representations of Rio1 localizing to transposons (YLRWTy2-1, YNLWTy1-2), LTR-retrotransposons (YCLWTy2-1, YGRWTy1-1) and telomere regions (TEL10L, TEL16R). (E) Visual representation of Rio1 associating with the mitochondrial genome.

ure S7E) Rio1 transcriptionally regulates the majority of its ORFs (94%) either in an indirect, off-DNA fashion or directly at the chromatin through short-lived physical interactions. Both observations are consistent with activity models established for other protein kinases that regulate gene transcription, including the yeast and human mitogen-activated protein kinases (97). Rio1 footprints were also distinguished across the chromatin landscape (Supplementary Table S8). Of the 297, 397 and 481 Rio1-binding sites that were identified across the nuclear genome in our repeat experiments, we found that 82 ± 2% of them did not affect transcript levels of the nearby ORF when Rio1 was depleted. A lack of association at the transcriptional level may indicate roles of Rio1 in basic chromatin activities (98–100). Indeed, Rio1 co-purified with histone H3 proteins Hht1 and Hht2 (85) and physically and genetically interacted with DNA helicases, repair enzymes, DNA replication regulators, cohesin, condensin and the chromosome-segregating centromere and kinetochore proteins (Figure 1; Supplementary Figure S2B and D). For example, Rio1 was identified at all 32 telomeres and telomere repeat sequences (Figure 4C and D) and at transposable gene elements (Figure 4C and D). Activities at these regions control genetic stability, chromosome length and cellular lifespan; as well as stress adaptation via transposition-mediated gene regulation, respectively. In contrast, the lack of Rio1 footprints at genes whose transcripts are significantly affected by the absence of Rio1 activity may indicate fast kinetics at the chromatin levels (Rio1 not captured) but also point to an involvement of Rio1 in RNA processing, turnover and stability.

Next, analysis of the mitochondrial ChIP-Seq reads identified Rio1 along the mitochondrial DNA (Figure 4E) consistent with regulating seven of its encoded messages (Figure 3; ChIP-Seq1-3 P-values = 5.0E-14, 1.0E-71 and 4.4E-48, respectively). Rio1-AID signals were prominent at the 15S rDNA and 21S rDNA promoter and transcribed regions. Our RNA-Seq experiments had revealed that Rio1 promotes 21S rDNA expression. The 21S pre-rRNA self-splices to produce the mature 21S rRNA, which together with the 15S rRNA becomes part of the mitochondrial ri-
The mitochondrial ChiP-Seq data extended the tally of genes that are under the direct control of Rio1 to at least 59 (52 nuclear + 7 mitochondrial). We emphasize that Rio1 regulates the transcription of both the nuclear and mitochondrial rDNA genes.

**Rio1 localizes to the nucleus, cytosol, mitochondria and vacuoles**

Our Rio1-protein, gene-transcription and genome-binding maps, as well its global genetic interaction diagram (96) indicate that Rio1 acts and localizes throughout the cell. Indirect IF widefield imaging of isolated nuclei previously distinguished Rio1 at the nucleus and the nucleolus (16). IF imaging of whole yeast cells endogenously expressing Rio1-GFP (the GFP tag did not affect cell fitness, Supplementary Figure S2C) identified the protein at the nucleus (DAPI weakly stains the nucleolus due to its high protein content) and the cytoplasm (compatible with Rio1 contributing to 40S maturation). No anti-GFP IF signals were identified in the *Rio1* strain.

To localize Rio1 with ultrastructural resolution, we submitted the *RI01-GFP* strain to anti-GFP cryo-immunogold EM (Figure 5B; the untagged *Rio1* strain acted as the negative control, Supplementary Figure S8). The efficiency with which the GFP epitope was identified by the anti-GFP antibody and gold-labeled protein A, was determined by the method of Griffiths (41) as 10% (standard of excellence), meaning that one gold signal represents 10 Rio1-GFP molecules. We distinguished Rio1-GFP in the nucleus (20-fold enrichment over the negative control), the cytosol (3-fold enrichment), mitochondria (lumen + membrane, 53-fold enrichment) and the vacuole (lumen + membrane, 75-fold enrichment). While it is difficult to compare IF imaging (Rio1 signals throughout the cytosol) and EM, we believe that the less-than-expected 3-fold enrichment of Rio1-GFP signals in the cytoplasm as observed by EM is due to poor gold (10 nm)-immune-complex formation of Rio1-GFP bound to the pre-ribosomes (stERIC hindrance).

The identification and enrichment of Rio1-GFP at mitochondria is consistent with its localization to the mitochondrial genome, as observed in our ChiP-Seq experiments (Figure 4E). Furthermore, as determined with MitoFates software (102), Rio1 contains a putative N-terminal mitochondrial pre-sequence MPP processing site (residues 41–50, predicted cleavage at residue 42) and a consensus Tom20 recognition motif (residues 90–95), which is compulsory for mitochondrial import. Rio1 physically interacts with mitochondrial membrane protein Sam37 (Figure 1A and Supplementary Figure S1) and with 37 other mitochondrial proteins (Supplementary Tables S1 and 2). Besides regulating the expression of seven mitochondrial ORFs, Rio1 is poised to act in mitochondrial (oxidative) signaling as it further determines the transcription of 123 nuclear genes whose proteins catalyze mitochondrial activities including metabolic, energy generating, calcium buffering, stress signaling and apoptotic mitochondrial processes (103, 104) (Supplementary Table S5).

The localization of Rio1 to the vacuole; the storage and recycling organelle that mediates the cellular adaptation to nutrient deprivation, osmotic shock and ROS, is consistent with Rio1 physically interacting with nine proteins involved in vacuolar fusion, vacuolar protease and phosphatase activity, vacuolar import and export, and the cytoplasm-to-vacuole targeting pathway (Figure 1A; Supplementary Tables S1 and 2). A functional connection between Rio1 and vacuole biology is reinforced by Rio1 regulating the expression of 32 genes involved in vacuolar processes (Supplementary Table S5), including two genes whose protein products interact with Rio1 at vacuoles (vacuolar proteinase PrBl1, v-SNARE vacuolar membrane protein Ykt6). In summary, our intracellular imaging data infer that Rio1 not only acts in the nucleus, nucleolus and the cytosol (as reported before), but also at mitochondria and the vacuole. The presence and activity of Rio1 at these organelles is supported by its global genetic diagram (Supplementary Figure S2D (96)) and our interactome, transcriptome and chromatin-binding maps.

**Establishing the activity network of Rio1**

While our findings expanded the functional spectrum of Rio1, the question became: how are Rio1, its gene/transcript and protein targets—localizing throughout the cell—functionally and physically connected? Indeed, Rio1 regulates its downstream processes largely by employing partner regulators (enzymes, transcription factors, etc.). Answering this question required the identification of intermediary ‘nodes’, including transcription factors known to directly act at the ORFs here identified based on the changes measured in their transcript levels, as Rio1 gene targets. To accomplish this task, we collected all up-to-date, experimentally validated yeast protein–protein, yeast protein–transcription factor, yeast protein–gene and transcription factor–gene interactions, as curated by BIOGRID (60, 61), IntAct (62), MINT (63) and TRANSFAC (64). Each of the 119 600 protein–protein and 14 010 protein/transcription factor–DNA contacts (represented by 6237 proteins; including 217 transcription factors) was then weighted via a Bayesian scheme to add statistical relevance to each interaction. Next, the ResponseNet algorithm (65–67) (freely accessible at http://netbio.bgu.ac.il/respnet/) implemented a maximum-probability, minimum-cost flow optimization approach to combine our own interaction and gene-expression data into the above catalogs to produce a top-to-bottom (Rio1-to-genes) signaling network (Supplementary Figure S9). Using this network, one can step backward from a chosen gene target to identify the upstream protein contacts (including functional nodes identified by the algorithm from the interaction catalogs) that link Rio1 to the gene. ResponseNet correctly identified protein kinase Ck2 (subunits Cka1 and Cka2) as a Rio1 activator, as reported (68) (Figure 6). The algorithm also identified seven transcription factors, with which Rio1 physically interacts (six identified in our Y2H screens, one was co-purified with Rio1 (83); indicated by yellow triangles in Figure 6). These transcription factors functionally associate Rio1 with 47 target genes (blue squares). Rio1-target genes such as GCN4, RPN4 and RAP1 encode key node transcriptional regulators that modulate the expression of metabolic proteins, the 26S proteasome subunits, and the structural
Figure 5. Rio1 localizes to the nucleus, cytosol, vacuoles and mitochondria. (A) Indirect wide-field IF imaging of endogenous Rio1-GFP localization in exponentially growing *Saccharomyces cerevisiae*. The untagged parent (*RIO1*) was analyzed as the negative control. Imaging was performed with Living Colours Full-Length anti-GFP polyclonal rabbit antibody and FITC-conjugated anti-rabbit secondary antibody. Rio1-GFP signals are shown in green. DAPI stains the nuclear DNA (red) and not the nucleolar rDNA since the high levels of nucleolar proteins prevent DAPI from intercalating into the rDNA array. The right images show zoom-ins of the inset G1 cell. (B) Cryo-immunogold EM images of exponentially grown yeast cells endogenously expressing Rio1-GFP. Single slices of gelatin-embedded yeast (50–60 nm thickness) were incubated with rabbit anti-GFP antibody and gold (10 nm)-labeled protein A, and then imaged. Rio1 was identified at the nucleus (N, black arrows), at the vacuolar lumen and membrane (V, blue arrows), at mitochondria (M, red arrows) and in the cytosol (C, green arrows). The labeling efficiency (LE) \( \approx 10\% \) (standard of excellence, quantitated using the method of Griffiths (41), means that one gold signal corresponds to 10 Rio1-GFP molecules. A negative control image of the untagged *RIO1* strain and statistical analysis of Rio1 localization in the *RIO1* and *RIO1-GFP* strains are shown in Supplementary Figure S8A and B, respectively.
Figure 6. The Rio1-Ck2 sub-network in *Saccharomyces cerevisiae*. The Rio1-Ck2 sub-network, which is part of Rio1’s global network (depicted in Supplementary Figure S9), comprises 106 differentially expressed genes (blue squares), 14 transcription factors; 7 of which physically interact with Rio1 (yellow triangles), while 7 were identified by the algorithm as intermediate nodes (white triangles). The two subunits of the protein kinase Ck2 (Cka1, Cka2), which physically interacts with Rio1 (68), are indicated by yellow circles.

ribsosomal subunits plus metabolic proteins, respectively (Supplementary Figures S10 and 11). By integrating a large collection of similar sub-networks, Rio1 can conduct its activities on a global level.

Our network analysis also highlights its multi-layered regulation by Rio1. First, Rio1 acts at the protein, gene and RNA levels (many of its physical interactors and gene targets are involved in RNA synthesis, pre-RNA processing, and regulation of mRNA decay). Second, 29 Rio1 gene targets encode proteins that in turn physically interact with Rio1 (listed in Supplementary Table S7). Third, eight transcription factors whose expression states are controlled by Rio1 (Gcn4, Mig2, Mot2, Rap1, Rlm1, Rox1, Rpn4 and Tup1) in turn modulate the expression of numerous Rio1-controlled genes, indicating that the Rio1 network is feedback regulated. Using our network one can experimentally assess how and/or via which partners Rio1 modulates a certain protein/gene/RNA or cellular activity.

**Rio1 and its network respond to nutritional availability**

Having mapped how Rio1, its interactors and gene targets are connected within its signaling network, we wondered which circumstance(s) would provoke Rio1 to act upon it. While Rio1 is a member of the Ribi regulon, which strongly responds to nutrient availability, we wondered whether its network is linked also to other cues. Hence we used Fisher’s exact test to compare Rio1’s transcriptome dataset with those of other regulatory networks in yeast. We found that the Rio1 gene regulon significantly overlapped with those of the heat (P-value = 1.4E-07), osmotic (P-value = 3.7E-56), nutritional (P-value = 7.2E-22) and oxidative (P-value = 7.3E-04) stress-response systems (107–111) (the transcriptomes are size-weighted in Figure 7A, individual genes are listed in Supplementary Table S10). This observation suggested that Rio1 and its network are physically and functionally integrated with these response networks. In addition, Rio1 might relay multiple cues into its system, allowing yeast to respond to a number of growth conditions. Given that the Rio1 regulon is highly enriched in genes coding for ribosome biogenesis and activity, we probed whether the overlap between the Rio1 and stress response regulons is simply due to their shared regulation of ribosome biology. When we removed ribosome-linked genes from all regulons and reanalyzed their overlap with the Rio1 regulon devoid of genes mediating ribosome formation and activity,
Rio1 responds to nutritional deprivation and crosstalks with Gcn4 at the transcriptional level. (A) The Rio1 transcriptome is enriched in transcripts of genes that are part of various stress-response systems. The main pie shows the percentage of the 818 Rio1-modulated genes that belong to specific stress transcriptomes (represented by the peripheral pies, which are size-weighted for the number of genes they contain) listed in Supplementary Table S10. The slices indicate the % of overlap at the gene level between the Rio1 and stress-response transcriptomes. P-values quantitating the significance of these overlaps (determined with Fischer’s exact test) are listed in the text. (B) Expression analysis of 6 Rio1-regulated reporter genes in yeast growing in complete synthetum medium (+ amino acids [AA] +2% glucose) or in synthetic medium lacking AA or glucose. Blue: gene whose transcript levels decrease due to Rio1 activity. Red: gene whose transcript levels increase due to Rio1 activity. White bars: Rio1-containing cells, black bars: Rio1-depleted cells. Transcript concentrations were measured by TaqMan-based RT-qPCR analysis and normalized to those of ACT1 (encoding actin, ACT1 mRNA levels are plotted in the upper right graph). Numbers were then correlated to those calculated for the mock-treated cells grown in complete synthetic medium. Error bars = standard errors. The RT-qPCR data are listed in Supplementary Table S11. The levels of confidence (values) are indicated by stars and were calculated with the unpaired, two-tailed student t-test. N.S. = not significant.

(C) Experimental outline and western hybridization blot evidencing the level of Rio1-AID, Gcn4-13Myc and OsTir1-9Myc (anti-Myc) in the strain that was grown exponentially in complete 2% glucose synthetic medium, and then shifted for 1 h to complete 2% glucose synthetic medium (+amino acids [AA] +2% glucose) or in synthetic medium lacking AA or glucose. Blue: gene whose transcript levels decrease due to Rio1 activity. Red: gene whose transcript levels increase due to Rio1 activity. White bars: Rio1-containing cells, black bars: Rio1-depleted cells. Transcript concentrations were measured by TaqMan-based RT-qPCR analysis and normalized to those of ACT1 (encoding actin, ACT1 mRNA levels are plotted in the upper right graph). Numbers were then correlated to those calculated for the mock-treated cells grown in complete synthetic medium. Error bars = standard errors. The RT-qPCR data are listed in Supplementary Table S11. The levels of confidence (values) are indicated by stars and were calculated with the unpaired, two-tailed student t-test. N.S. = not significant.

(D) Rio1 and Gcn4 mRNA levels measured by TaqMan-based RT-qPCR analysis in Rio1-AID, PADH1-OsTIR1-9Myc, gcn4-13Myc, and osTir1-9Myc strain that was grown exponentially in complete 2% glucose synthetic medium, and then shifted for 1 h to complete 2% glucose synthetic medium containing or lacking AA, that was further provided with 500 μM of auxin or mock solution. 3-Phosphoglycerate kinase Pgk1 acted as the loading control (as did the exogenous auxin receptor OsTir1-9Myc). D) Rio1 and Gcn4 mRNA levels measured by TaqMan-based RT-qPCR analysis in Rio1-AID, PADH1-OsTIR1-9Myc (named Rio1-AID) and Rio1-AID, PADH1-OsTIR1-9Myc, gcn4-13Myc (named Rio1-AID, gcn4-Δ) cells grown in complete, 2% glucose synthetic medium, followed by a 1 h shift to complete, 2% glucose medium (blue bar, black underline), 2% glucose medium lacking AA (orange bar, black underline), complete 2% glucose medium provided with 500 μM of auxin (blue bar, red underline) or 2% glucose medium lacking AA but containing 500 μM of auxin (orange bar, red underline). Rio1 and Gcn4 transcript levels were normalized to those of ACT1 and then to their respective values measured in the cells grown in complete 2% glucose medium lacking auxin (value = 1). Error bars = standard errors. The levels of confidence (values) are indicated by stars and were calculated with the unpaired, two-tailed student t-test. (E) Graphic summary of the data shown in panels (C) and (D).
we found that the connection between the oxidative stress regulon and that of Rio1 became non-significant (Fisher’s exact test; P-value = 0.09). This finding indicated that the overlap between both regulons is due to their shared control of ribosome biology. The overlaps between Rio1 and the heat, osmotic, and nutritional regulons remained highly significant (P-values = 1.7E-11, 3.2E-23 and 2.9E-12, respectively), indicating a shared response to extracellular cues that covers but also extends way beyond regulating ribosome production and activity.

Since the Rio1 transcriptome shares 18% of its genes with nutritional stress regulons (Figure 7A), we decided to examine the response of Rio1 to sugar and amino acid availability. Specifically, we chose six Rio1 target genes as response readouts that represent various cell biological activities (Supplementary Table S5 and Figure S5D). They include the Rio1 downregulated LIN1 (encodes a U5 snRNP protein that is also involved in regulating cohesion), ECM29 (encodes a protein that assists in the association of the core and regulatory particles of the 26S proteasome) and PDR12 (encodes a plasma membrane ATP-binding cassette transporter), next to the Rio1 upregulated GLN1 (encodes L-glutamine synthetase 1, which catalyzes the formation of L-argininosuccinate from L-citrulline and L-aspartate). L-argininosuccinate synthetase 1, which catalyzes the formation of L-argininosuccinate from L-citrulline and L-aspartate). Three Rio1-AID yeast cultures were grown exponentially in complete 2% glucose synthetic medium. Each culture was then split in six. The cells were washed and transferred to synthetic medium lacking amino acids or glucose and were then split in four, the cells washed and transferred for 1h to 2% glucose synthetic medium containing or lacking amino acids, and provided with 500 µM of auxin or a mock solution (graphically summarized in Figure 7C). The experiments were performed in triplicate. While the shift from complete medium to mock-treated amino acid-deprived medium did not affect Rio1 protein levels (lane 1 versus lane 3 in anti-AID western blot, Figure 7C), the number of its transcripts fell dramatically (blue bar -auxin versus orange bar -auxin; Figure 7D), suggesting that Rio1 mRNA translation and/or Rio1 protein stability increased during amino acid starvation. Depleting Rio1 during amino acid deprivation (confirmed by the absence of Rio1-AID signals in the western blot; lane 2 versus lane 4) lead to a decrease in Rio1 transcript levels (blue bar +auxin versus orange bar +auxin), implying transcriptional auto-repression or increased Rio1 transcript turnover under nutritional stress conditions. Depleting Rio1 in complete medium strongly reduced Rio1 transcripts (but decreased transcript turnover under nutritional stress conditions). In yeast grown in complete medium we did not detect the Rio1 footprint in the Rio1 promoter sequence. As such, Rio1 may regulate its own transcription off-DNA or act with high kinetics, preventing us from capturing the interaction with its own promoter.

In yeast grown in complete medium we did not detect the Gcn4 protein (lane 1 in anti-Myc western blot, Figure 7C), as expected, due to its high turnover in rich conditions (half-life ~2 min (113-115). The same was true for cells grown in complete medium and depleted of Rio1 (lane 2 in anti-Myc western blot). In both cases, we nevertheless detected Gcn4 transcripts (but Gcn4 transcript levels were 80% lower in the cells depleted of Rio1 (blue bar –auxin versus blue bar +auxin). This finding suggests that despite the active turnover of Gcn4, Rio1 upregulates Gcn4 expression and/or stabilizes the Gcn4 transcripts in yeast grown in complete medium, confirming our RNA-Seq results. Taken together, we corroborate that in rich medium Gcn4 mRNA translation diminishes (113) and that Gcn4 becomes actively turned over (114,115). Upon amino acid deprivation, the Gcn4 protein accumulated (lane 1 versus lane 3 in anti-Myc western blot) while Gcn4 transcript levels dropped with 60% (blue bar -auxin versus orange bar -auxin). Shifting the cells from complete to amino acid-depleted medium in the absence of Rio1 did not further affect Gcn4 protein levels (lane 2 versus lane 4). However, the latter nutritional downshift led to an increase in Gcn4 transcript levels (blue bar +auxin versus orange bar +auxin),...
indicating reduced GCN4 mRNA translation and/or improved Gcn4 stability (half-life ~10 min (114)).

To examine whether Gcn4 affects RIO1 expression, as suggested by Rio1’s network (Supplementary Figure S10), we grew RIO1-AID and RIO1-AID gcn4Δ strains exponentially in complete synthetic 2% glucose medium. The cultures were then split in four, the cells washed and transferred for 1 h to 2% glucose medium containing or lacking amino acids, and provided with 500 μM of auxin or with a mock solution. The experiments were performed in triplicate. RT-qPCR analyses revealed that Gcn4 activates RIO1 expression in complete medium (first blue bar – auxin versus third blue bar – auxin) while Gcn4 represses RIO1 in medium lacking amino acids (blue bar + auxin versus orange bar + auxin) and Gcn4 represses RIO1 in medium lacking amino acids (blue bar + auxin versus orange bar + auxin) and Gcn4 represses RIO1 in medium lacking amino acids (blue bar + auxin versus orange bar + auxin), reinforcing their cross-regulatory relationship (all above observations are summarized in Figure 7E).

To determine whether Rio1 and Gcn4 also independently modulate the target genes they share (Supplementary Table S9), we measured in the RIO1-AID and RIO1-AID gcn4Δ strains the transcription of 10 reporter genes under the above nitrogen downshift conditions (the RT-qPCR data are tabulated in Supplementary Table S12 and are plotted in Supplementary Figure S13A). It is well-known (116) that during amino acid starvation, Gcn4 activates the expression of their shared target genes to ensure a swift, global response to nutritional stress.

The contributions of Rio1 to pre-rRNA processing and ribosome production and translation activity, while an additional 97 are involved in protein folding and degradation (a combined 323 genes = 39% of the Rio1 regulon). Simi-

**DISCUSSION**

To survive and reproduce in a constantly changing environment, yeast must quickly assess and transmit the nature and levels of available nutrients into its metabolic, growth and cell division programs. To maximize fitness under the conditions sensed, yeast has evolved signaling networks including those centered around the Ras/protein kinase A, the AMP-activated kinase and the TORC kinase complexes, that guide transcriptional, translational, post-translational, metabolic and developmental decisions (18). The presence of nutrients determines proliferation, a resource and energy-demanding process that primarily depends on the cell’s biosynthetic capacity, provided by ribosomes (117). Our present study has identified Rio1 as the upstream regulator of a new, essential and conserved nutrient-response network. Rio1 governs its network by involving a host of regulators and intermediate factors that it modulates either directly or indirectly at the protein, RNA and/or gene levels. Whether Rio1 acts upon its network members (proteins, ORFs, RNAs) and pathways as a kinase and/or ATPase remains to be determined. The purpose of Rio1 and its network is to promote growth and division when conditions are favorable, and to restrain them during nutrient deprivation. In this respect, the Rio1 network is functionally analogous to that managed by the conserved Tor1 kinase complex (TORC1). Indeed, the Tor1 kinase regulates at the protein and gene levels 35S rRNA transcription, pre-rRNA processing, ribosome protein expression and assembly, mRNA processing, protein stability, nutrient transport and autophagy (118). The Tor1 and Rio1 networks share 68 target genes (Tor1 regulates an additional 398 genes (119)). However, this overlap is not statistically significant (Fisher’s exact test, P-value = 6.4E-02).

Since Rio1 and the Tor1 kinase complex do not physically interact, and since Rio1 does not affect TOR1 expression (and **vice versa**), both kinase networks sustain the nutrient response in parallel fashion.

While we demonstrated that carbon and nitrogen levels are upstream cues, comparative analyses suggested that Rio1 and its network may manage additional stresses (Figure 7A). In that respect, it may be more broad-acting than the TORC1 network. While Rio1 and Tor1 act in parallel, the Rio1 network is closely integrated with other response networks driven by additional kinases (Figure 1B) and key transcription factors. The latter include the networks managed by Gcn4 [67 genes shared with Rio1, 195 additional], Rpn4 [62, 92] and Rap1 [104, 99] (Fisher’s exact test P-values = 1.2E-09, 4.1E-19 and 2.3E-43, respectively) (18,117,120,121) (Supplementary Figure S13 and Table S9).

From the Rio1–protein interaction map, its regulon and genetic diagrams, it is evident that Rio1 controls growth foremost by influencing protein synthesis and turnover. Indeed, of its 818 gene/transcript targets, 226 are dedicated to ribosome production and translation activity, while an additional 97 are involved in protein folding and degradation (a combined 323 genes = 39% of the Rio1 regulon). Simi-
Figure 8. The human RIOK1–protein interaction map. The interaction network comprises 352 proteins that were identified in co-purifications reported in 14 publications (Supplementary Table S13). RIOK1 and its partners are connected by green lines while protein interactions between the RIOK1 interactors themselves are shown by gray lines in the background. The RIOK1 interactors were functionally clustered using the Gene Ontology term ‘Biological Process’ that is associated with each protein. The \( P \)-values of the functional enrichments are: ribosome biogenesis and activity = 4.2E-25, RNA biology and regulation = 2.4E-54, protein folding and stability = 8.6E-03, cell cycle and chromosome segregation = 1.3E-02, metabolism = 5.8E-21, transcription, chromatin assembly and remodeling = 1.7E-23.

larly, Rio1 physically interacts with 35 proteins involved in ribosome biogenesis, activity and protein governance.

Typical Ribi gene products help ribosomes become operational protein-synthesis machines (pre-rRNA processing, folding, nuclear export of pre-ribosomal particles to the cytoplasm, maturation and assembly events, etc.). As demonstrated previously, Rio1 serves the Ribi program by regulating 35S rDNA transcription, pre-rRNA processing and 40S ribosomal subunit maturation. However, we find that Rio1 also contributes by regulating the expression of 20% of the Ribi regulon members, including ORFs that encode components of the small subunit processome (122,123) and proteins that mediate ribosome assembly, maturation, export, translation initiation and termination (Supplementary Table S5). Rio1 also physically interacts with proteins contributing to ribosome biogenesis and mRNA translation in events beyond those associated with Rio1 activity today. Since Rio1 strongly genetically interacts with the Dom34:Hbs1 complex (94), which dissolves stalled ribosomes (124), and also physically interacts with Zuo1, a ribosome-associated chaperone for nascent polypeptide chains (91), Rio1 could well be involved in surveying the quality of ribosomes and the proteins they deliver. Being a Ribi member, we for sure did not expect Rio1 to transcriptionally regulate the RP regulon, encoding the structural RPs. Their promoters share binding sites for RP-specific transcriptional activators, including Rap1, which we identified as a Rio1 gene target. Hence, Rio1 may command ribosome expression in part directly (footprints identified in promoters of ORFs encoding RPs, Figure 4A) and by regulating \( RAP1 \) transcription and/or \( RAP1 \) mRNA stability (Supplementary Figure S11B).

Nitrogen availability not only decides on ribosome production and protein synthesis, but also on protein turnover (restrained under rich conditions, active during amino acid deprivation). Consistent with this, we find that Rio1 down-
regulates the expression of all subunits of the 26S proteasome under nitrogen-rich conditions. This likely occurs via Rpn4, the transcription factor for all proteasomal proteins, which we identified as a Rio1 gene target (Supplementary Figure S11A). Besides determining its expression, rio1 may also promote proteasome formation as it regulates the expression of 26S proteasomal assembly factor Rpn6. Furthermore, Rio1 and its network manage the transcription of enzymes constituting the ubiquitin pathway. Its localization to vacuoles (peptidase- and proteinase-catalyzed protein turnover compartments), physical interaction with and control of genes involved in autophagy and cytosol-to-vacuole pathways are in line with Rio1 supervising protein governance at a global scale. Fittingly, Rio1 physically interacts with and regulates the expression of numerous chaperones.

Ribosome production, mRNA translation and cell growth are highly demanding from a substrate and energy point of view. Also, available nutrients need to be uptaken and transported internally. Hence, it is not surprising that part of Rio1’s network (11 protein interactors, 306 gene targets = 37% of its regulon) is dedicated to metabolism (nutrient uptake and transport, carbohydrate, amino acid, lipid, purines, pyrimidines and vitamins) and energy production (glycolysis, mitochondrial respiration, ATP synthase–ATPase complex subunits), as is its localization to the mitochondria. Importantly, the majority of these genes (83%) are upregulated together with ribosome production and protein synthesis under rich conditions.

While Rio1 stimulates growth and survival, its also promotes physiology (protein movement, transport) and cell division. Rio1 was already shown to ensure a timely entry into the cell cycle by regulating directly RNA polymerase I activity at the rDNA array. In addition, repressing rDNA transcription allows for rDNA condensation, which finalizes the chromosome segregation process in late anaphase (16). During DNA replication, repression of rDNA transcription by Rio1 also reduces local collisions between RNA polymerase I and the replisome. This minimizes rDNA double strand breaks, hyper-recombinations within and between sister rDNA arrays, resulting in a loss of or increase in rDNA units. Importantly, ribosome biosynthetic activity is linked to the rDNA gene copy number within the rDNA array (125,126). As such, one might hypothesize that the transcriptional regulation of RNA polymerase I by Rio1 could lead to a controlled increase or decrease in rDNA repeats to enhance or reduce ribosome production capacity depending on nutritional resources. We have experimentally confirmed that Rio1 acts positively toward replication fork stability/progression at the rDNA (Figure 2B and C). Since Rio1 physically and functionally interacts with various proteins mediating DNA repair and recombination, the role of its network in ensuring genetic stability during cell division is likely more multifaceted than currently appreciated. Its localization to kinetochores (physically interacts with conserved subunit Mcm21CENP-O) implies an important role in chromosome transmission and may explain the increased level of aneuploidy, measured in yeast suffering from reduced Rio1 activity (88).

Telomere-length homeostasis depends on a large genetic network that is regulated in part by Rio1 gene-target RAPI (127). This network is disrupted by environmental stress signals, resulting in altered telomere length and adverse effects on lifespan. While Rio1 regulates RAPI expression, other Rio1 target genes (including DAPI, HTL1, OPI1, SDC1, STMI and XRNI) are involved in telomere maintenance and silencing. In addition, Rio1 physically interacts with the DNA helicase Y′-Help1 (128) and the conserved protein Elg1 (129), which protect telomere length and stability, suggesting that the Rio1 contributes to telomere stability and timely aging.

During our study, we also probed the regulation of RIO1 expression and found it to be auto-regulated and sustained by nutrient-stress transcription factor Gcn4, which determines the expression of amino acid biosynthetic genes. Similarly, Rio1 modulates Gcn4 expression, revealing cross-regulation, as was suggested by our algorithm that assembled the Rio1 network (Supplementary Figure S10). This observation is especially striking since control of Gcn4 activity was considered to occur only at the level of Gcn4 transcript translation and Gcn4 stability and turnover (112,130). Rio1 could regulate Gcn4 expression via transcription factors Hap4 and Fkh2 (Y2H interactors of Rio1), which localize to the Gcn4 promoter (131,132). In turn, the Gcn4 consensus binding sequence 5′-TGACTC-3′ (133–135) lies 90 bp upstream of RIO1’s transcription initiation site. Furthermore, while RIO1 expression is reduced under nutritionally poor conditions, Rio1 protein stability increases. The same is true for Gcn4. Future inquiries into the Rio1-Gcn4 axis promise to significantly advance our understanding of the nitrogen starvation response in yeast and species beyond.

Rio1 protein numbers (536 molecules/cell (136)) are in line with those measured for other network kinases, including Tor1 (225–589 molecules/cell (136,137)). By interacting with a portfolio of regulators, kinases, phosphatases, transcription factors, etc. Rio1—similar to other network kinases—can amplify its activity to manage its network.

For most nutrient signaling pathways, the signal receptors and transduction processes are barely understood (138). When amino acid levels increase in the lysosome (the proteins(s) sensing this change are unknown), human mTORC1 is activated by recruitment to the lysosome surface (equivalent of the yeast vacuole) (139). The AMP-activated protein kinase responds to increased AMP:ATP ratios as a surrogate for glucose abundance (140). Could Rio1 similarly sense and transduce energy levels into its network as it may also act as an ATPase (so far reported only for pre-40S ribosomal subunit maturation) (8,9) and since Rio1 localizes to the ATP-generating mitochondria? Does Rio1 signaling depend on other sensor kinases (including Ck2, Sch9, Psk1 and Rim15) or protein phosphatases (including Pho8, Pah1, Cdc14, Psy2 and Yvh1) with which it physically or genetically interacts? Answering these questions represent important challenges for the future.

We believe that our singular datasets and integrated network (analyzable and manipulatable at http://netbio.bgu.ac.il/respnet/ by adding the Rio1 protein and gene targets) represent valuable resources that will advance the understanding of biological processes now associated with Rio1 activity. For example, yeast depleted of Rio1 displays a chain-like morphology after two to three cell cycles (Supplementary Figure S3G). We found that Rio1 physically in-
teracts with septin ring component Cdc12 and with the morphogenesis checkpoint kinase Hsl1, which are required for cytokinesis (141) and for coupling bud formation to cell cycle progression (142), respectively. A lack of Riol1-based regulation of both proteins could explain the chain-like phenotype of the Riol1-AID depleted strain, typical of a cytokinesis defect. The covertsensitivity of heterozygous riolΔ yeast to drugs interfering with sphingolipid and ergosterol synthesis (20) is coherent with our finding that Riol1 controls the transcription of genes catalyzing sphingolipid and ergosterol synthesis (Supplementary Table S5).

In human cells, RIOK1 expression is activated by the proto-oncogenic transcription factor c-Myc (143) whose amplification in ~20% of all cancers correlates with metastasis and poor prognosis (144). In turn, RIOK1—as part of the PRMT5 complex—promotes the translation of c-Myc transcripts (26). Hence, it is not surprising that RIOK1 is overexpressed in myriad malignancies (145). c-Myc activates genes involved in ribosome biogenesis, mitochondrial activity (respiration, glycolytic flux), metabolism, nucleotide and lipid synthesis, and cell-cycle progression; thereby enhancing the ability of a cancer cell to transform, grow and proliferate (144,146). Since the cellular processes controlled by c-Myc significantly overlap with those identified here for Riol1 (and RIOK1), overexpression of RIOK1 by c-Myc could be an important contributor to the transformation capacity of c-Myc.

DATA AVAILABILITY
The RNA-Seq and ChIP-Seq data are available at GSE70287 (GEO).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Authors’ contribution: M.G.I. performed most of the biological experiments. M.B. analyzed the protein-interaction, genetic-interaction and gene-expression datasets to produce the weighted interactome and transcriptome maps. O.B. and E.Y.-L. performed the ResponseNet analyses. L.G. and W.C. analyzed the RNA-Seq and ChIP-Seq data, respectively. C.G. and M.D.M. performed the genetic interaction studies. V.I. did the ChIP-chip experiment, while C.V. and R.V. localized Riol1-GFP via indirect IF imaging.

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