Understanding the *in vivo* functions of Holliday junction resolvase Yen1

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UNDERSTANDING THE *IN VIVO* FUNCTIONS OF HOLLIDAY JUNCTION RESOLVASE YEN1

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ABSTRACT

Elaborate replication fork recovery pathways support the duplication of the genome under replication stress. Nucleases and helicases take center stage in these pathways, but our understanding of the molecular underpinnings remains incomplete. The Holliday junction resolving nucleases Mus81-Mms4 (human MUS81-EME1) and Yen1 (GEN1) act redundantly to remove replication-associated homologous recombination repair intermediates, safeguarding chromosome segregation. They are cell cycle regulated, so that Mus81-Mms4/MUS81-EME1 activity peaks first, making it the major resolvase in yeast and human. Yen1/GEN1 subsequently provides a catchall activity for the resolution of recombinational joint molecules that escape the attention of Mus81-Mms4.

In this work, we used budding yeast as a model to explore whether additional, Mus81-independent functions of Yen1 exist, potentially outside the context of canonical Holliday junction resolution. To this end, we investigated the reported genetic interactome of *YEN1*. We disproved a purported genetic interaction between *YEN1* and *PBY1*, linking wrongly assigned *PBY1* interactions to *MUS81-MMS4* instead. More significantly, addressing a synthetic sick interaction between *YEN1* and the conserved nuclease-helicase *DNA2*, we identify a first unique function of Yen1 in targeting replication, as opposed to recombination, intermediates. Furthermore, we uncover a novel role for the elusive Dna2 helicase activity in the recovery of stalled replication intermediates. Our findings provide new insight into the replication stress response in eukaryotes.

LIST OF ABBREVIATIONS

9-1-1 :	Ddc1/Rad17/Mec3 complex
BIR :	Break-induced replication
CDK :	Cyclin-dependent kinase
CO :	Crossover
CPT :	Camptothecin
CSF :	Common fragile sites
D-loop :	Displacement loop
DDK :	Dbf4-dependent kinase
dHJ :	Double-Holliday junction
DSB :	Double-strand break
dsDNA :	Double-stranded DNA
HJ :	Holliday junction
HR :	Homologous recombination
HU :	Hydroxyurea
IR :	Ionizing radiation
JM :	Joint molecules
LOH :	Loss of heterozygosity
MMC :	Mitomycin C
MMS :	Methyl methanesulfonate
NCO :	Non-crossover
NHEJ :	Non-homologous end joining
NES :	Nuclear export signal
NLS :	Nuclear localization signal
P-body :	mRNA processing body
PI3K :	Phosphoinositide 3-kinase
RF :	Replication fork
RFB :	Replication fork barrier
RPA :	Replication Protein A
SAC :	Spindle assembly checkpoint
SDSA :	Synthesis-dependent strand annealing
ssDNA :	Single-stranded DNA
STR :	Sgs1/Top3/Rmi1 complex

THESIS OUTLINE

This PhD thesis is based on the following publication and manuscript:

Ölmezer G., Klein D., Rass U. (2015). DNA repair defects ascribed to *pby1* are caused by disruption of Holliday junction resolvase Mus81 – Mms4. *DNA Repair 33, 17–23*

Ölmezer G., Levikova M., Klein D., Falquet B., Fontana G.A., Cejka P., Rass U. Replication intermediates that escape Dna2 activity are processed by Holliday junction resolvase Yen1. *Manuscript in revision (Nature Communications)*

This thesis consists of five chapters. In chapter 1, I summarize the current knowledge on the pathways maintaining genome integrity under replication stress. The focus is on *Saccharomyces cerevisiae*, the model organism used in this study. Conservation of the pathways discussed is extensive between yeast and human, and frequent references to the human system are made.

Chapters 2 and 3 are experimental chapters, and the respective title pages state the people who contributed to the work. In chapter 2, we show that the disruption of Holliday junction resolvase Mus81-Mms4 is responsible for a set of negative genetic interactions wrongly assigned to *PBY1*. The work presented in this chapter has been published in the journal *DNA Repair*. Chapter 3 focuses on the role of conserved Dna2 nuclease-helicase and Holliday junction resolvase Yen1 in protecting cells from replication stress. The work presented in this chapter is in revision for publication in *Nature Communications*.

Chapter 4 presents additional results obtained during my PhD studies.

In the last chapter, chapter 5, I summarize the main conclusions arising from the work presented, and highlight future questions.

TABLE OF CONTENTS

1 Chapter 1: Introduction

Below, I will give an introduction to some of the key topics that form the background to the experimental concepts and data presented in the following chapters. This includes a description of the basic concepts of cell cycle, genome instability, replication stress, and pathways maintaining replication fork stability, with an emphasis on homologous recombination (HR). Subsequently, I will describe the role of Yen1 in the resolution of Holliday junctions (HJs) and highlight Yen1's genetic interactions, with special attention to the nuclease-helicase Dna2.

1.1 The Eukaryotic Cell Cycle

The mitotic cell cycle can be divided into distinct phases, and the phase transitions are controlled by regulatory mechanisms and checkpoints (Hartwell and Weinert, 1989). The first phase is G1 (gap 1), where cells produce proteins and grow in size in preparation for genome duplication, which occurs during S phase (synthesis phase), the second cell cycle phase. Alternatively, cells can reversibly exit the cell cycle and persist in a quiescent state (G0 phase), for example upon encountering unfavorable growth conditions or nutrient starvation. Within G1 phase, gating mechanisms exists, which restrict the commitment to S phase. Once the restriction point is crossed, however (for example START in yeast), cells commit to S phase. For this to happen, certain requirements must be met, for example sufficient cell size and mass. In addition, there is the G1 DNA damage checkpoint, which, if activated, retains cells in G1 phase, providing repair time and ensuring that S phase is not entered in the presence of DNA damage (Hartwell, 2002).

Within S phase, the entire genome must be faithfully replicated. In eukaryotes, the task is distributed between many different DNA synthesis centers, each with an "origin of replication". The eukaryotic genome contains an excess of replication origins that are distributed along the chromosomes. In G1 phase, replication origins are licensed and the MCM replicative helicase is loaded. Yet, only a subset of origins engages in replication in a given S phase. DNA synthesis is initiated after the recruitment of elongation components and activation of MCM, which is regulated by cyclin-dependent kinases (CDK) and Dbf4-dependent kinase (DDK). Origins can only fire once per cell cycle, and

their time of firing differs. In other words, some origins are early replicating while others fire later. The temporal regulation of DNA replication is critical for preventing exhaustion of initiation factors and the flexible use of origins allows cells to respond to changing replication dynamics (Méchali, 2010).

Upon origin firing, bidirectional replication forks (RFs) emanate from the origin, each engaged with a multi-subunit complex of replication elongation factors. These "replisomes" associate with the replicative helicase and catalyzes DNA synthesis. The MCM helicase unwinds the parental strands and DNA polymerase α, associated with the Primase, synthesizes short RNA-DNA primers, which provide the free 3'-OH end for the incorporation of complementary dNTPs. Replicative polymerases ε and δ complete the action of DNA polymerase α and mediate further elongation of the leading and the lagging strands, respectively. Once all active RFs encounter an oncoming fork from a neighboring origin, or the end of the chromosome, the entire chromosome is replicated, and the parental DNA has been converted into two nascent sister chromatids (Bell and Dutta, 2002).

Given the complicated DNA metabolic processes during genome replication, it is not surprising that cells are more vulnerable to DNA insults during S phase. A particular problem arises from the unwinding of the parental DNA, since single-stranded DNA (ssDNA) is more fragile than double helical DNA (Hustedt et al., 2013). DNA damage on the DNA template can lead to mutations and impairs the replication machinery, if not attended to properly (explained in more detailed later). The intra-S phase checkpoint is responsible for monitoring the integrity of the DNA and progression of RFs during replication, and coordinates DNA repair, replication, and cell cycle progression.

Once bulk DNA synthesis is completed, cells initiate a second stage of growth and biosynthesis called G2 (gap2) phase. Presence of DNA damage is also audited by a DNA damage checkpoint at this point, as in G1 phase, and entry into mitosis is halted until after DNA repair (Hustedt et al., 2013).

Finally, mitosis (M phase) has four distinct stages - prometaphase, metaphase, anaphase and telophase – which the cell traverses prior to cell division, or cytokinesis

(Nigg, 2001). During the initial stages of M phase, chromosome condensation leads to chromosome compaction, facilitating the subsequent pulling apart of the sister chromatids at anaphase. Via the kinetochores, each of the two sister chromatids attach to opposite poles of the spindle. Transition from metaphase to anaphase is regulated by the spindle assembly checkpoint (SAC). Anaphase is initiated only when all chromosomes are attached to spindle poles and align at the mid-zone of the cell. Upon anaphase onset, one last step is initiated by cleaving cohesion, which holds the sister chromatids together, having been loaded during S phase, to allow segregation. Spindle fibers then pull the sister chromatids to opposite cell poles at telophase. Mitosis divides the cell into two, giving rise to two daughter cells with near-identical DNA content.

1.2 Genome Integrity

The genome is constantly exposed to endogenous and exogenous sources of DNA damage, and cells have to mediate an adequate response, harnessing the appropriate DNA repair mechanisms, orchestrated by the cell cycle checkpoints (reviewed in (Aguilera and Gómez-González, 2008)). Failed repair results in mutation or gross chromosomal rearrangements (Aguilera and García-Muse, 2013).

Genome instability is a hallmark of cancer cells and contributes to aging (Magdalou et al., 2014). A major factor in genome instability is "replication stress", stress conditions that impair the progression of replication. Therefore, elucidating how cells respond to replication stress is key in understanding genome integrity and human pathologies associated with genome instability.

1.3 Replication Stress –Causes and Consequences

Accurate transmission of hereditary information relies on error-free DNA repair, and the complete and faithful replication of the genome. Repair and replication intermediates that persist post-replicatively must be timely resolved to allow proper chromosome segregation at mitosis (Mankouri et al., 2013). Thus, replication stress, which may be induced by endogenous or exogenous factors, acts locally at sites of active DNA synthesis, but its effects can have global consequences of gross chromosomal instability.

At the heart of replication stress is the perturbation of RF progression. RFs have to traverse each chromosome in their entirety. However, there are a variety of obstacles that the replication machinery has to overcome on its way. These obstacles might cause transient pausing of forks, in which case the replisome remains in position, so that replication can restart once the cause of stalling is overcome. Longer delays, on the other hand, can cause fork collapse, a potential source of genome instability if not properly processed (Aguilera and García-Muse, 2013).

1.3.1 The causes of replication stress

Inappropriate origin usage: In order to ensure whole genome duplication, the number of origins fired in a given S phase must be correctly titrated (Yekezare et al., 2013). Since excess origins are licensed in G1, cells can compensate for any shortfall in replication that may be caused by replication stress. Oncogene-induced imbalances in origin licensing and firing, however, have been linked to replicative stress and genome instability (Hills and Diffley, 2014). Thus, overexpression of cyclin E, cyclin D, or MYC in cells causes hyper-replication phenotypes, perhaps as a result of overexpression of origin licensing factors along the RB-E2F pathway. As a consequence, dNTP pools become depleted and the likelihood for replication stalling is increased. On the other hand, oncogene-induced dormant origin paucity impairs the ability of cells to react to replication stress and is thought to contribute to genome instability in cancer (Hills and Diffley, 2014).

Low levels of dNTPs: DNA replication requires a large reservoir of dNTPs and histones. Low levels of dNTPs slow down the progression of RFs. Hence, DNA polymerases cease to incorporate nucleotides, but the replicative helicase continues to unwind the parental template strands. This uncoupling results in extensive ssDNA stretches at the fork, a hallmark feature of replication stress (Magdalou et al., 2014). In laboratory conditions, dNTP depletion can be mimicked using hydroxyurea (HU), a drug that inhibits the biosynthesis of dNTPs (Eklund et al., 2001). HU treatment leads to replication slow down in cells; longer periods of exposure cause DNA double-strand breaks (DSBs), indicating that stalled forks are prone to breakage (Petermann et al., 2010).

In addition, DNA synthesis is accompanied with *de novo* nucleosome assembly along the nascent DNA strands. This depends upon effective histone incorporation and

modification. Defective chromatin assembly during replication is another factor altering replication dynamics, thereby causing replication stress (Aguilera and García-Muse, 2013).

Obstructions to fork progression: DNA lesions, tightly bound proteins, and secondary structures within the template DNA are among the factors impeding the progression of RFs (Mankouri et al., 2013). Moreover, the replisome might stall upon encountering other ongoing DNA metabolic processes, most prominently transcription. Transcription might induce topological stress on both sides of an advancing RNA polymerase. DNA binding-proteins, such as transcription factors, or chromatin loops further elevate the local torsional stress, forming a potent obstruction to fork progression (Gaillard and Aguilera, 2015). In yeast, natural replication pausing sites are found in the genome, and some are linked to transcription. These sites include tRNA genes, highly transcribed RNA Polymerase II (Pol II) genes, and the replication fork barrier (RFB) in the rDNA (Azvolinsky et al., 2009; Deshpande and Newlon, 1996; Greenfeder and Newlon, 1992; Sanchez et al., 1998). The rDNA RFB ensures that transcription and replication move in the same direction (Sanchez et al., 1998). However, eventually RFs must move on or fuse with an oncoming fork to ensure full genome duplications.

Replication stress hotspots: Throughout the genome, some DNA regions are associated with frequent fork stalling events, even in the absence of exogenous damage. These regions comprise sites intrinsically difficult to replicate, such as centromeres, telomeres, or the rDNA, because they contain repetitive DNA elements, complex secondary structures or tightly bound proteins. Due to the presence of topological stress, highly transcribed genes also cause elevated levels of fork stalling (see above). If enhanced stalling occurs in regions with a low density of origins, finishing replication in time for mitosis can become a challenge (Magdalou et al., 2014; Mankouri et al., 2013). Stalling events in these regions can lead to underreplication and DNA breakage, as seen at common fragile sites (CSFs). Both, breakage-induced HR repair intermediates, or unresolved replication intermediates in challenging, late-replicating DNA regions, can cause sister chromatid non-disjunction and mitotic problems (Rass, 2013).

1.3.2 The consequences of replication stress

Frequent RF stalling events compromise the completion of DNA replication in S phase. In fact, recent evidence suggests that chromosomes with underreplicated regions or sister chromatid entanglements may rather frequently persist into mitosis (Lukas et al., 2011; Magiera et al., 2014; Torres-Rosell et al., 2007). The failure to complete DNA replication or sister chromatid disjunction before the onset of mitosis may be due to either late/delayed replication or innate structural difficulties at certain loci, obstructing replication and subsequently segregation. As a result, intertwined sister chromatids remain physically linked. Upon segregation, these links manifest themselves as anaphase bridges between the segregating sister chromatids (Mankouri et al., 2013). Anaphase bridges are potent sources of genome instability. Increasing mechanical tension on chromatids during mitosis might lead to chromosome breakage and uneven segregation. Cells meet this challenge using late activated mechanisms to detach the physically linked sister chromatids (Minocherhomji et al., 2015; Pedersen et al., 2015). For example, the activation of structure-specific nucleases ensures the removal of branched DNA intermediates and sister chromatid joint molecules (JMs), effectively dealing with the consequences of underreplication and induced recombinational repair, despite the fact that incomplete DNA replication and recombination intermediates appear not to be detected by checkpoints (Baxter, 2014). Prolonged metaphase arrest may cause mitotic catastrophe and cell death or senescence. In multicellular organisms, cells that bypass senescence and therefore transmit DNA damage through mitosis, may drive tumorigenesis and/or aging (reviewed in (Baxter, 2014; Halazonetis et al., 2008)).

1.3.3 The cellular response to replication stress

Eukaryotic cells employ checkpoint functions to control their advance through the cell cycle. If a checkpoint is not satisfied, cell cycle progression is halted to dedicate more time for stage-completion and prevent premature entry into the next phase (Hartwell and Weinert, 1989). The intra-S phase checkpoint is particularly important for genome integrity, regulating dNTP levels and replication initiation events even in unchallenged conditions (reviewed in (Hustedt et al., 2013)). Furthermore, the DNA replication checkpoint responds to replication stress by slowing the progression through S phase and promoting tolerance/repair events, which ensures cell survival. Incidentally, checkpoint

response proteins are often found to be mutated in cancer and human syndromes associated with genome instability (Kastan and Bartek, 2004).

Checkpoint signal transduction is initiated by two apical phosphoinositide 3-kinase (PI3K)-related protein kinases; Mec1 and Tel1 (ATR and ATM in human, respectively) (Friedel et al., 2009). Recruitment and activation of Mec1 and Tel1 have both local and global effects through a phosphorylation cascade of several downstream factors. Genetic studies show that *MEC1* and *TEL1* are partially redundant, even though Tel1/ATM is specifically required for telomere maintenance and DNA damage response in G1, whereas Mec1/ATR function is particularly important during S and G2 phases (Ira et al., 2004).

Checkpoint induction: While there are several forms of DNA damage, most of the lesions in S phase are processed to generate ssDNA, which is rapidly coated with ssDNA binding protein RPA (replication protein A). RPA-coated ssDNA recruits Mec1 kinase via interaction with its constitutive binding partner, Ddc2 (ATRIP in human) (Zou and Elledge, 2003). Tel1 kinase is recruited and activated at DSBs, whose ends are bound by Mre11-Rad50-Xrs2 (MRX; MRN in human) complex. The MRX complex, then, promotes resection, yielding RPA-coated ssDNA that further stimulates Mec1 activity (Finn et al., 2011).

For checkpoint induction, the recruitment of Mec1-Ddc2 is not sufficient on its own. The damage sensor 9-1-1 checkpoint clamp (Ddc1, Rad17 and Mec3 in *S.cerevisiae*) and its loading factor, the Rad24-RFC complex are required to be bound to double-strand DNA (dsDNA)-ssDNA junction structures, that can arise from lagging strand DNA synthesis, nucleotide excision repair or resection of DSBs. This brings Mec1-Ddc2 in close contact with the Ddc1 factor of the 9-1-1 complex, which activates Mec1 kinase by phosphorylation. In addition to 9-1-1 complex, Dbp11 and Dna2 are further damage sensors contributing to the activation of Mec1 kinase (Kumar and Burgers, 2013).

Checkpoint mediators Mrc1 & Rad9: At sites of DNA lesions, Mec1/Tel1 phosphorylate mediator proteins, Mrc1 and Rad9, which transduce the checkpoint signal to the effector kinases Rad53 and Chk1 (CHK2 and CHK1 in human, respectively). While Rad9 responds

to DNA damage in G1 and G2, Mrc1 functions during DNA synthesis (Alcasabas et al., 2001). Mrc1 is part of the replisome and activates Rad53 during replication stress by recruiting the kinase to stalled forks and enhancing its interaction with Mec1. In contrast, Rad9 is phosphorylated by Mec1 after its recruitment to damaged DNA through histone modifications. Rad9 binds to Rad53, increasing the local concentration of the kinase, thereby triggering its auto-phosphorylation and activation (Gilbert et al., 2001). Furthermore, Sgs1 helicase, which is constitutively associated with RFs, contributes to direct phosphorylation of Rad53 at stalled forks (Hegnauer et al., 2012). In addition, Mec1 and Tel1 can activate both Rad53 and Chk1 directly (Sweeney et al., 2005).

The diffusible effector kinases Rad53 and Chk1 allow the master kinases Mec1/ATR and Tel1/ATM to act globally and mount a full-blown cellular response to DNA lesions. At the local level, Mec1 and Tel1 phosphorylate histone H2A (H2AX in human) at sites of DNA damage. The phosphorylated form, γ-H2A, then, recruits and stabilizes DNA repair and checkpoint proteins, maintaining the checkpoint-signaling cascade and orchestrating repair (Finn et al., 2011).

Targets of intra-S phase checkpoint: The global DNA damage response targets several factors involved in maintenance of fork integrity, repair of lesions, prevention of gross chromosomal rearrangements, and control of the cell cycle progression. The main targets are described in greater detail below.

- Cell cycle regulation. The effector kinases arrest the cell cycle upon checkpoint activation though different mechanisms in different species. In *S. pombe* and human, CHK1 and CHK2 attenuate the transition to G2/M by blocking the activation of CDK. In *S. cerevisiae*, the mitotic regulator Pds1/securin, which needs to be degraded for the mitotic exit network to mediate the transition to anaphase, is stabilized by the intra-S phase checkpoint. Consequently, cells are halted at metaphase with short intra-nuclear spindles (Hustedt et al., 2013).

- dNTP pool regulation. In yeast, Rad53 and another kinase, Dun1, upregulate the expression of DNA repair genes, and genes involved in nucleotide biosynthesis to replenish the dNTP pools, which is essential for cell viability (Zhao and Rothstein, 2002).

- Replication origin control. Replication origins are temporally regulated and their firing allow cells to respond to the changing dynamics of DNA replication. Rad53 suppresses firing of late origins by targeting CDK- and DDK-dependent pathways. Blocking of late origin firing is thought to help preserve rate-limiting replication factors for when replication resumes after the damage is removed (Santocanale and Diffley, 1998; Zegerman and Diffley, 2010). In contrast, the replication checkpoint mediates firing of local origins near the site of replication stalling, which is thought to help compensate for any replication shortfall.

- Fork maintenance. The control of the cell cycle progression, gene expression and origin firing by the intra-S phase checkpoint are important responses in dealing with replication stress. Interestingly, mutants which cannot inhibit late-origin firing do not show sensitivity to replication stress induced by HU (Zegerman and Diffley, 2010). Furthermore, nocodazole-induced metaphase arrest does not rescue the lethality of *rad53* or *mec1* mutants upon treatment with high doses of HU or methyl methanesulfonate (MMS), a DNA alkylating agent (Tercero and Diffley, 2001). These observations led to the notion that the crucial function of the intra-S phase checkpoint resides in the maintenance of RFs, so that later restart or fork convergence remain an option. Indeed, HU or MMS-treated *mec1* mutants fail to restart DNA replication due to losing replisome components from early origins (Cobb et al., 2005; 2003; Tercero et al., 2003). Moreover, checkpoint mutants display an accumulation of ssDNA and aberrant DNA intermediates, such as regressed forks, following fork stalling (Lopes et al., 2001; Sogo et al., 2002). Even though the exact mechanism is not yet clear, these evidences suggest that the association of replisome components is stabilized by intra-S phase checkpoint-mediated phosphorylation events, reinforcing replisome integrity. One model suggests that Mec1 keeps the DNA polymerases engaged with stalled fork, whereas Rad53 ensures replication restart by preserving the MCM helicase in an active state (Cobb et al., 2003; 2005).

Several lines of evidence indicate that both Mec1 and Rad53, independently from each other, regulate the activity of certain nucleases and helicases at stalled forks. This regulation safeguards stalled RFs, which would otherwise form pathological DNA

intermediates, impairing fork restart after the removal of stress factors. As an example, deletion of the nuclease *EXO1* largely rescued the sensitivity of *rad53* mutants to DNA-damaging agents (Segurado and Diffley, 2008). Furthermore, Exo1 is shown to be inhibited by the DNA damage checkpoint, preventing the accumulation of ssDNA at stalled RFs (Cotta-Ramusino et al., 2005). Similarly, the intra-S phase checkpoint in *S. pombe* targets Dna2 nuclease-helicase, which is thought to limit ssDNA formation after HU treatment by inadvertent degradation of nascent DNA ends at regressed forks (Hu et al., 2012). Moreover, Cds1, the Rad53 homolog in fission yeast, targets Mus81-Eme1 structure-specific nuclease, promoting its release from chromatin upon fork stalling (Kai et al., 2005).

Another function of the intra-S phase checkpoint is to coordinate repair events at RFs, avoiding unwanted reactions at its ssDNA component. To prevent illegitimate recombination at stalled forks, for instance, the intra-S phase checkpoint ensures these DNA structures are refractory to recruitment of recombination proteins (Alabert et al., 2009; Barlow and Rothstein, 2009; Lisby et al., 2004). Collectively, these data suggest that the Mec1- and Tel1-mediated checkpoint cascade aims to retain RFs within a state of replication competence and/or preserve them for fusion with a convergent fork.

In most of the cases, blocked RFs can restart without further assistance after the respective impediment has been removed (Cobb et al., 2003; Lopes et al., 2001). Moreover, several pathways have evolved to assist replication resumption, depending on the constitution of the lesion and structure of the DNA intermediate formed upon stalling. HR plays a pivotal role in facilitating the recovery of stalled RFs. For example, recombinational repair can be used to reassemble a RF following strand invasion in an origin-independent manner. When fork recovery pathways are delayed, however, RFs become more prone to collapse, causing increased DNA breaks and ssDNA gaps (Petermann and Helleday, 2010). Nevertheless, as mentioned before, unrestrained recombination might as well result in genome instability. Therefore, in order to prevent the generation of deleterious rearrangements, cells, with the help of checkpoint surveillance, need to coordinate the various repair options carefully.

1.4 Homologous Recombination

Various repair pathways have evolved to counteract lesions originating from various sources to maintain the integrity of the DNA-encoded hereditary information. DNA repair mechanisms are specialized for different types of damage, and may be more prominent in certain cell cycle stage. Among the most deleterious lesions are DNA DSBs, which, if repair fails, lead to chromosomal loss or gross chromosomal rearrangements. Repair of DSBs occurs via two pathways: HR and non-homologous end joining (NHEJ).

HR plays an essential role in DSB repair occurring during both meiosis and mitosis. HR repair relies on an intact repair template of identical or near-identical (homologous) DNA sequence, establishing base pairing with the damaged double helix to prime repair synthesis to restore the integrity of the broken strands (reviewed in (Pâques and Haber, 1999; Symington et al., 2014)). During the meiotic program in eukaryotes, repair of deliberate DSBs by HR allows reciprocal exchange of genetic material between the maternal and paternal homologous chromosomes. In mitosis, DSB repair by HR is predominant in S phase, where sister chromatids are available to serve as a template for repair. In yeast cells, Rad52-mediated HR is extremely effective and the major DSB repair pathway in S and G2 phase.

Molecular aspects of DSB-initiated HR: The commitment to HR is made upon nucleolytic degradation of the 5′ terminated DNA strands at DSBs. This process, known as "end-resection" (reviewed in (Cejka, 2015)), generates 3′ overhangs, the substrate of HR. End-resection entails redundant pathways, involving the MRX complex, Sae2 (CtIP in human), Exo1 and Sgs1-Dna2 (Bloom's helicase-DNA2 in human) (Cejka et al., 2010; Mimitou and Symington, 2008; Niu et al., 2010). In yeast, as in higher organisms, MRX and Sae2 promote initial short-range resection, while Exo1 and the Sgs1-Dna2 complex redundantly mediate long-range resection.

End-resection produces 3′ ends, which are first covered with RPA and later with Rad51 (RAD51 in human) recombinase to form nucleoprotein filaments. Rad51-DNA filaments mediate strand exchange between ssDNA and a donor duplex of homologous sequence, forming a displacement loop (D-loop). Efficient strand exchange requires the activity of several mediator proteins, such as Rad52 (or BRCA2 in human), which promote

the loading of Rad51 onto RPA-coated ssDNA (New et al., 1998). Within the D-loop, DNA repair synthesis is primed at the donor template to restore the sequence information lost at the DSB. Following DNA synthesis, the D-loop may dissociate and the displaced (elongated) strand may anneal with the 3′ tail of the other break end at the DSB; this process is called synthesis-dependent strand annealing (SDSA) (Pâques and Haber, 1999). As a matter of fact, SDSA is thought to be the main pathway into which DSBs are channeled in mitotic cells, preventing the formation of more complex JM HR intermediates (Bzymek et al., 2010).

In an estimated 10% of cases, the displaced strand of the repair template anneals with the 3′ terminated strand at the other side of the break (a process referred to as second-end capture), and repair synthesis is initiated (Bzymek et al., 2010). This strengthens the interaction between the broken molecule and the sequence donor, and ligation of the JM that is formed can eventually lead to double Holliday junction (dHJ) formation (Liu and West, 2004; Pâques and Haber, 1999). Four-way HJ intermediates represent covalent links between the recombining molecules, and must be severed prior to chromosome segregation to avoid genome instability.

Processing of recombination intermediates: JMs that arise as intermediates of HR are of a broad spectrum, comprising D-loops, single intact or nicked HJs, and dHJs (Pâques and Haber, 1999). Pathways, tightly regulated through the cell cycle, process these JMs, generating two possible outcomes with regard to the separated recombinant duplex molecules: crossover (CO) products involve the reciprocal exchange of flanking markers, and non-crossover (NCOs) products, which differ from the original DNA molecules only by a gene conversion tract in the vicinity of the DSB that has been repaired.

In mitotic cells, NCO outcomes are preferred (Bzymek et al., 2010; Ira et al., 2003), while CO formation, recombination between homeologous sequences, and excessive HR are avoided. Thus, helicases, such as Srs2 and Mph1 function as anti-recombinases to counteract the formation of Rad51 nucleoprotein filaments and D-loops, respectively, limiting HR and favoring NCO repair via SDSA (Ira et al., 2003; Mazón and Symington, 2013; Pfander et al., 2005). HJ resolution is largely mediated by “dissolution”, a pathway catalyzed by the STR complex (BLM complex in human), which comprises the Sgs1

helicase, type I topoisomerase Top3 and the cofactor Rmi1 (Cejka and Kowalczykowski, 2010; Wu and Hickson, 2003). STR promotes convergent branch migration of dHJ intermediates. This generates a hemicatenate that can be removed by Top3, resulting exclusively in NCO products. Alternatively, structure-specific nucleases can resolve HJs, yielding both CO and NCO products (Wechsler et al., 2011; Wyatt et al., 2013). Our initial understanding of HJ resolution came from the studies on *E.coli* HJ resolvase RuvC (West, 1997). The RuvC homodimer introduces symmetrical nicks across the branch point of HJs to produce nicked duplex molecules that can be readily ligated. While RuvC is not found in eukaryotes, multiple conserved resolvases, which follow the RuvC paradigm to varying degrees, have been identified from yeast to human, highlighting the importance of HJ resolution in genome stability (Rass, 2013; Schwartz and Heyer, 2011).

Three eukaryotic structure-specific endonucleases of the HJ resolvase-type have been found: Mus81-Mms4 (MUS81-EME1/EME2 in human), Slx1-Slx4 (Slx1-FANCP), and Yen1 (GEN1) (reviewed in (Wyatt and West, 2014)). These are members of distinct nuclease families, showing no similarity in the primary structure; yet, all of them can cleave JMs resulting from DSB repair. In yeast, disruption of the Mus81-Mms4 or Slx1-Slx4 resolvases causes synthetic lethality in *sgs1* cells. This implies that resolution pathways become essential when HJ dissolution is removed (Fabre et al., 2002; Mullen et al., 2001). However, when resolution and dissolution are available, the STR dissolvasome represents the major pathway of HJ removal. STR is active throughout S phase, whereas the activities of the HJ resolvases are tightly regulated, with activity peaks towards the end of S phase/G2 and in mitosis. This intriguing temporal regulation likely serves to protect branched DNA intermediates present in S phase from unintended cleavage, while redundancy provides a mechanism to ensure that eventually all chromosomal DNA links are severed in time for chromosome segregation (Matos et al., 2013; Sarbajna and West, 2014; Szakal and Branzei, 2013).

1.4.1 HR as a facilitator of DNA replication

In addition to its well-described role in DSB repair, HR plays a central role in the recovery of stalled forks (**Fig. 1-1**). In contrast to the canonical repair of two-ended DSBs, the substrates of HR in replication involve single dsDNA ends or ssDNA gaps (Rass, 2013).

Thus, Rad52-mediated HR reactions facilitate the restart of broken RFs via strand-invasion, allowing replicative DNA synthesis in an origin-independent manner via "break-induced replication" (BIR). Moreover, evidence is accumulating that RF remodeling and reversal is an important means of RF preservation and replication restart (Neelsen and Lopes, 2015). In such cases, structural preservation or remodeling at forks is mediated by DNA helicases and HR factors (Petermann and Helleday, 2010). One model proposes that the reversed end at a remodeled RF may be engaged by the HR machinery and subsequently invade the parental DNA upstream of the fork. Of note, HR factors such as BRCA2 and RAD51 have been implicated in protecting stalled RFs from degradation, allowing replication restart in absence of strand exchange (Neelsen and Lopes, 2015; Schlacher et al., 2011). Finally, lesion-bypass by the replisome can leave ssDNA gaps behind the fork, which can be repaired by HR-dependent post-replicative repair. This is an error-free alternative to translesion synthesis by low-fidelity polymerases, which mediate DNA damage tolerance at the cost of mutations (Branzei and Foiani, 2008; Minca and Kowalski, 2010).

In many of these cases, HR events at forks entail the formation of physically linked intermediates that require resolution prior to segregation. Null mutants of both STR complex components and HJ resolvases were shown to be sensitive to agents that compromise RF progression, while not being particularly sensitive to DSB-inducing ionizing radiation (IR) (Blanco et al., 2010; Interthal and Heyer, 2000; Mullen et al., 2001). Initially a surprise, given the canonical function of the resolvases in DSB repair, this suggests that the critical function of these enzymes in mitotic cells is to process recombination intermediates downstream of HR-associated fork repair/restart pathways.

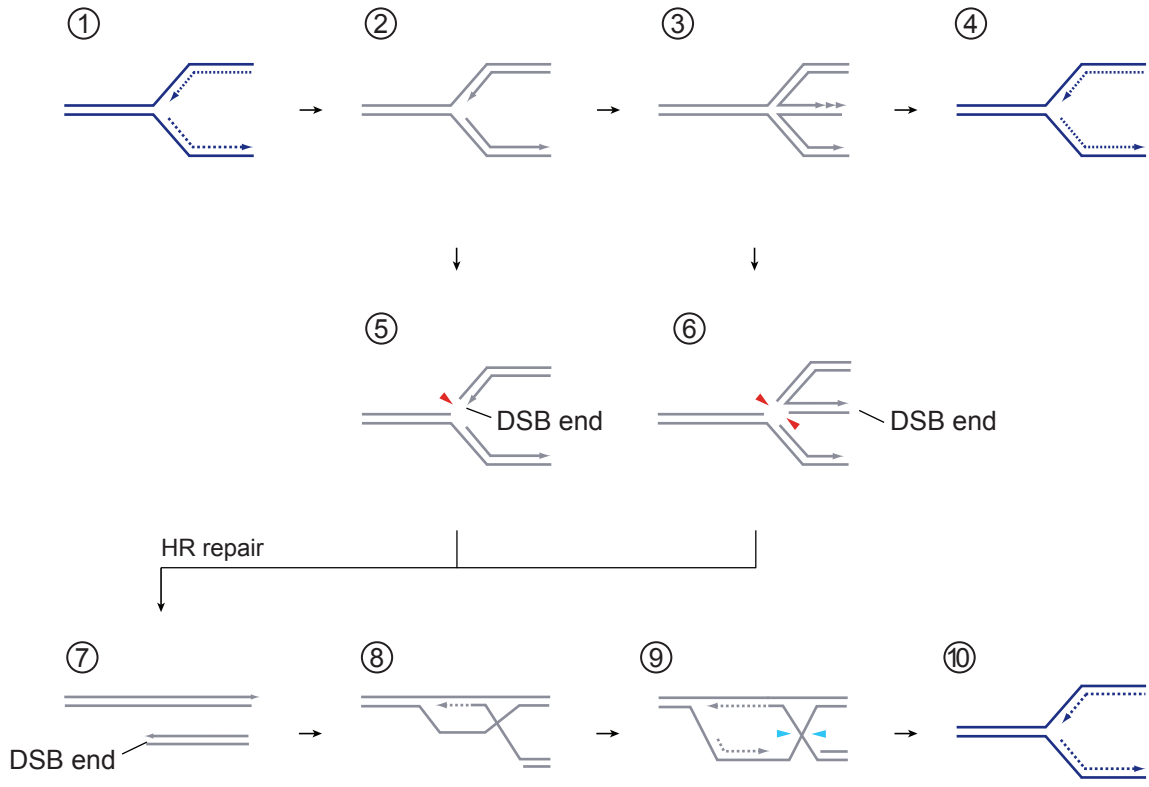

Figure 1-1 Mechanisms of RF recovery. 1, RF progression can stall due to various obstructions (detailed description in the text); 2, leading to inactive forks (coded in gray); 3, Fork regression anneals the nascent strands, forming a "chicken-foot" structure. 4, if the impediments can be removed, HJ branch migration can mediate restart. Alternatively, 5, fork cleavage by structure-specific endonucleases (red arrowhead) on three-way junctions or 6, four-way junctions 7, produce a single-ended DSB. 8-10, Rad52-mediated HR/BIR reactions facilitate strand-invasion, restarting DNA synthesis in an origin-independent manner. Of note, 9, initiation of repair causes the formation of a single HJ, which needs to be resolved before mitosis. Adapted from (Rass, 2013).

RF cleavage: It has long been assumed that dead-end replication intermediates formed upon fork stalling within difficult-to-replicate areas may require resolution by structure-specific endonucleases (**Fig 1-1, 1-2, *5,7-10***). Early evidence from yeast supported a role for the Slx1-Slx4 nuclease in cleaving converged fork intermediates within the rDNA to promote termination in the absence of Sgs1-Top3 (Fricke and Brill, 2003). Human MUS81-EME1 has been repeatedly associated with increased DSB formation upon exposure of cells to drugs, such as DNA polymerase inhibitor aphidicolin, HU, interstrand crosslinking agent mitomycin C (MMC), or oncogene overexpression (Fernandez-Capetillo et al., 2004; Murfuni et al., 2013; Neelsen et al., 2013; Niedernhofer et al., 2004; Wang et al., 2011). MUS81-EME1-mediated DSB formation was shown to correlate, in some cases, with subsequent fork restart and cell survival. Moreover, MUS81-EME1

promotes fragile site expression in human cells, whereas its absence causes anaphase bridges (Naim et al., 2013; Ying et al., 2013). Recently, it has been demonstrated that recruitment of SLX4-associated MUS81-EME1 to CFSs promotes deliberate fork cleavage and late (mitotic) DNA repair synthesis by DNA polymerase POLD3 (Minocherhomji et al., 2015). Analogous functions for Yen1/GEN1 have not been described.

Fork regression: Long stretches of ssDNA at stalled RFs are thought to lead to a re-annealing of the parental strands and annealing of the nascent strands with one another (**Fig 1-1, *1-3***). This causes fork-backtracking, which generates a four-way DNA intermediate resembling HJs, called "chicken-foot" structure (Petermann and Helleday, 2010). Recent work has identified a variety of situations in which fork reversal (and subsequent restart) can be observed in human cells, implying that an older suggestion by Higgins *et al.,* (1976), that fork reversal is a preferred strategy as a mechanism that prevents excessive ssDNA accumulation and ensures efficient DNA damage bypass, by allocating more time for template repair instead of postreplicative repair pathways (Neelsen and Lopes, 2015), may be correct. Besides, fork reversal offers replication restart in a DSB-independent manner, avoiding the risk of illegitimate recombination (Rass, 2013). It is not clear how and if the replisome is preserved at reversed forks. If yes, HJ branch migration offers a simple way for resetting reversed forks and restart DNA synthesis (**Fig 1-1, *3-4***). If the replisome disintegrates, however, HR may offer a way to rebuild a replication bubble by strand invasion (as explained in more detail earlier) (**Fig 1-1, *3, 6-10***). An alternative strategy may be reversed fork protection (involving HR factors such as BRCA2 and RAD51, as mentioned above) to enable fork fusion with an oncoming, active RF.

In *E.coli*, RF regression is an active mechanism mediated by the RecG helicase, stabilizing both stalled and damaged forks (McGlynn and Lloyd, 2002). Even though there is no RecG homolog identified in eukaryotes, several helicases/translocases were shown to catalyze fork regression reactions and HJ branch migration. These include RecQ family helicases BLM and WRN (Machwe et al., 2011; 2006; Ralf et al., 2006), Mph1 (Zheng et al., 2011), Rad54/RAD54 (Bugreev et al., 2006; 2011), Rad5/HLTF (Blastyák et al., 2010; 2007), and SMARCAL1 (Bétous et al., 2012). In yeast, fork reversal has been detected first

in checkpoint-deficient cells (Lopes et al., 2001; Sogo et al., 2002). More recently, fork reversal was detected in wild-type yeast cells treated with a topoisomerase inhibitor, and in replication mutants (Chaudhuri et al., 2012; Fumasoni et al., 2015). Evidence from the human system suggests that fork reversal is benign and part of the cell's replication response (Neelsen and Lopes, 2015). Fork reversal, although mechanistically still unclear, followed by restart is thought to constitute an alternative mechanism to deliberate fork cleavage in coping with replication perturbations. Indeed, MUS81-dependent fork cleavage was shown to functionally compensate for the depletion of WRN helicase in both Werner's syndrome cells and WRN-knockdown HeLa cells. WRN depletion caused spontaneous and HU-induced DSBs, which were largely dependent on MUS81-mediated incisions. Simultaneous depletion of WRN and MUS81 resulted in poor cell survival, suggesting that inhibition of one fork-remodeling pathway promotes use of the other. Furthermore, HR was shown to form a parallel recovery pathway in MUS81-depleted Werner's syndrome cells, as additional depletion of RAD51 caused severe killing after HU treatment (Murfuni et al., 2012; 2013).

1.5 Holliday Junction Resolvase Yen1 – Regulation and Genetic Interactors

Yen1 (GEN1) is a Rad2/XPG family structure-specific nuclease, possessing a bipartite XPG nuclease domain, consisting of an N-terminal (XPG-N) and an internal (XPG-I) motif, a helix-two-turn-helix (HnH) DNA binding domain and nuclear localization signal (NLS) motifs at the C-terminus (Eissler et al., 2014; Ip et al., 2008; Kosugi et al., 2009; Rass et al., 2010). Similar to other members of the XPG family, 5' flaps and RF structures are among the substrates of Yen1/GEN1. Additionally, Yen1/GEN1 cleaves four-way DNA substrates by introducing symmetrical nicks across the junctions, yielding two nicked products ready to be ligated (Rass et al., 2010). By this characteristic pattern of incision, Yen1 and GEN1 are the HJ resolvases in eukaryotes that are biochemically most similar to *E.coli* RuvC (Ip et al., 2008; Rass et al., 2010).

Budding yeast Yen1 nuclease was identified in a biochemical screen for HJ resolving enzymes alongside the other structure-specific endonucleases Mus81-Mms4 and Slx1-Slx4 (Ip et al., 2008). While Yen1 has been shown to contribute to meiotic and mitotic CO formation after DSB formation (Ho et al., 2010; Zakharyevich et al., 2012), defects

associated with loss of *YEN1 in vivo* transpire only in the absence of Mus81-Mms4 (Blanco et al., 2010). Consistently, Yen1 acts redundantly with Mus81-Mms4 in the resolution of toxic HR intermediates generated during the repair of damaged forks in mitotic yeast cells (Blanco et al., 2010; García-Luis et al., 2014; Ho et al., 2010). While *yen1* cells do not exhibit overt sensitivity to RF-stalling drugs, such as HU and MMS, *yen1 mus81* (or *mms4*) double mutant cells exhibit hypersensitivity to a level that far exceeds the sensitivity exhibited by *mus81* (or *mms4*) single mutant cells. The double mutant accumulates G2/M cells with unsegregated DNA when grown in low concentrations of MMS. Furthermore, in the presence of low MMS concentrations, in which cells can enter anaphase, *yen1 mus81* cells form anaphase bridges, leading to increased chromosome missegregation and reduced viability (García-Luis and Machín, 2014). Further deletion of *SLX1* did not exacerbate these defects, suggesting that Mus81-Mms4 and Yen1, but not Slx1-Slx4, jointly promote faithful chromosome segregation in anaphase following HR-mediated repair of fork-associated lesions. Intriguingly, the missegregation defects observed in *yen1 mus81* cells could be reversed when cells were arrested in telophase of the same cell cycle, and either one of the nuclease was then re-expressed (García-Luis and Machín, 2014). This result indicates that either HJ resolvases alone is sufficient to disentangle sister chromatids that are linked by HR intermediates. The overlapping function of Mus81-Mms4 and Yen1 is downstream of Rad52-mediated HR because *RAD52* exhibits epistasis with *MUS81-YEN1* (Blanco et al., 2010; Ho et al., 2010).

During the course of the work presented herein, it has emerged that Yen1 activity is tightly regulated by a series of phosphorylation/dephosphorylation events, constraining the nuclease spatially and biochemically (Blanco et al., 2014; Eissler et al., 2014; García-Luis et al., 2014; Kosugi et al., 2009). Yen1 is a direct target of the S phase CDK, Cdc28-Clb5 complex (Loog and Morgan, 2005; Ubersax et al., 2003). The protein is enriched in nucleus at G1 phase. Upon phosphorylation by CDK, NLS motifs become inaccessible and Msn5-mediated nuclear export of the protein is enhanced (Kosugi et al., 2009). During S and G2/M, Yen1 is thus found in a diffuse state in the cytosol (Blanco et al., 2014; Kosugi et al., 2009; Matos et al., 2011). Subcellular compartmentalization of Yen1 nuclease is in parallel to biochemical inhibition, also mediated by CDK-dependent phosphorylation at multiple sites in the central region of Yen1 (Blanco et al., 2014; Matos et al., 2011). At

anaphase, Cdc14 phosphatase is released from the nucleolus and removes the inhibitory phosphorylation of Yen1, unlocking its nuclease activity and allowing nuclear entry (Blanco et al., 2014; Eissler et al., 2014; García-Luis et al., 2014). Thus, Yen1 is put in a position where it can act on intermediates that have persisted until anaphase onset, providing a last opportunity to ensure faithful chromosome segregation.

Key in understanding Yen1 regulation was the use of unique forms of Yen1, Yen1^{on} and Yen1^{9A}, which were generated by mutating the serine residues at nine CDK consensus sites to alanine (Blanco et al., 2014; Eissler et al., 2014). These versions of Yen1 are refractory to CDK-mediated inhibitory phosphorylation and do not require activation by Cdc14 phosphatase. Thus, Yen1^{on} and Yen1^{9A} are constitutively active and nuclear.

Expression of Yen1^{on} rescued the MMS sensitivity of *mus81* and partially suppressed the defects of *sgs1* single mutants. In addition, premature activation of Yen1 could rescue the synthetic lethality of *sgs1 mus81* double mutant, suggesting that Yen1 is capable of processing structures that are targeted by Mus81-Mms4 and Sgs1 *in vivo* (Blanco et al., 2014). Nevertheless, Yen1^{on} gives rise to DNA damage sensitivity and elevated levels of CO products (causing loss of heterozygosity (LOH)) (Blanco et al., 2014; Eissler et al., 2014). These observations suggest that tight control over Yen1 activity, by means of nucleocytoplasmic shuttling and biochemical inactivation, serves two purposes. First, inhibiting Yen1 in S phase avoids unscheduled and deleterious cleavage of replication intermediates. Secondly, late activation of Yen1 restricts LOH as the nuclease promotes CO formation (Blanco et al., 2014).

Within GEN1, the N-terminal XPG nuclease domains and DNA-binding domain are common with the yeast counterpart (Rass et al., 2010). GEN1 possesses a long-unstructured C-terminal domain carrying eight CDK consensus target sites and a nuclear export signal (NES) (Chan and West, 2014). The protein is subject to phosphorylation by CDK at eight target sites. However, both GEN1^{8A} and *in vitro* dephosphorylated GEN1 displayed wild type levels of HJ resolution activity, indicating that CDK-phosphorylation is not involved in biochemical regulation of the enzyme (Chan and West, 2014; Matos et al., 2011). Conversely, spatial exclusion from the nucleus acts as the main regulator of GEN1 activities, which depends on the NES found at the unstructured C-terminal. During

interphase, GEN1 is enriched in the cytoplasm. The chromatin association of GEN1 is allowed only after nuclear envelope breakdown in prometaphase (Chan and West, 2014; Matos et al., 2011). A constitutively nuclear form of GEN1, GEN1^{nuc}, causes more frequent CO events in cells. In addition, the expression of GEN1^{nuc} could compensate for the combined loss of MUS81 and BLM. However, GEN1^{nuc}-expressing cells were not more sensitive to DNA-damaging agents, unlike yeast expressing Yen1^{on}, reflecting a potential difference in the substrate specificity of yeast Yen1 and human GEN1 (Blanco et al., 2014; Chan and West, 2014).

1.5.1 The Mus81-Mms4 complex

Mus81 and Mms4 are XPF family endonucleases (Ciccia et al., 2008). Despite the ERCC4 nuclease domain found in both, only Mus81 retains its catalytic activity, whereas the ERCC4 domain in Mms4 is more divergent and inactive. The heterodimer can process 3' flaps, RF analogs, D-loops and nicked HJs (Boddy et al., 2001; Ciccia et al., 2003; Constantinou et al., 2002; Kaliraman et al., 2001). Interestingly, fully fledged HJs are poor substrates for this nuclease *in vitro* (Ehmsen and Heyer, 2008; Fricke and Brill, 2003; Osman et al., 2003). In human cells, MUS81-EME1 associates with SLX1-FACNP in a multi-nuclease complex (also containing XPF-ERCC1) that exhibits activity towards covalently closed HJ substrates (Wyatt et al., 2013).

Recent work demonstrated that in both meiotic and mitotic cells, the biochemical activity of Mus81–Mms4 complex is tightly regulated through the cell cycle, as is the case for Yen1 (Matos and West, 2014; Matos et al., 2011). While the nuclease complex shows little activity during G1 and S phase, two cell cycle kinases, Cdc5 and Cdc28/CDK, increasingly phosphorylate Mms4 at G2/M phase, which in turn boost the biochemical activity of the Mus81-Mms4 (Gallo-Fernandez et al., 2012; Matos et al., 2013; Szakal and Branzei, 2013). The late activation of Mus81-Mms4 complex ensures the removal of persistent intermediates that have escaped the attention of the STR complex (Gallo-Fernandez et al., 2012; Matos et al., 2011; Szakal and Branzei, 2013). Nonetheless, Mus81-Mms4 activation has been shown to have multiple layers. Upon Cdc5-mediated phosphorylation at G2/M, Mus81-Mms4 binds to a complex with the scaffold protein Dbp11 and Slx4 nuclease, rendering the nuclease more efficient in the resolution

recombination intermediates (Gritenaite et al., 2014). Moreover, the binding of Mus81-Mms4 to the Dbp11-Slx4 complex is subject to checkpoint control and can be disrupted in response to replicative stress.

1.5.2 Pby1

Pby1 is a putative tubulin tyrosine ligase, which has been implicated with cytoplasmic mRNA processing bodies (P-bodies) (Sweet et al., 2007). The C-terminus of Pby1 contains a nucleotide-binding ATP-grasp domain with homology to tubulin tyrosine ligases (Galperin and Koonin, 1997). Mammalian tubulin tyrosine ligases were shown to modify α tubulin *in vivo* by adding tyrosine residues to their C-terminus. Although the purpose of this modification is unclear, it is thought to be important for microtubule dynamics (Sweet et al., 2007). The protein was shown to co-localize with another P-body protein, Dcp2, to cytoplasmic foci under glucose starvation. In addition, Pby1 physically interacts with two mRNA de-capping proteins, Edc3 and Dcp1, reinforcing the association of the protein with mRNA turnover mechanisms (Gavin et al., 2006). However, loss of Pby1 does not lead to any apparent defects in P-body associated processes such as mRNA decay and mRNA storage (Sweet et al., 2007).

In contrast to the physical interaction data, overwhelming genetic evidence suggests a role of *PBY1* in DNA repair. 53 unique negative interactions have been reported for *PBY1*, mainly from large-scale studies that employed the yeast knock-out collection (Giaever et al., 2002; Winzeler et al., 1999). These interactions include a number of genome stability genes such as *SGS1*, *TOP3*, *RMI1*, *ELG1*, *SLX5*, *SLX8*, *POL32* and *YEN1* (Costanzo et al., 2010; Tong, 2004; Wilmes et al., 2008). Deletion of these genes has been shown to cause defects during DNA replication or in the later stages of HR, indicating that Pby1 might buffer such defects. Consistent with this notion, large-scale studies have reported that *pby1* cells are sensitive to replication blocking agents; such as HU, camptothecin (CPT) and MMS (Parsons et al., 2003; Hartman and Tippery, 2004; Kapitzky et al., 2010; Tkach et al., 2012). Based on the chemical-genetic interaction profile reported for *PBY1*, it has been proposed that Pby1 might mediate important post-translational modifications in the DNA damage response (Parsons et al., 2003; Svilar et al., 2012).

1.5.3 Dna2 nuclease-helicase

Dna2 is an evolutionarily conserved nuclease–helicase, which contributes to genome integrity through multiple DNA metabolism pathways. The gene is essential in yeast and is required for embryonic development in mice (Budd and Campbell, 1995; Lin et al., 2013; Liu et al., 2000; Masuda-Sasa, 2006). To date, there have been numerous studies addressing Dna2, implicating the protein most prominently in Okazaki fragment maturation, DNA end-resection during HR-dependent DSB repair, and, most recently, in a similar end degradation reaction at reversed DNA RFs that is thought to promote replication restart after fork arrest in *S. pombe* and human (Cejka, 2015; Hu et al., 2012; Levikova and Cejka, 2015; Thangavel et al., 2015).

All Dna2 homologs possess a nuclease and a helicase domain, while the unstructured N-terminus found in *S. cerevisiae* (amino acid residues 1 to 499) and *S. pombe* is not conserved in higher eukaryotes (Bae et al., 2001b). The endonuclease domain, which is located in the central region of *S.cerevisiae* DNA2 (amino acid residues 500 to 700), is thought to confer the essential function of Dna2 because complete inactivation of the nuclease leads to cell death (Lee et al., 2000). The nuclease domain of Dna2 displays homology to RecB family nucleases (Aravind et al., 1999). Biochemical data suggests the Dna2 nuclease is a ssDNA endonuclease that cleaves both 5′ and 3′ ssDNA overhangs while showing no catalytic activity for dsDNA, ssRNA or dsRNA substrates (Bae, 2000; Bae et al., 1998). ssDNA binding protein RPA promotes the cleavage of 5′ overhangs and inhibits the 3′ ssDNA cleavage by Dna2 *in vitro* (Cejka et al., 2010; Niu et al., 2010; Zhou et al., 2015). Thus, it is probable that 5′ terminated strands are the relevant Dna2 substrates *in vivo*.

A role for Dna2 in Okazaki fragment maturation. Studies addressing the physical and genetic interaction partners of Dna2 identified several members of the lagging strand synthesis network (Budd and Campbell, 1997; Budd et al., 2005; Formosa and Nittis, 1999). Nascent strand DNA synthesis proceeds continuously on the leading strand, and discontinuously on the lagging strand, due to the 5′ to 3′ directionality of DNA polymerases (reviewed in (Kang et al., 2010)). The polymerase α–primase complex initiates lagging strand synthesis by generating RNA-DNA primers at regular intervals

along the DNA template, which are further extended by polymerase δ into distinct DNA segments called "Okazaki fragments". RNA found at 5′ termini of Okazaki fragments needs to be removed prior to sealing of any remaining nicks by DNA ligase I during Okazaki fragment maturation. Initially, Okazaki fragment maturation was attributed to the cooperative work of Fen1 and RNase HI (encoded by the *RAD27* and *RNH35* genes in yeast, respectively). Nevertheless, *RAD27* and *RNH35* are dispensable for cell viability in *S.cerevisiae*, which predicted that they might not be the only enzymes mediating Okazaki fragment maturation. Intriguingly, Dna2, which in turn is an essential protein, was shown to act on a common substrate with Rad27, namely 5′ tailed ssDNA. Moreover, overexpression of *RAD27* suppressed the temperature sensitivity of Dna2 nuclease-impaired mutants, while *DNA2* overexpression partially suppressed the growth impairment of *rad27*Δ cells. In addition, evidence has been presented for a physical interaction between Dna2 and Rad27, implying that they might cooperate in processing Okazaki fragments (Budd and Campbell, 1997). Further work led to the suggestion of the "two-nuclease model" for Okazaki fragment processing, according to which the length of the 5′ flaps generated through strand displacement by polymerase δ determines the pathway of processing (Bae et al., 2001a). Short flaps are taken care of by Rad27, supported, as suggested by the synthetic lethality of *exo1 rad27* double mutants, by related Rad2 family nuclease Exo1 (exonuclease 1) (Tishkoff et al., 1997). In contrast, longer flaps, which become coated with RPA, are refractory to cleavage by Rad27. Therefore, they require shortening by Dna2, which would dissociate RPA to allow further processing by Rad27 to create ligatable nicks (Bae et al., 2001a). Recent data shows that Dna2 nuclease is capable of cutting RPA-covered 5′ flaps at the base, which would suggest that Dna2 alone can complete Okazaki fragment trimming to allow maturation (Levikova and Cejka, 2015). *In vivo*, Dna2 is not thought to contribute to Okazaki fragment processing to a quantitatively large extent, but the protein may tend to occasional long flaps that have escaped the attention of the Rad27 nuclease activity (Budd et al., 2011).

A role for Dna2 in DSB repair. DSBs can give rise to detrimental chromosomal rearrangements if not properly repaired (Symington et al., 2014). The Rad52-mediated HR pathway in yeast necessitates DNA end-resection to produce 3′ overhangs on which Rad51 can polymerize. Extensive resection is mediated by either Exo1 nuclease or by the

combined action of Dna2 nuclease-helicase and Sgs1 helicase. Of note, and although Dna2 possesses a helicase domain, in DNA end resection only the Dna2 nuclease is required and the helicase activity necessary to separate the strands in order to provide a suitable Dna2 substrate is contributed by Sgs1 (Ira et al., 2004). In parallel, Exo1 functions as a stand-alone resection exonuclease, independently of helicase activities. For Sgs1-Dna2-mediated resection, RPA plays a regulatory role. Sgs1 helicase unwinds double strands, revealing DNA single strands that become coated with RPA. RPA promotes the incision of 5′ terminated ssDNA by Dna2 while hindering the degradation of 3′ terminated ssDNA, thereby enforcing the polarity required for HR-dependent DSB repair (Cejka et al., 2010; Niu et al., 2010). RPA also promotes end resection by human DNA2 nuclease, which cooperates with Bloom's syndrome helicase BLM, the human homolog of Sgs1, showing that the interplay between Sgs1 helicase and Dna2 nuclease is conserved through evolution (Daley et al., 2014; Sturzenegger et al., 2014).

A role for Dna2 in RF recovery. A role of Dna2 nuclease in preventing fork reversal upon RF arrest was suggested in a study that identified *S. pombe* Dna2 as a downstream target of Cds1 (CHK2 homolog) checkpoint kinase (Hu et al., 2012). Phosphorylation of Dna2 by the intra-S phase checkpoint after HU treatment of cells appeared to stabilize the binding of Dna2 to chromatin and prevented the formation of chicken-foot structures. In addition, nuclease-deficient Dna2 mutants exhibited elevated levels of reversed RFs, implying that *S. pombe* Dna2 nuclease acts to recover stalled forks upon intra-S phase checkpoint activation (Hu et al., 2012).

The elusive role of the Dna2 helicase. The conserved helicase motifs in the C-terminus are shared by all Dna2 homologs. DNA2 is a member of the superfamily I helicases with seven characteristic motifs named I, Ia, and II-VI (Budd et al., 1995). Motif I and II are Walker A and B motifs responsible for ATP hydrolysis. Disruption of the conserved ATP binding motif GKT within the Walker A domain in yeast Dna2 abolishes its helicase activity and dramatically reduces cell viability, although it has remained controversial whether the Dna2 helicase represents an essential activity (Budd et al., 1995; Formosa and Nittis, 1999). The Dna2 helicase (amino acid residues 1050 to 1522 in yeast) is greatly stimulated by the presence of DNA with ssDNA overhangs and RPA (Bae, 2000). While

ATPase activity is readily observed, the helicase activity of Dna2 is somewhat cryptic because it is limited by its extremely potent nuclease activity, which quickly deprives the helicase of its substrate (Levikova et al., 2013). Intriguingly, the nuclease and the helicase activity of Dna2 both require 5′ tailed DNA as the substrate to load onto, highlighting the possibility that *in vivo* the two catalytic functions must be finely tuned. To date, there has been no clear functional assignment for the Dna2 helicase, although it has been suggested that it might facilitate Okazaki fragment processing by removing secondary structures from long RNA-DNA flaps. However, it was shown that both Dna2 protein and helicase-dead mutant *dna2 K1080E* are capable of cleaving 5′ flap substrates containing short hairpins when RPA is present, suggesting that in cells RPA likely eliminates the need for the helicase in any potential contribution of Dna2 to Okazaki fragment processing (Bae et al., 2002).

Despite the uncertainty surrounding the physiological role of Dna2 helicase, a study by Formosa and Nittis has described phenotypes that differentiate helicase mutants from mutants with amino acid changes outside the helicase domain, suggesting that the yeast Dna2 endonuclease and helicase might engage in separate roles *in vivo* (Formosa and Nittis, 1999). Thus, point mutations outside the helicase domain of Dna2 tended to cause temperature-sensitivity, while helicase mutants displayed robust growth at temperatures that are non-permissive for non-helicase mutants, but exhibited MMS sensitivity. The MMS sensitivity phenotype was accompanied, without exception, by synthetic lethality with a *CTF4* deletion. In this study, Formosa and Nittis described in some detail a helicase mutant called *dna2-2*, which encodes a R1253Q mutation at an invariant arginine residue in helicase motif IV, providing some insight on the implications of Dna2 helicase impairment. Mutant *dna2-2*, which is able to grow both at low and high temperature (11 °C and 38 °C, respectively), shows features shared to some extent by most of the Dna2 helicase mutants described, namely MMS sensitivity and synthetic lethal interactions with *ctf4*. Cells harboring the *dna2-2* allele proved capable of bulk DNA synthesis but exponentially growing cultures accumulated G2/M stage cells with a 2N DNA content. The paper mentions (as data not shown) that *dna2-2* cell cycle delays were dependent upon the DNA damage checkpoint mediator *RAD9*, whose deletion also suppressed the synthetic lethality of *ctf4 dna2-2* double mutants. Another study provided evidence of

increased fork pausing at the natural RFB within the rDNA of *dna2-2* cells with a concomitant accumulation of converging forks and HJ intermediates (Weitao, 2003). Collectively, these observations imply a role for Dna2 helicase in DNA repair and/or the response to replication problems. Importantly, in chapter 3, we show that the nuclease activity of *dna2 R1253Q* (*dna2-2*) is intact by *in vitro* analysis of the purified protein (Ölmezer et al., manuscript in revision). Moreover, the *dna2-2* allele is sufficient to promote DNA end resection during DSB repair (Zhu et al., 2008). Together, these data suggest that the Dna2 helicase has specific functions in the replication process, as explained in more detail below.

Dna2 as a checkpoint activator. The N-terminus of yeast Dna2 lacks catalytic activity and appears to be unstructured (Bae et al., 2001a). Deletion of N-terminal 405 amino acids (*dna2 405NΔ*) does not interfere with the endonuclease and ATPase/helicase activities of Dna2 *in vitro* (Bae et al., 2001a). Kumar and Burgers demonstrated that two aromatic residues within the Dna2 N-terminal domain help activate checkpoint kinase Mec1 *in vitro* and *in vivo* (Kumar and Burgers, 2013). This supportive function in checkpoint activation is restricted to S phase and redundant with the actions of the 9-1-1 complex and Dbp11. Due to this redundancy, the temperature sensitivity of *dna2 405NΔ* cannot be sufficiently explained by Dna2's checkpoint activation role. Further work suggested that the N-terminus of Dna2 is involved in regulation of Dna2 functions. Dna2 lacking a 45 kDa N-terminal fragment was impaired in RPA binding and therefore lost the stimulatory effect that RPA normally has on the catalytic activity of Dna2 *in vitro* (Bae et al., 2003; 2001a). Interestingly, the temperature sensitive *dna2 405NΔ* allele was rescued by overexpression of *dna2 405NΔ*, Rad27 (Bae et al., 2001b) or all three subunits of RPA (Bae et al., 2003). Altogether, these data highlight the importance of N-terminal region of Dna2 for the interaction with RPA.

Regulation and localization of Dna2. Dna2 is subject to multiple layers of post-translational regulation. Dna2 is a direct target of Cdk1, which controls its nucleocytoplasmic shuttling through phosphorylation (Kosugi et al., 2009). While Dna2 is cytoplasmic-diffuse in G1 phase of the cell cycle, phosphorylation by Cdk1 leads to translocation into the nucleus upon S phase entry, enabling Dna2 to act on nuclear DNA.

In yeast, CDKs coordinate the timely onset of HR events, ensuring that DNA end-resection takes place predominantly during S and G2/M phase, when a homologous template is available in form of the sister chromatid (Aylon et al., 2004; Ira et al., 2004). Promoting the long-range resection role of Dna2, Cdk1 phosphorylates Dna2 at multiple sites, facilitating its recruitment and activity at DSBs (Chen et al., 2011). Then, checkpoint kinase Mec1 further phosphorylates Dna2 but the functional consequences of this event remain to be determined (Chen et al., 2011). In *S. pombe*, Dna2 phosphorylation by the checkpoint effector kinase Cds1 tethers the protein to DNA following HU treatment, a step thought necessary for Dna2 to prevent RF reversal reactions by degradation of the reversed arm (Hu et al., 2012).

In yeast, DNA damage response/repair proteins recruited to the sites of damage can be observed as discrete foci observed by light microscopy (Lisby et al., 2004). Under both unperturbed and HU-induced replication stress conditions, Dna2 forms nuclear foci, mainly during S phase, potentially marking troubled RFs that require the attention of Dna2 (Tkach et al., 2012; Yimit et al., 2015). When cells are treated with phleomycin, a radiomimetic drug that can induce DSBs, S phase Dna2 foci co-localize with Rad52 and other proteins of the Rad52 epistasis group. This would be expected given that Dna2 is involved in end resection during Rad52-mediated HR repair (Cejka, 2015). Dampened expression of Dna2 in yeast, achieved by using a tetracycline-controlled promoter, results in increased levels of spontaneous DNA damage marked by Ddc2 foci, and elevated levels of chromosome arm loss (Cheng et al., 2012). Altogether, this evidence underlines the central role of Dna2 in DNA replication and repair.

Cellular roles of human DNA2. Human DNA2 is localized in both nucleus and mitochondria, contributing to genome integrity in both compartments (Duxin et al., 2012; 2009). Depletion studies of human DNA2 show analogies to yeast in many aspects. Confirming the genome stability role, depletion of DNA2 in human cells causes cell cycle arrest, micronuclei formation and aneuploidy (Duxin et al., 2012). Similar to yeast Dna2 helicase mutants, DNA2-depleted U2OS cells are proficient in bulk DNA synthesis, exhibiting similar replication dynamics as control cells; however, completion of S phase is delayed and there is marked CHK1 phosphorylation. Cells accumulate in G2 phase, prior

to mitosis, with elevated levels of γ-H2AX, RPA foci and phospho-ATM. Similar to *RAD9* deletion in yeast, inhibition of CHK1 in DNA2-depleted U2OS cells leads to better cell cycle progression; however, this results in an increased number of inter-nuclei chromatin bridges. These results were the first evidence in human for genome instability phenotypes caused by depletion of DNA2. Based on several lines of evidences, Duxin *et al.* (Duxin et al., 2012) made the point that these phenotypes are likely not due to telomere dysfunction or an Okazaki fragment processing problem caused by depletion of DNA2. First, depletion of DNA2 did not change RF progression rates, while longer DNA track lengths were observed in cells expressing shFEN1, implying a faster replication progression than in wild type cells. Second, maturation of newly replicated DNA was slowed upon depletion of LIG1 or FEN1, two undisputed Okazaki fragment-processing factors, but not upon depletion of DNA2. More importantly, shDNA2 did not further slow down the maturation of newly replicated DNA in FEN1-depleted cells. Lastly, defects, such as cell cycle arrest and increased γ-H2AX levels, caused by DNA2 knockdown were not observed when FEN1 was knocked down and could not be rescued by ectopic FEN1 expression. This suggests that either DNA2 acts only on a small subset of long flaps generated during Okazaki fragment processing, or they are processed by another nuclease in human. Alternatively, DNA2 could promote completion of DNA replication by assisting resolution of replication intermediates in human, i.e. by serving a role distinct from Okazaki fragment processing.

The precise function of the nuclease and the helicase of human DNA2 remain to be elucidated. Nevertheless, the observation that the aberrant cell cycle profile of U2OS cells expressing shDNA2 could not be rescued by complementation with nuclease-deficient or helicase-deficient DNA2 alone suggests that both activities of human Dna2 are somehow coupled *in vivo* and are both required to maintain genome stability (Duxin et al., 2012).

Recent reports suggest that fork reversal in metazoans is a more frequent (or at least more obvious) event than in yeast, occurring extensively both during unperturbed S phase and as a response to various genotoxic treatments (Berti et al., 2013; Chaudhuri et al., 2012; Zellweger et al., 2015). Similar to the fission yeast homolog, human DNA2 nuclease was also shown to respond to the regression of RFs, driving replication restart

(Thangavel et al., 2015). For this function, the DNA2 nuclease – independently of the helicase – cooperates with WRN helicase, promoting genome stability under unperturbed conditions or prolonged replication stress conditions caused by HU, CPT and MMC (Berti et al., 2013).

In human, RAD51 plays an important role in protection and restart of stalled forks (Costanzo, 2011; Schlacher et al., 2011). RAD51 loading onto RPA-coated ssDNA at perturbed forks prevents deleterious nucleolytic degradation and primes forks for HR pathway-dependent repair. MRE11, EXO1 and DNA2 are the main nucleases identified to date to exert controlled resection at stalled RFs. A recent report showed that a novel factor called BOD1L stabilizes RAD51 at troubled forks to block DNA2-dependent over-resection (Higgs et al., 2015), a role that might be shared with FANCD2 in repair of inter-strand crosslinks (Karanja et al., 2014). Moreover, a dominant negative RAD51 mutant cell line, which is proficient in HR but defective in ICL repair was shown to suffer from extensive DNA degradation by DNA2 and WRN (Wang et al., 2015), indicating the involvement of human DNA2 nuclease in fork recovery pathways.

In order to maintain genome integrity, *DNA2* appears to promote multiple processes in collaboration with distinct sets of proteins and by putting to use its nuclease and/or helicase domains. Control over Dna2 is mediated by the cell cycle and checkpoint kinases. Recent findings suggest that the essential functions of Dna2 are conserved in evolution. *DNA2* is frequently upregulated in cancer and mutations have been found in human genetic disorders (Dominguez-Valentin et al., 2013; Shaheen et al., 2014; Strauss et al., 2014). Further studies on *DNA2* will not only illuminate the key metabolic pathways for DNA replication and repair, but will also help in understanding how replication stress responses are linked with human disease.

1.6 Rationale for Thesis

In this thesis I describe my work to uncover the *in vivo* functions of HJ resolvase Yen1 using the model organism *S. cerevisiae*. HJ resolution is a key aspect of the replication stress response, and the conserved factors that mediate it contribute to genome stability.

When I embarked on my studies, Yen1 was described as a "backup" nuclease, processing recombination intermediates that escape Mus81-Mms4 (Blanco et al., 2010; Ho et al., 2010). We challenged the notion that Yen1 is a "backup" for Mus81-Mms4, hypothesizing that unique functions of Yen1 exist, which so far had remained unidentified. Such unique functions, we surmised, may shed new light on the cell's replication stress response, and help explain the evolutionary conservation of Yen1.

YEN1 is characterized by a relatively small set of genetic interaction partners derived from high-throughput studies (listed at http://www.yeastgenome.org/). Moreover, most of these interactions only transpire in the absence of *MUS81-MMS4* (Agmon et al., 2011; Blanco et al., 2010; Ho et al., 2010). Two genetic interactors of *YEN1*, namely *PBY1* and *DNA2*, were described to be apparent in the presence of *MUS81-MMS4* (Budd et al., 2005; Tkach et al., 2012). With the goal of finding a unique role for Yen1, we focused on a close examination of these two genetic interactions.

My research, documented in this thesis, demonstrates that Yen1 uniquely acts on replication intermediates emerging in Dna2 helicase-defective cells, revealing critical functions for both enzymes in the replication stress response. Our findings resonate with studies that have emerged during the course of my work. For example, detailed descriptions of the cell cycle regulation of Yen1 and Mus81-Mms4 have been published (reviewed in (Blanco and Matos, 2015)). These studies relate to, and helped explain, the involvement of the G2/M checkpoint that we find in the Dna2-Yen1 axis (chapter 3). Furthermore, the Dna2 nuclease activity was implicated in a fork recovery pathway, both in *S. pombe* and human, corroborating that Dna2 is a prominent player during replication stress (Berti et al., 2013; Hu et al., 2012; Thangavel et al., 2015), as also suggested herein with regard to the Dna2 helicase activity. More generally, the concept of fork recovery is evolving fast. Older concepts considering certain DNA structures as pathologic are being challenged by new evidence showing that RF reversal is a benign intermediate step in fork restart (Neelsen and Lopes, 2015). In this context, nucleases seem to play a broader role than preciously assumed. New studies demonstrate that the structure-specific nuclease MUS81-EME1/EME2 can resolve replication intermediates to promote recovery (Minocherhomji et al., 2015; Murfuni et al., 2012; 2013). We provide evidence that this

more flexible, context-dependent use of nucleases extends to Yen1. A view is emerging, that cells employ a complex network of helicases and nucleases to promote RF recovery and faithful chromosome segregation. The two-tiered response involving the Dna2 helicase activity and HJ resolvase Yen1 reveals a new aspect within this important genome integrity network.

2 Chapter 2: DNA repair defects ascribed to *pby1* are caused by disruption of Holliday junction resolvase Mus81 – Mms4

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Brief communication

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ABSTRACT

PBY1 continues to be linked with DNA repair through functional genomics studies in yeast. Using the yeast knockout (YKO) strain collection, high-throughput genetic interaction screens have identified a large set of negative interactions between *PBY1* and genes involved in genome stability. In drug sensitivity screens, the YKO collection *pby1*Δ strain exhibits a sensitivity profile typical for genes involved in DNA replication and repair. We show that these findings are not related to loss of Pby1. On the basis of genetic interaction profile similarity, we pinpoint disruption of Holliday junction resolvase Mus81-Mms4 as the mutation responsible for DNA repair phenotypes currently ascribed to *pby1*. The finding that Pby1 is not a DNA repair factor reconciles discrepancies in the data available for *PBY1*, and indirectly supports a role for Pby1 in mRNA metabolism. Data that has been collected using the YKO collection *pby1*Δ strain confirms and expands the chemical-genetic interactome of *MUS81-MMS4*.

1. Introduction

The *Saccharomyces* genome deletion project has constructed a collection of single mutant yeast strains by replacing each gene with a kanamycin resistance marker (*KanMX*) [1]. This unique genetic tool has been used extensively for genome-scale analyses, providing an important source of functional data for all ~6000 genes in budding yeast. By converting single mutant strains into collections of haploid double mutant strains, genetic interactions have been studied in a systematic and quantitative manner [2]. Synthetic sick and synthetic lethal (SSL) interactions describe cases in which the fitness of the double mutant is more severely reduced than would be expected from the individual single mutant phenotypes. This type of genetic interaction serves as a strong indication that two gene products promote related, compensatory pathways within the same biological process, providing important clues as to the *in vivo* function of gene products [2–5].

The available genetic data on *PBY1*/*YBR094W* strongly suggest that Pby1 may function in DNA repair. The *Saccharomyces* Genome Database (SGD) [6] currently lists 53 SSL and conditional negative interactions from 15 different studies [7–21], predominantly with genes involved in genome stability (Table S1). "Cellular response to DNA damage" stimulus and "DNA repair" are the most significantly overrepresented biological process ontology terms among the 53 negative genetic interactors (for a full list of biological processes and the associated genes see Table S2). Moreover, the genetic interaction profile, i.e., the sum of genetic interactions determined for *PBY1*, shows a considerable overlap with profiles of genes with known functions in DNA repair and replication. Finally, the YKO collection *pby1*Δ strain has repeatedly been shown to exhibit hypersensitivity to agents that induce DNA damage and replication stress, including DNA alkylating agent methyl methanesulfonate (MMS), ribonucleotide reductase inhibitor hydroxyurea (HU), and topoisomerase I poison camptothecin (CPT) [17,22–30]. In light of the chemical-genetic interaction pattern, it has been hypothesized that Pby1, which contains a nucleotide-binding ATP grasp domain with homology to tubulin-tyrosine ligase [31], might be important for an as yet unknown protein modification mechanism in the cellular response to DNA damage [21,23]. However, a potential function of Pby1 in DNA repair has not been addressed directly.

In stark contrast to these considerations, Pby1-GFP fusion proteins have been shown to localize to cytoplasmic processing (P) bodies [26,32], which represent sites of mRNA storage, translational suppression, and mRNA decay [33]. Furthermore, Pby1 has been co-purified with mRNA de-capping proteins Edc3 and Dcp1 [34–37], but a specific function of Pby1 in mRNA turnover could not be demonstrated [32].

Abbreviations: YKO, yeast knockout; P body, cytoplasmic processing body; SSL, synthetic sick/synthetic lethal; SGD, *Saccharomyces* Genome Database; MMS, methyl methanesulfonate; HU, hydroxyurea; CPT, camptothecin; DRYGIN, Data Repository of Yeast Genetic Interactions.

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1568-7864/

Here, we show that *PBY1* has been erroneously associated with DNA repair and discuss the implications for Pby1 and the Holliday junction resolvase Mus81-Mms4, whose disruption we find to underlie DNA repair phenotypes currently thought to relate to *PBY1*.

2. Results and discussion

2.1. DNA repair phenotypes ascribed to pby1 mutants are not related to disruption of PBY1

To investigate a potential function of Pby1 in DNA repair, we confirmed that the YKO collection *pby1*Δ strain exhibits sensitivity to DNA damage and replication stress induced by MMS, CPT, or HU (Fig. 1A). In parallel, we independently replaced *PBY1* with a *URA3* deletion construct in the same strain background. To our surprise, this second *pby1*Δ isolate was no more sensitive to MMS, CPT, and HU than the parental wild-type strain (Fig. 1A). The YKO collection *pby1*Δ strain proved resistant to Geneticin (data not shown), indicating that the *KanMX* marker gene had been inserted into the genome. However, a diagnostic PCR reaction for *PBY1* yielded a product (~2.3 kb) that matched the *PBY1* gene rather than *KanMX* (~1.6 kb expected), while replacement of *PBY1* with *URA3* was confirmed (~1.6 kb product) (Fig. 1B). This indicated that the *PBY1* locus is intact in the *pby1*Δ strain from the YKO collection, and suggested that its sensitivity to DNA damage was due to an acquired mutation or insertion of *KanMX* at a DNA damage sensitivity locus somewhere else in the genome.

2.2. Genetic interaction profile similarity identifies disruption of MMS4 as the relevant mutation for phenotypes ascribed to pby1

We reasoned that the genetic interaction profile now attributed to *PBY1* should reflect the unidentified DNA damage sensitivity locus apparently disrupted in the YKO collection *pby1*Δ strain. To pinpoint the locus, we queried the Interactome Database (http://interactome-cmp.ucsf.edu/) for genetic interaction profile similarity with *PBY1*. Searching a dataset enriched for genes involved in DNA replication, repair, and chromosome segregation [12], identified *MUS81* and *MMS4* as the two genes that most closely resembled *PBY1*. A reciprocal search identified *PBY1* among the top five genes with a profile similar to those of *MUS81* and *MMS4* (Fig. 1C). Searching the Data Repository of Yeast Genetic interactions (DRYGIN; http://drygin.ccbr.utoronto.ca/index.html) [38] produced a similar result: *PBY1* correlated most closely with *YBR100W* and *YBR099C* (data not shown), two dubious ORFs which overlap with the 3′-end of *MMS4* (see Fig. 2).

MUS81 and *MMS4* encode the two subunits of the Mus81-Mms4 Holliday junction resolvase, which plays a key role in replication-associated DNA repair reactions and homologous recombination [39]. To test directly whether the genetic and drug-hypersensitivity profiles reported for *pby1* might be due to *KanMX* insertion within *MMS4* or *MUS81*, we performed diagnostic PCR. As shown in Fig. 1B, the YKO collection *pby1*Δ strain yielded an aberrantly large PCR product for the *MMS4* locus (~3.5 kb rather than ~2.1 kb), while the *MUS81* locus was intact (~1.8 kb PCR product).

Disruption of *MUS81* or *MMS4* causes well-documented defects in the response to replication blocking agents MMS, CPT, and HU [40,41]. We found that the level of sensitivity to these drugs exhibited by the YKO collection *pby1*Δ strain closely resembled that of strains lacking *MUS81* or *MMS4* (Fig. 1A).

To further verify that the aberrant *MMS4* locus that we detected within the commercially available YKO *pby1*Δ strain, rather than a deletion of *PBY1*, is responsible for the negative genetic interactions that have been reported for *pby1*, we tested one such interaction, with *yen1* [8], in exemplary fashion. Disruption of *YEN1* is known to cause synthetic growth defects in *mus81/mms4* mutants in unperturbed and replication stress conditions [42,43], as Yen1 and Mus81-Mms4 serve overlapping functions in the resolution of Holliday junctions [39]. In contrast, we found that the combined disruption of *YEN1* and *PBY1* does not lead to any overt SSL phenotypes. Doubling times for *pby1*Δ, *yen1*Δ, and *pby1*Δ *yen1*Δ cells were similar to wild-type (±5 min), and *pby1*Δ *yen1*Δ cells did not exhibit conditional SSL interactions in the presence of HU, CPT, or MMS (data not shown).

Collectively, these observations are consistent with the notion that all genetic interactions and DNA damage sensitivities that have been derived using YKO collection *pby1*Δ and derivate strains are in fact due to disruption of *MMS4* that results in dysfunction of the Mus81-Mms4 Holliday junction resolvase.

2.3. The YKO pby1Δ strain is a ybr099cΔ strain with a deletion/insertion mutation in MMS4

DNA sequencing analysis of the *MMS4* locus within the YKO *pby1*Δ strain revealed a *KanMX* insertion with the opposite orientation (Fig. 2). Furthermore, the locus showed a 378 nt deletion that coincides with *YBR099C*, the *MMS4*-overlapping ORF that had been identified by DRYGIN on the basis of genetic profile similarity (see above). This suggested a targeted deletion of *YBR099C* rather than an inadvertent insertion of *KanMX* into the *MMS4* locus. Indeed, when we retrieved the deletion primers designed for *YBR099C* from the *Saccharomyces* genome deletion project website (www-sequence.stanford.edu/group/yeast_deletion_project/strain_a_mating_type.txt), we found that the barcode tag (a sequence unique for each individual deletion) matched our sequencing results for the *MMS4-KanMX* borders. We conclude that the YKO collection *pby1*Δ strain has been misannotated, and is in fact a perfectly constructed *ybr099c*Δ strain, in which disruption of *MMS4* is the relevant mutation.

2.4. Revision of available PBY1 data supports a role for Pby1 in mRNA metabolism

The misannotation of the *mms4* mutant YKO collection *ybr099c*Δ strain as *pby1*Δ has led to the erroneous association of *MMS4*-related data with *PBY1*. Firstly, deletion of *PBY1* does not cause sensitivity to DNA damage or replication stress inducing agents (Fig. 1A). Observations of sensitivity to genotoxins of presumed *pby1*Δ cells [17,22–30] relate to disruption of *MMS4*, and reflect the role of the Mus81-Mms4 resolvase in DNA repair. Secondly, most, but not all, genetic interactions documented for *PBY1* are related to *MMS4*. An overview of reported negative *PBY1* interactions is presented in Table 1. A review of the literature shows that 14 out of 15 studies [7–21] have identified negative genetic interactions on the basis of the YKO collection *pby1*Δ strain or derivatives thereof, i.e., in the presence of wild-type *PBY1* and a disrupted *MMS4* gene. Consistently, more than half of the genes identified in these studies have also been found as negative interactors of *MMS4* and/or *MUS81* (Table 1 and Table S1). In contrast, Wilmes et al. [11] have identified 11 SSL interactions for *PBY1*, and only one of them has previously been reported for *MUS81-MMS4*. This study has used an independently generated *pby1*Δ query strain, and hits that have been scored exclusively by Wilmes et al. are therefore likely to represent *bona fide PBY1* genetic interactions. Although the genes interrogated by this study were enriched for RNA processing factors, it is interesting that several genes involved in mRNA decay and translation exhibited SSL interactions with *PBY1* (Table 1). Thus, by revealing that most genetic interactions currently annotated to *PBY1* are false positives, we remove the misleading link between Pby1 and DNA repair. By homing in on high confidence *PBY1* inter-

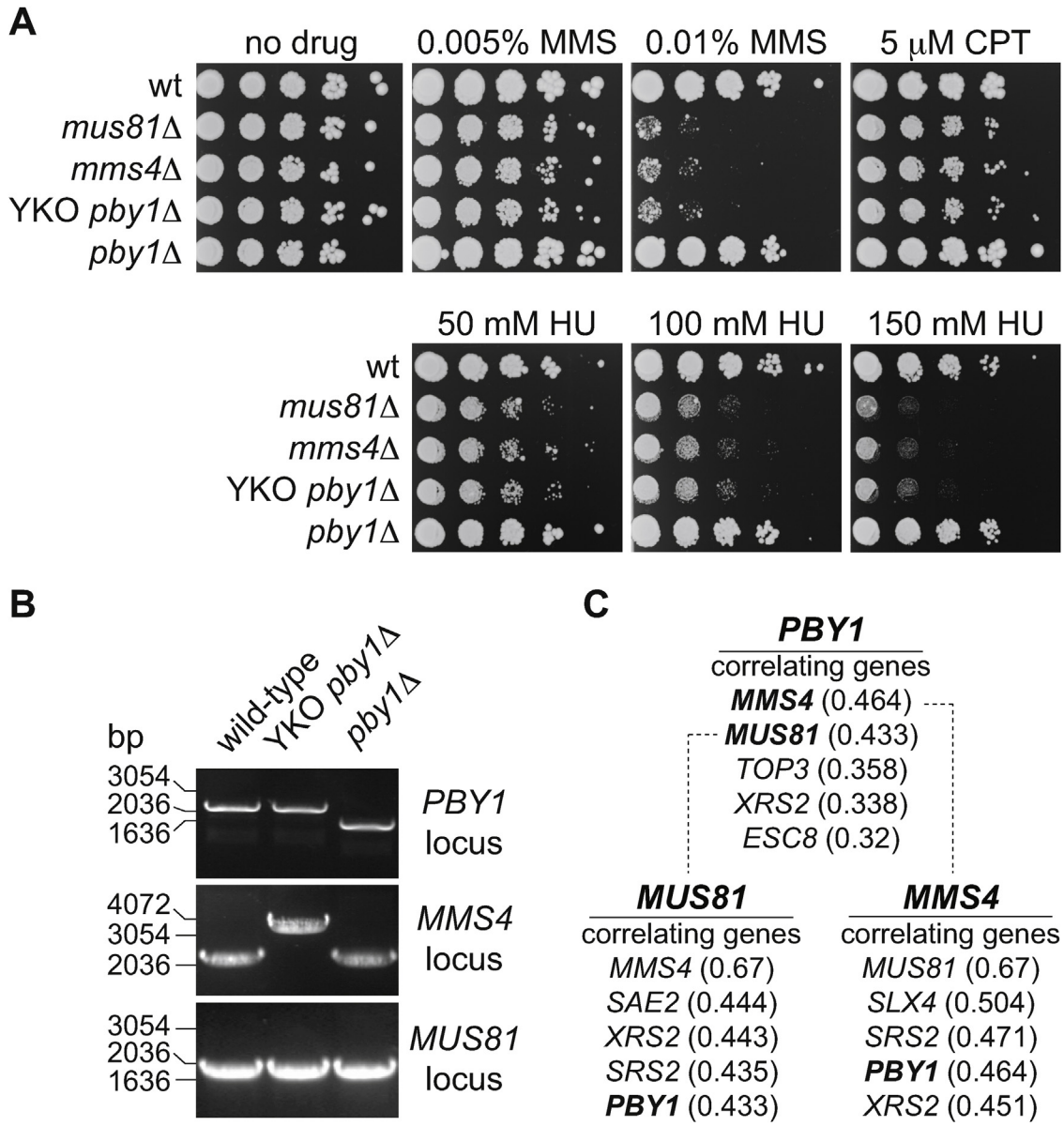

Fig. 1. Disruption of the Holliday junction resolvase *MUS81-MMS4* and not *PBY1* causes the DNA damage sensitivity of the YKO collection *pby1*Δ strain. (A) The indicated strains were grown to mid-log-phase, normalized and spotted in 10-fold serial dilutions onto YPAD plates containing the indicated amounts of MMS, CPT, or HU. (B) Analysis of the *PBY1*, *MMS4*, and *MUS81* genomic loci by diagnostic PCR. Primers were designed to anneal within 100 bp up- and downstream of the respective genomic locus. PCR products were resolved by agarose gel electrophoresis. The positions and sizes of marker DNA fragments are indicated. (C) Similarities between the genetic interaction profiles of *PBY1* and *MUS81*/*MMS4*. The Krogan Laboratory Interactome Database was queried for genes with similarities to *PBY1*, *MUS81*, or *MMS4*, and the top five correlating genes are listed for each with correlation coefficients in brackets.

actors, we indirectly provide support for a role of Pby1 in mRNA metabolism, which is in line with the protein's physical connection to P bodies [12,26,32,34–37].

2.5. Data gathered using the YKO collection pby1Δ strain contains novel genetic interactions of the MUS81-MMS4 Holliday junction resolvase

As shown in Table 1, several genes implicated in genome stability, whose deletion caused synthetic defects in combination with *pby1*Δ, have not been found previously to interact genetically with *MMS4* and/or *MUS81*. These genes represent strong candidates for novel SSL interactions of the *MUS81-MMS4* Holliday junction resolvase. They include DNA glycosylase *MAG1* [44,45], helicase *HRQ1*, homologous to disease-linked RECQ4 [46–48], negative regulator of homologous recombination *SLX5* [49,50], and INO80 chromatin remodeling complex subunit *IES2* [51,52]. We directly addressed one of these candidates, *IES2*. The interaction between *ies2*Δ and *pby1*Δ (using the YKO *pby1*Δ strain) has been detected on the basis of a moderate but significant synthetic decrease in colony size for the double mutant [13]. Consistently, we found that combinatorial deletions of either subunit of the *MUS81-MMS4* Holliday junction resolvase and *IES2* led to a reduction in mean colony size of ~25% compared to wild-type and the respective single mutants (Fig. 3A). Moreover, both *mus81*Δ *ies2*Δ and *mms4*Δ *ies2*Δ double mutant cells exhibited moderate and strong SSL interactions in the presence of MMS and HU, respectively; no synthetic hypersensitivity was found upon exposure to CPT (Fig. 3B). Simi-

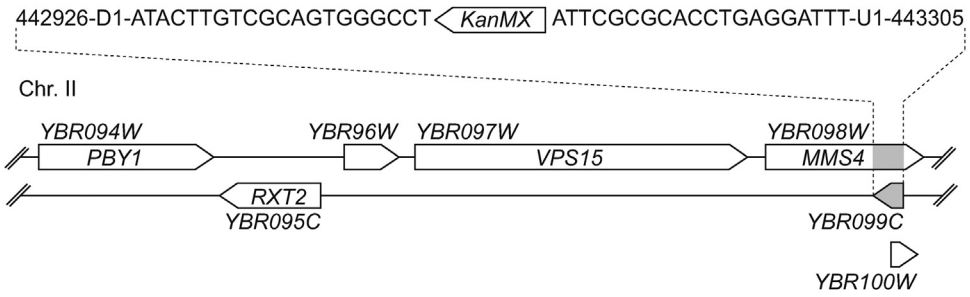

Fig. 2. Schematic depiction of a region of chromosome II spanning from the 5′-end of *PBY1/YBR094W* on the Watson strand (coordinate 432036) to the 3′-end of *MMS4/YBR098W* (coordinate 443590), as found in the YKO collection *pby1*Δ strain. The *PBY1* locus is intact, while the *KanMX* deletion module has replaced nearby dubious ORF *YBR099C* on the Crick strand. This leads to disruption of *MMS4* on the Watson strand (deleted region in grey). DNA sequencing showed that the *KanMX* insertion is flanked by the sequences designed by the genome deletion project consortium for the deletion of *YBR099C*, including generic D1 and U1 sequences [60] and a unique barcode (sequence given in full), indicating that the *pby1*Δ strain is misannotated and should be labeled *ybr099c*Δ. *YBR100W* is no longer classified by SGD as an independent ORF and has been merged with *MMS4*.

Table 1
Overview of reported SSL interactions for *PBY1*.

Study reporting *PBY1* SSL interactions	Number of *PBY1* SSL interactions	Overlap with *MUS81/MMS4*[a]		Non-overlapping with *MUS81/MMS4*[b]	
Tong et al., 2004 [8]	19	14	(73.7%)	5	
Collins et al., 2007 [12]	17	10	(58.7%)	7	(*SLX5*)*
Costanzo et al., 2010 [13]	11	7	(63.6%)	4	(*IES2*)*
Pan et al., 2006 [18]	3	3		0	
Leung et al., 2014 [17]	3	2		1	(*HRQ1*)*
Dixon et al., 2008 [14]	2	2		0	
Beltrao et al., 2009 [10]	2	0		2	
Tong et al., 2001 [7]	1	1		0	
Ballaoui et al., 2003 [9]	1	1	(60.6%)	0	
Huang et al., 2005 [16]	1	1		0	
Hanna et al., 2007 [15]	1	1		0	
Liu et al., 2010 [19]	1	0		1	
Sharifpoor et al., 2012 [20]	1	0		1	
Svilaret al., 2012 [21]	1	0		1	(*MAG1*)*
Wilmes et al., 2008 [11]	11	1	(9.1%)	10	(*DCS1*, *STO1*, *TIF35*, *TRM7*, *MEF1*, *MSK1*, *TMA23*)**

[a] Specifies the subset of *PBY1* SSL interactions listed as SSL interactions of *MUS81* and/or *MMS4* by SGD. Percent overlap for a given study or group of studies (those reporting ≤3 SSL interactions) is given in brackets.
[b] Specifies the subset of *PBY1* SSL interactions not listed as SSL interactions of *MUS81* and/or *MMS4* by SGD. Genes in brackets are involved in genome stability (*) or mRNA decay and translation (**) and represent candidate interactors of *MUS81/MMS4* and *PBY1*, respectively.

lar observations were made after deletion of *IES2* within the YKO *pby1*Δ strain, consistent with the notion that partial disruption of *MMS4* caused by deletion of *YBR099C* confers a *mus81/mms4* null phenotype (data not shown). The drug-dependent SSL interactions are in good agreement with the sensitivity pattern of *ino80* mutant cells, which are affected by MMS and HU, but – in contrast to *mus81* or *mms4* mutants – not by CPT [53,54]. They suggest that Mus81-Mms4 acts in non-overlapping fashion with Ies2 in the repair of broken replication forks after replication run-off at CPT-induced DNA single-stranded breaks, while Ies2 and Mus81-Mms4 promote compensatory pathways in the recovery and repair of stalled replication forks. This is consistent with an increase in foci formed by homologous recombination proteins in *ino80* mutant cells after HU treatment [55], and the role of Mus81-Mms4 in the resolution of late recombination intermediates [39]. This example demonstrates that the data currently ascribed to *PBY1* holds relevant new information on the interactome and function of *MUS81-MMS4*.

It is noteworthy that a designated *YBR099C* deletion exists in yeast strain collections and has been used in high-throughput genetic interaction studies. Having established that deletion of *YBR099C* in the YKO *pby1*Δ strain causes a *mus81/mms4* null phenotype, data collected with *ybr099c* mutants can serve as an additional source to enrich the *MUS81-MMS4* interactome. *YBR099C* SSL interactions are not curated by SGD, but a DRYGIN search returned 31 negative genetic interactions, 24 of which (77.4%) match and confirm known *MUS81* and/or *MMS4* SSL interactors, while 7 are not currently associated with *MUS81-MMS4* (Table S3).

3. Materials and methods

3.1. Yeast strains

Saccharomyces cerevisiae wild-type strain BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) [56] and BY4741-derived YKO collection deletion strains for *PBY1* (*pby1*Δ::*KanMX4*; clone ID 3233; desig-

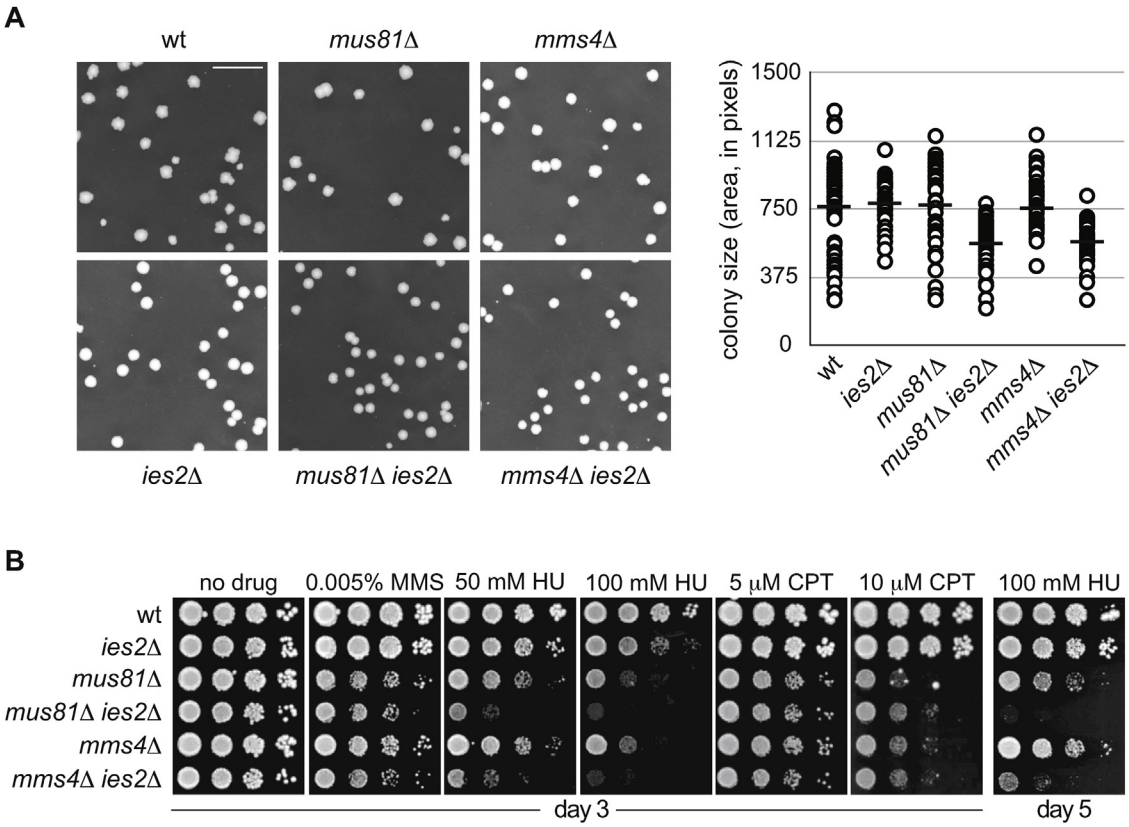

Fig. 3. SSL interactions between the Holliday junction resolvase *MUS81-MMS4* and the INO80 complex subunit *IES2*. (A) Representative images (scale bar: 1 cm) and quantitation of colony size variation among the indicated strains grown for 3 days on YPAD medium. Black circles represent individual colony size measurements ($n \geq 45$), horizontal black lines the mean colony size for each strain. (B) The indicated strains were grown to mid-log-phase, normalized and spotted in 10-fold serial dilutions onto YPAD plates and YPAD plates containing the indicated amounts of MMS, HU, or CPT.

nated as "YKO *pby1*Δ" in this study), *MMS4* (*mms4*Δ::*KanMX4*; clone ID 3237), and *YEN1* (*yen1*Δ::*KanMX4*; clone ID 174), were purchased from Open Biosystems/GE Healthcare. Strains YRL65 (*pby1*Δ::*URA3*), YRL66 (*pby1*Δ::*URA3 yen1*Δ::*KanMX4*), YRL221 (*mus81*Δ::*HIS3 ies2*Δ::*URA3*), YRL222 (*mms4*Δ::*KanMX4 ies2*Δ::*URA3*), YWL169 (*mus81*Δ::*HIS3*) and YWL170 (*yen1*Δ::*KanMX4 mus81*Δ::*HIS3)* [42] were derived from BY4741 using standard methods [57,58]. Diagnostic PCR reactions shown in Fig. 1B were done with the following DNA primers: *PBY1*, 5′-CGCTCTGACTTGATCTCT and 5′-CAGTATTTTAGGATGGGTTC; *MMS4*, 5′-GCGTTGAAGTACCCTTTT and 5′- AAGGAGGGGGAAATAGAG; *MUS81*, 5′-CAAAGGATTGATACGAACAC and 5′-GCAGGGATGACTATATTTC.

3.2. Assessment of growth and drug sensitivity

Doubling times were determined as described [42]. For drop assays, cells grown to mid-log phase were normalized to 10^7 cells/ml, and 2 μl drops of 10-fold serial dilutions were spotted onto YPAD plates, or YPAD plates containing different concentrations of MMS, HU, or CPT. Unless stated otherwise, plates were incubated for 3 days at 30 °C before imaging. Colony size variation was documented using a Canon EOS 550D camera with a Canon macro lens EF-S 60 mm and quantified using CellProfiler software (www.cellprofiler.org) [59].

3.3. PBY1 interaction data and online tools for data analysis

Genetic interaction data for *PBY1*, *MMS4*, and *MUS81* was retrieved from SGD. The SGD Gene Ontology Slim Mapper tool (http://yeastgenome.org/cgi-bin/GO/goSlimMapper.pl) was used to assign biological process terms to negative genetic interactors of *PBY1*. The Krogan Laboratory Interactome Database (University of California, San Francisco) and DRYGIN (Laboratory of C. Boone, University of Toronto) are freely available online tools.

4. Conclusion

We demonstrate that *PBY1* is not a novel DNA repair gene and disambiguate its database record. Phenotypes associated with the YKO collection *pby1*Δ strain are in fact related to deletion of *YBR099C* and the resulting disruption of the Holliday junction resolvase gene *MMS4*. DNA damage sensitivity and genetic interaction data collected with the presumed *pby1*Δ strain from the YKO collection resembles, strengthens, and expands the available data for *MUS81-MMS4*.

The strategy we use to pinpoint the causal mutation for purported *pby1* interactions and phenotypes is based on genetic interaction profile similarity. It is generally applicable to troubleshoot "bad strains" within the widely used YKO strain collection, in which an unidentified mutation may lead to the accumulation of misleading data and discrepancies in the database records.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2015.05.006

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3 Chapter 3: Replication intermediates that escape Dna2 activity are processed by Holliday junction resolvase Yen1

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Author Contributions:

G.Ö. and U.R. planned and analyzed the experiments. G.Ö. performed the experiments with help from D.K. B.F. supported the two-dimensional gel analyses, and G.F. the microscopic work. M.L. and P.C. purified and analyzed Dna2 *in vitro*. U.R. wrote the paper.

Abstract

Cells have evolved mechanisms to protect, restart, and repair perturbed replication forks, allowing full genome duplication, even under replication stress conditions. Interrogating the interplay between nuclease-helicase Dna2 and Holliday junction (HJ) resolvase Yen1, we find the Dna2 helicase activity acts parallel to homologous recombination (HR) in promoting DNA replication and chromosome detachment upon replication fork stalling. Yen1, but not the HJ resolvases Slx1-Slx4 and Mus81-Mms4, safeguards viable chromosome segregation by removing replication intermediates that escape Dna2 activity. Post-replicative DNA damage checkpoint activation in Dna2 helicase-defective cells causes terminal G2/M arrest by precluding repair by Yen1, whose activation requires entry into anaphase. These findings explain the exquisite replication stress sensitivity of Dna2 helicase-defective cells, and identify a non-canonical role for Yen1, distinct from HJ resolution, in the processing of replication intermediates. The involvement of Dna2 helicase activity in completing replication may have implications for *DNA2*-associated pathologies, including cancer and Seckel syndrome.

Duplication of the genome requires the passage of DNA replication forks along the entire length of every chromosome. If segments of DNA remain unreplicated, physical links between the nascent sister chromatids persist, which can lead to aberrant chromosome segregation[1]. Replication fork collapse, characterized by replisome inactivation and DNA breakage, induces recombinogenic DNA lesions and gross chromosomal instability[2]. Consistently, replication stress, which increases the risk of replication fork stalling, arrest, and collapse, has been recognized as a driver in cancerogenesis[3]. Cells respond to replication stress by activating the S phase checkpoint, which triggers a cascade of downstream events aimed at preserving the replication machinery at troubled replication forks until DNA synthesis can resume[4]. Replication restart also involves fork remodeling, nucleolytic processing of stalled replication intermediates, and HR reactions[5-7]. Thus,

full genome duplication and proper chromosome segregation is dependent upon a complicated network of replication and repair proteins that remains incompletely understood.

A protein implicated in multiple aspects of DNA replication and repair is the conserved nuclease-helicase Dna2[8]. Essential in yeast[9], *DNA2* is required for embryonic development in mice[10], and its downregulation leads to chromosomal instability[11-15]. The enzymatic activities of Dna2 reside in a RecB-like nuclease domain[16] with single-stranded DNA (ssDNA)-specific endonuclease activity[17], and a C-terminal superfamily 1 helicase domain[9]; in yeast, Dna2 has an additional, unstructured N-terminal domain that serves a redundant function in S phase checkpoint activation[18] (Fig. 1a).

The nuclease activity of Dna2, in particular, has been linked with a number of molecular pathways. *In vitro*, Dna2 cuts DNA 5'-flaps bound by RPA, which are refractory to cleavage by Rad27 (FEN1 in human), and it has been proposed that Dna2 is ideally suited to promote lagging strand synthesis by supporting Rad27 in the removal of 5'-flaps from Okazaki fragments during replication[19,20]. Yet, Rad27 is very efficient in removing nascent 5'-flaps from Okazaki fragments before they reach the length required to bind RPA[21,22], and nuclease-deficient *dna2* mutant yeast cells lack the severe trinucleotide repeat instability phenotype seen in Okazaki fragment processing-defective *rad27*Δ cells[23,24]. Most 5'-flaps are therefore thought to be removed from maturing Okazaki fragments by Rad27 *in vivo*, while occasional long flaps may attract RPA, potentially necessitating an involvement of Dna2.

During DNA double-strand break repair, Dna2 cooperates with the helicase Sgs1 (Bloom's syndrome helicase BLM in human), promoting DNA end-resection redundantly with Exo1 to facilitate HR. Stimulated by RPA, the Dna2 nuclease degrades the 5'-terminated single strand as the duplex is unwound by Sgs1[25]. Similarly, the Dna2 nuclease has been implicated in *S. pombe* in the processing of stalled replication fork intermediates through degradation of the regressed DNA branch emanating from reversed replication forks as the newly synthesized DNA strands become

displaced and anneal with one another to form a chicken-foot structure[26,27]. An analogous reaction, mediated by the DNA2 nuclease in conjunction with Werner's syndrome helicase WRN promotes replication restart in human cells[28], while failure to properly control DNA2-mediated DNA resection at stalled forks leads to excessive DNA degradation and genome instability[29,30].

The physiological role of the Dna2 helicase activity, as opposed to the nuclease activity, has remained unclear. There is currently no evidence that the helicase activity contributes to the degradation/resection of DNA ends at reversed forks or DNA double-strand breaks. A supportive role in Okazaki fragment processing has been proposed, where the helicase might help to straighten out 5'-flaps capable of forming secondary structures to promote degradation by the Dna2 nuclease. However, the Dna2 helicase activity is dispensable *in vitro* for the processing even of those 5'-flaps that may contain fold-back structures, when RPA is present[31,32]. Interestingly, a number of Dna2 mutants affected within the conserved SF1 helicase motifs I-VI (Fig. 1a) confer growth defects accompanied by sensitivity to the DNA alkylating agent methyl methanesulfonate (MMS)[15,33]. This phenotype is not generally shared with mutants affected in the N-terminal domain[33] or nuclease domain[34], indicating that Dna2 helicase-specific functions in the repair of DNA damage or in the response to damage-induced replication stress exist.

Intriguingly, a genetic screen[35] uncovered a synthetic sick interaction, characterized by slow growth, between *dna2-2*, an allele that encodes a Dna2 variant with a single amino acid change (R1253Q) in the helicase domain[15], and structure-specific *RAD2*/*XPG* superfamily nuclease *YEN1*, indicating a potential functional interplay. Yen1, and its human ortholog GEN1, are HJ resolvases[36]. These enzymes are best known for their role in processing late HR intermediates, such as fully double-stranded DNA (dsDNA) four-way HJ junctions, which they resolve by symmetric nicking on either side of the branch point[37]. Eukaryotes use three conserved HJ resolvases, Yen1/GEN1, Mus81-Mms4/human MUS81-EME1, and Slx1-Slx4/human SLX1-FANCP to remove recombination intermediates that form during replication-associated DNA repair

processes in mitotic cells[38]. Mounting evidence suggests that Mus81-Mms4/MUS81-EME1 also targets unproductive replication intermediates, effectively breaking stalled replication forks to allow HR-dependent replication restart or repair[39]. In human cells, MUS81-EME1 promotes the expression of chromosomal fragile sites, which is thought to represent controlled breakage of underreplicated DNA at the time of mitosis to limit sister chromatid non-disjunction[40,41]. At present, there is no evidence for a similar role of Yen1/GEN1 in targeting replication – rather than recombination – intermediates.

Here, we analyze the interplay between Dna2 and Yen1 to reveal new aspects of the cellular response to replication stress. We find that the Dna2 helicase activity acts upon replication fork stalling, promoting full genome duplication along a pathway parallel to HR-mediated replication fork recovery. If the Dna2 helicase fails to respond properly to stalled replication forks, replication intermediates remain and give rise to post-replicative chromosomal DNA links that preclude chromosome segregation. Resolution is uniquely dependent upon the actions of Yen1, which identifies a first non-redundant function of Yen1 in protecting cells from mitotic catastrophe after replication stress.

Results

Dna2 R1253Q, encoded by *dna2-2*, is helicase defective and nuclease proficient. Using budding yeast, Campbell and colleagues have conducted a large-scale genetic screen[35] using *dna2-2* and the nuclease-defective allele *dna2-1* (P504S)[15], which identified 37 synthetic sick/synthetic lethal interactions, predominantly with genes involved in DNA replication and repair. Many interactions were shared between *dna2-2* and *dna2-1*, but a synthetic sick interaction with *YEN1* was unique to *dna2-2*. Dna2 variant R1253Q is affected at an invariant arginine in helicase motif IV of the SF1 helicase domain, suggesting that the Dna2 helicase and Yen1 may function in related pathways. However, since single amino acid changes in Dna2 have been described that impact both the nuclease and ATPase/helicase activities[42], and because Dna2 R1253Q has never been isolated and

analyzed biochemically, we first assessed directly the mutant protein's ATPase/helicase and nuclease activities. Dna2 R1253Q was purified to near-homogeneity following overexpression in *S. cerevisiae* (Fig. 1b), and tested alongside wild type Dna2, and well-established nuclease-dead and helicase-dead variants, Dna2 E675A and Dna2 K1080E[42], respectively.

When wild type Dna2 was incubated with 5'-tailed DNA, the activity of the ATPase/helicase domain was readily detected, before the potent Dna2 nuclease could degrade the ssDNA tails, so that the ATPase was no longer stimulated and ATP hydrolysis subsided; the nuclease-dead Dna2 variant E675A exhibited persistent ATPase activity[42,43] (Fig 1c). In contrast to wild type and Dna2 E675A, Dna2 R1253Q showed no ATPase activity, and was indistinguishable from previously-characterized[42,43] ATPase/helicase-dead variant Dna2 K1080E (Fig. 1c and Supplementary Fig. 1). *In vitro*, Dna2 exhibits ssDNA-specific nuclease activity on 5'-tailed or 3'-tailed DNA substrates, while RPA stimulates its nuclease and enforces 5'-3' directionality, which is likely the relevant polarity *in vivo*[25]. In the presence of RPA, Dna2 R1253Q degraded 5'-tailed DNA in a manner similar to wild type Dna2, showing that the R1253Q mutation does not interfere with the nuclease activity (Fig. 1d). In line with the observed lack of ATPase activity, we did not find evidence of DNA unwinding by Dna2 R1253Q, an activity that was readily detected for nuclease-deficient mutant Dna2 E675A (Fig. 1d). Quantification of the nuclease/helicase assays showed that Dna2 R1253Q was as efficient as wild type and Dna2 helicase mutant K1080E in degrading 5'-tailed DNA (Fig. 1e). Finally, and in accord with previous studies using other Dna2 helicase mutants[31,32], Dna2 R1253Q was fully proficient in removing 5'-flaps from dsDNA by cleavage at the flap base, in a reaction that mimics the potential role of Dna2 in Okazaki fragment processing (Fig. 1f). These results show that the *dna2-2* allele confers a helicase-specific defect and does not impinge on the activity of the Dna2 nuclease.

Dna2 helicase-defective cells suffer dual growth inhibition by checkpoint activation and loss of *YEN1*. Having established that the R1253Q mutation selectively inactivates the helicase activity of

Dna2, we introduced the mutation into cells by replacing the *DNA2* gene with the *dna2-2* allele to investigate the effect of Dna2 helicase deficiency *in vivo*. While Dna2 protein levels were unaffected (Supplementary Fig. 2a), the R1253Q mutation caused MMS sensitivity, as expected for *dna2-2* cells[15] (Supplementary Fig. 2b). Under unperturbed conditions, the *dna2-2* strain exhibited a plating efficiency similar to wild type. In contrast, viability dropped sharply for the *dna2-2 yen1*Δ double mutant to ~35% of wild type levels (Fig. 2a). Doubling time measurements revealed that the *dna2-2* mutation was associated with a mild slow growth phenotype, extending doubling times by ~10 min (103 min versus 92 min for wild type). Upon deletion of *YEN1*, the growth phenotype was much more severe, with an increase in doubling time of ~50 min for the double mutant (143.5 min). Consistent with previous results[35], we did not observe a synthetic growth defect when *YEN1* was deleted in Dna2 nuclease-mutant *dna2-1* cells (data not shown), indicating that the genetic interaction between *YEN1* and *DNA2* relates specifically to the Dna2 helicase activity. Contrary to a reported temperature-dependent lethal interaction between *YEN1* and *DNA2*[35], we found double mutant cells were viable at elevated temperature (37°C) (Supplementary Fig. 2c), although doubling times for *dna2-2* and *dna2-2 yen1*Δ were further increased by ~20 min and ~5 min, respectively.

Microscopic inspection of exponentially growing *dna2*-2 cultures revealed an accumulation of cells in G2/M phase of the cell cycle, and this effect was further accentuated upon deletion of *YEN1*. Morphological examination showed that *dna2-2* cultures contained ~4% large dumbbell-shaped cells. In *dna2-2 yen1*Δ cultures, this sub-fraction was more extensive, accounting for ~8% of cells, and ~5% of cells exhibited morphological changes such as bud elongation and the formation of short chains of elongated cells (Fig. 2b). Finally, the vast majority of G2/M cells within the *dna2-2* and *dna2-2 yen1*Δ cultures (≥ 70%), but not within wild type or *yen1*Δ cultures, contained unsegregated nuclear DNA positioned near the bud neck (Fig. 2b).

Analysis by flow cytometry showed that *dna2-2* and *dna2-2 yen1*Δ cells progressed through a single cell cycle with apparently normal kinetics upon synchronous release into S phase after α-factor pheromone-induced G1 arrest. Later, an accumulation of G2/M cells occurred during exponential growth (Fig. 2c). This indicates that the helicase activity of Dna2 is largely dispensable for bulk DNA synthesis, but that Dna2 helicase-deficient cells have a tendency to arrest at the G2/M transition, as noted previously[15]. Importantly, replication and the G2/M transition phenotype was unaffected by the presence or absence of Yen1. The accumulation of G2/M cells may result from elevated levels of stochastic DNA damage, since we detected low-level phosphorylation of the checkpoint kinase Rad53 in *dna2-2* and *dna2-2 yen1*Δ cells in unperturbed conditions, which was suppressed upon deletion of the DNA damage checkpoint mediator *RAD9* (Fig. 2d). Moreover, the levels of G2/M cells in either strain were much reduced in the absence of *RAD9*, and bud-elongation and cell-chain formation was no longer observed (Fig. 2e and data not shown). The extended doubling times for the *dna2-2* and *dna2-2 yen1*Δ strains were reduced upon *RAD9* deletion, albeit not to wild type levels (96 min and 122 min, respectively). Significantly, the viability of both *dna2-2* and *dna2-2 rad9*Δ cells was indistinguishable from the *rad9*Δ control, whereas the severe reduction of viability we had observed upon loss of *YEN1* in the *dna2-2* background was only mildly suppressed by *RAD9* deletion (Fig. 2f). Together these data suggest that growth defects in *dna2-2 yen1*Δ cells arise from two separate sources: (1) Dna2 helicase dysfunction causes cells to accumulate DNA lesions during unperturbed growth, triggering Rad9-dependent DNA damage checkpoint activation and a delay at the G2/M transition. (2) Yen1 cannot prevent these defects, so that they manifest themselves similarly in *dna2-2 yen1*Δ double mutant and *dna2-2* single mutant cells. Yet, the absence of Yen1 is toxic to *dna2-2* cells, indicating that Yen1 acts downstream, resolving a catastrophic DNA metabolic event that ensues when the Dna2 helicase is non-functional.

The dependency of Dna2 helicase-defective cells on Yen1 is strongly increased under replication stress conditions. Dna2 helicase deficiency sensitizes cells to DNA alkylating agent MMS[15,33]. To

test whether loss of Yen1 has an additional effect on the MMS sensitivity of Dna2 helicase-defective cells, we exposed *dna2-2 yen1*Δ cells to increasing amounts of the drug. As expected[44], loss of *YEN1* alone did not result in overt MMS sensitivity. In contrast, *dna2-2 yen1*Δ cells proved to be several orders of magnitude more sensitive than *dna2-2* cells in drop assays (Fig. 3a). This phenotype was not restricted to MMS, and similar results were obtained with topoisomerase I poison camptothecin (CPT) and ribonucleotide reductase inhibitor hydroxyurea (HU). These drugs have disparate mechanisms of action, but their effects (DNA damage, accumulation of trapped Top1 cleavage complexes throughout the genome, and nucleotide depletion, respectively) all inhibit the progression of replication forks, suggesting that the functional overlap of Dna2 and Yen1 relates to replication fork stalling. This also suggests that endogenous replication problems are responsible for the growth defects of *dna2-2* and *dna2-2 yen1*Δ cells in unperturbed conditions.

Plasmid-based expression of Yen1 suppressed the HU sensitivity phenotype of *dna2-2 yen1*Δ cells. This suppression was strictly dependent upon the nuclease activity of Yen1, demonstrating that Yen1 protects *dna2-2* cells through nucleolytic cleavage of otherwise toxic DNA intermediates (Fig. 3b).

Dna2 helicase-defective cells accumulate DNA damage immediately following acute replication stress and depend upon Yen1 for recovery. To investigate the immediate effects of replication stress on Dna2 helicase-defective cells, we next performed mitotic time-course experiments (Fig. 4). Synchronized *dna2-2* and *dna2-2 yen1*Δ cells were released into S phase under mild replication stress conditions in the presence of 50 mM HU, which impairs, but does not block, replication. After 2 hours, cells were shifted back to drug-free medium. DNA synthesis was monitored by flow cytometry, while DNA replication/DNA damage checkpoint activation was assessed by Western blot analysis of the phosphorylation status of Rad53. As expected, wild type and *yen1*Δ cells exhibited slowed replication progression in the presence of HU. Interestingly, *dna2-2* and *dna2-2*

*yen1*Δ cells progressed through S phase at a pace similar to wild type. As shown in Fig. 4a, all strains showed S phase checkpoint activation in the presence of HU, and S phase checkpoint silencing occurred with normal kinetics across strains, followed by completion of bulk DNA synthesis in drug-free medium. 120 min after removal of HU, wild type and *yen1*Δ cells underwent cell division. In contrast, *dna2-2* and *dna2-2 yen1*Δ cells exhibited a reemergence of Rad53 phosphorylation at this time and remained in G2/M with a 2N DNA content. This biphasic Rad53 phosphorylation pattern, with an unexpected second wave of checkpoint activation in G2/M phase, required both the presence of the *dna2-2* allele and replication stress in the preceding S phase (i.e. it was not discernible above background in control experiments without HU; Supplementary Fig. 3). One interpretation of these observations is that the Dna2 helicase is involved in an immediate response to replication fork stalling, preventing the emergence of DNA structures that signal DNA damage in G2/M.

The presence of Yen1 could not protect Dna2 helicase-defective cells from G2/M checkpoint activation and cell cycle arrest after acute replication stress. However, in the presence of Yen1, the G2/M arrest proved more transient. Thus, G1 cells with a 1N DNA content started to appear 240 min after removal of HU, and continued to appear through overnight incubation in the *dna2-2* culture, while the *dna2-2 yen1*Δ strain produced very few G1 cells, as judged by flow cytometry (Fig. 4a). Microscopic analysis showed that within the *dna2-2 yen1*Δ culture ~11% of cells had segregated their nuclear DNA (~6% of double-nucleated cells with two DAPI-stained DNA masses distributed between mother cell and bud, and ~5% G1 cells), while the *dna2-2* culture contained a significantly higher number of cells, ~32%, with segregated DNA (~12% double-nucleated and ~20% G1 cells) after overnight incubation (Fig. 4b). This correlated with a significantly lower lethality scored for *dna2-2* mutants (~28% viability compared to wild type) than for the *dna2-2 yen1*Δ double mutant (< 1% viability compared to wild type) (Fig. 4c). We conclude that Yen1 promotes mitotic exit with viable chromosome segregation in Dna2 helicase-defective cells recovering from acute replication stress.

Dna2 helicase dysfunction causes post-replicative chromosomal links that require resolution by Yen1 during mitosis. Given that Dna2 helicase defective cells activate the G2/M checkpoint in response to endogenous and exogenous replication stress, and that G2/M arrested *dna2-2* cells recover slowly from acute replication stress in a Yen1-dependent manner, we next examined the effect of the G2/M checkpoint on recovery in greater detail. A potential explanation for the slow-recovery phenotype relates to recent work showing that Yen1 activity is cell cycle-regulated, with cyclin-dependent kinase-mediated phosphorylation lowering its catalytic activity and inhibiting access to the nucleus in S and G2 phase[45-47]. Upon anaphase onset, Cdc14-dependent dephosphorylation activates Yen1 and allows the protein to accumulate inside the nucleus during mitosis. To test whether Yen1 cell cycle control is manifest in Dna2 helicase-defective cells recovering from acute replication stress, we expressed and monitored a functional (Supplementary Fig. 4a) version of Yen1, tagged with enhanced green fluorescent protein (EGFP), in *dna2-2* and wild type cells. The expected bi-phasic checkpoint activation of *dna2-2* cells in response to acute HU treatment was recapitulated in the presence of Yen1-EGFP (Supplementary Fig. 4b), and the fusion protein exhibited the characteristic cell cycle-dependent subcellular localization pattern of untagged Yen1 (Fig. 5a)[45-48]. Importantly, when cells accumulated as large-budded G2/M cells after HU wash-out, a subset of cells was double-nucleated with a nuclear Yen1-EGFP signal, indicating that mitotic entry had occurred. This subset of cells was markedly larger in case of the wild type strain after 2 h in drug-free medium (Fig. 5b). After 4 h in drug-free medium, the fraction of wild type G2/M cells diminished as cells underwent mitosis. In contrast, *dna2-2* cells remained mostly in G2/M, with Yen1-EGFP in the cytoplasm and a single nucleus at the bud neck, as expected for DNA damage checkpoint-mediated pre-anaphase arrest. Thus, targeting of Yen1 to the nucleus through the actions of Cdc14[45-47] remained largely blocked, showing that unscheduled DNA damage checkpoint signaling in *dna2-2* cells is associated with retention of Yen1 in the cytoplasm, and that this may represent a major impediment to the recovery of Dna2 helicase-defective cells from replication stress.

To test this idea further, we next disrupted the G2/M DNA damage checkpoint by deletion of *RAD9*, allowing unrestrained anaphase entry, and thus Yen1 activation, in Dna2 helicase-defective cells. Upon acute replication stress treatment, checkpoint activation during S phase occurred normally in the absence of Rad9, consistent with signaling in response to replication fork stalling, rather than DNA damage, through the intact Mec1-Ddc2/Mrc1/Rad53-dependent pathway. In contrast, unscheduled Rad53 phosphorylation in G2/M phase after HU wash-out was abolished in *dna2-2 rad9*Δ and *dna2-2 yen1*Δ *rad9*Δ cells (Fig. 5c). Dna2 helicase-defective cells now progressed to cell division with kinetics similar to those exhibited by the *rad9*Δ control strain, and we were able to study the effects of Yen1 by microscopic inspection. This revealed two important phenotypes associated with concomitant loss of Dna2 helicase function and Yen1. 60 min after removal of HU, *dna2-2 yen1*Δ *rad9*Δ samples contained roughly 3-fold higher levels of early anaphase cells characterized by an elongated nucleus stretched through the bud neck, as compared to *dna2-2 rad9*Δ and *rad9*Δ samples. Concomitantly, there was a delay in the appearance of G1 cells containing a single nucleus (Fig. 5d). This suggests that chromosome segregation and cytokinesis are physically impeded in the absence of Yen1. Consistently, exclusively in *dna2-2 yen1*Δ *rad9*Δ cells, we observed prominent chromosomal DNA bridges that span the bud neck and connect the segregating masses of nuclear DNA (13.6% and 8% of the double-nucleated cells affected 180 min and 240 min after HU wash-out, respectively) (Fig. 5e). In some instances this phenotype could be seen in cells approaching abscission, as indicated by a narrowing bud neck.

When we determined the effect of checkpoint disruption on cell viability, we found that deletion of *RAD9* increased the viability of Dna2 helicase-defective cells after acute replication stress treatment 3-fold, reaching levels very similar to those observed for the *rad9*Δ control strain. In the absence of *YEN1*, viability was also improved, but did not reach more than ~14% of the viability of the *rad9*Δ control (Fig. 5f). Checkpoint activation in Dna2 helicase-defective cells after acute replication stress therefore appears futile, and eliminating the G2/M checkpoint

enabled a highly effective Yen1-dependent survival pathway, while allowing a small subset of cells to survive in a Yen1-independent manner. As expected, checkpoint disruption had no beneficial effect when cells were exposed to chronic replication stress (Supplementary Fig. 5), consistent with improved survival being linked specifically to allowing Yen1 access to post-replicative lesions in Dna2 helicase-defective cells. These observations resonate with previous findings showing that the lethality of some temperature-sensitive *dna2* alleles, and of *dna2*Δ, can be suppressed by deleting *RAD9*[15,49,50], linking this phenomenon, at least for the Dna2 helicase-defective *dna2-2* allele, to the removal of the inhibitory effect of the G2/M DNA damage checkpoint on Yen1 activation.

To test whether Yen1 activation, not mitotic entry *per se*, is sufficient for Yen1 to resolve aberrant DNA intermediates that arise in Dna2 helicase-defective cells, we used a constitutively active form of Yen1, referred to as Yen1^{on} (Supplementary Fig. 6a). Yen1^{on} is permanently active and nuclear[45] due to amino acid changes at nine phosphoacceptor sites, which abolishes CDK control over Yen1. We expressed Yen1^{on} from a galactose-inducible promoter in G2/M in *dna2-2 yen1*Δ cells recovering from acute, HU-induced replication stress in the presence of nocodazole. Strikingly, and in contrast to cells harboring an empty-vector control, Yen1^{on}-expressing cells did not exhibit unscheduled DNA damage checkpoint activation during nocodazole-induced G2 arrest (Supplementary Fig. 6b). Furthermore, transient expression of Yen1^{on} in *dna2-2* cells recovering from acute HU treatment led to a significant increase in cell viability (~3.3-fold ± 0.24 s.e.m., n = 2), as determined by colony outgrowth.

Collectively, these results suggest that Dna2 helicase-defective cells fail to respond adequately to replication stress, leading to post-replicative DNA damage signaling and chromosome entanglements. Upon anaphase entry, Yen1 promotes the survival of Dna2 helicase-defective cells by resolving post-replicative chromosomal DNA links, allowing proper chromosome segregation.

Yen1 acts along a pathway that is distinct from canonical HJ resolution to promote growth of Dna2 helicase-defective cells. Yen1 is known for its role in removing persistent HJ DNA structures that accumulate as Rad52-dependent HR intermediates. Previous findings also suggest an increased requirement for Rad52-dependent DNA repair by HR in Dna2 helicase-defective cells. Thus, *dna2-2 rad52*Δ cells without overt growth defect at 30 °C, but temperature sensitivity at a restrictive temperature of 37 °C, have been described[51]. We generated *dna2-2 rad52*Δ cells and found that compared to *dna2-2* (103 min) and *rad52*Δ (112 min), double mutant cells grew slowly, even at 30 °C, with a doubling time of 144 min. Furthermore, loss of Rad52 led to pronounced synthetic hypersensitivity of *dna2-2* cells to HU (Fig. 6a). Finally, we observed a significant increase of spontaneous Rad52 foci indicative of HR[52] in *dna2-2* cells in unperturbed conditions (Fig. 6b), whereas Dna2 focus formation is elevated in *rad52*Δ cells[53]. This suggests that Dna2 and Rad52-dependent HR represent parallel and compensatory pathways in the response to replication stress.

Elevated levels of HR repair could explain why loss of Yen1 is detrimental to *dna2-2* cells. If so, *dna2-2 mus81*Δ cells should exhibit an even stronger growth defect than *dna2-2 yen1*Δ cells, given that Mus81-Mms4 is activated, in a CDK-dependent manner, prior to Yen1 activation at anaphase onset. Reaching an activity peak in its hyperphosphorylated state in G2/M, Mus81-Mms4 is thus the major nuclease in removing HR intermediates in budding yeast[54]. Notwithstanding, we found that in contrast to loss of Yen1, disruption of Mus81-Mms4, or the Slx1-Slx4 HJ resolvase, did not increase the doubling time of *dna2-2* cells in unperturbed conditions. In the presence of HU or MMS, deletion of *SLX1* had no effect on the sensitivity of Dna2 helicase-defective cells. Deletion of *MUS81*, which in itself results in replication stress sensitivity, added to their sensitivity (Fig. 6c and data not shown), consistent with a requirement for Mus81-Mms4 in the resolution of excessive HR intermediates in *dna2-2* cells. However, *dna2-2 yen1*Δ cells were significantly more sensitive to HU or MMS than *dna2-2 mus81*Δ cells (0.2% versus 45% cell survival at 20 mM HU as determined by colony outgrowth) (Fig. 6c,d and data not

shown), despite the fact that defects related to HJ resolution as a consequence of Yen1 loss have been shown to transpire only in the absence of a functional Mus81-Mms4 resolvase[44,55]. Therefore, there is a pathway, distinct from canonical HJ resolution, which uniquely requires Yen1 for the removal of DNA intermediates that are apparently not amenable to cleavage by Mus81-Mms4, in *dna2-2* cells. Indeed, the toxicity caused by loss of Yen1 cannot be explained by an accumulation of HR intermediates alone, as we found that the synthetic sick relationship between *DNA2* and *YEN1* is maintained in cells deleted for *RAD52*, which cannot engage in HR reactions. In fact, we were unable to generate a *dna2-2 rad52*Δ *yen1*Δ triple mutant by tetrad dissection (data not shown), and have confirmed an essential requirement for Yen1 in *dna2-2 rad52*Δ cells using a plasmid-based assay (Fig. 6e). These results contrast with an epistatic relationship that exists between *RAD52* and the HJ resolution pathway defined by *MUS81-MMS4* and *YEN1*[44], and imply that the structures that are targeted by Yen1 in order to maintain the viability of DNA2 helicase-defective cells derive from perturbed replication intermediates in a HR-independent manner.

Yen1 resolves persistent replication intermediates in Dna2 helicase-defective cells. To address the question whether replication fork stalling in Dna2 helicase-defective cells gives rise to an accumulation of DNA intermediates that might become targets for Yen1, we turned to the natural replication fork barrier (RFB)[56] within the ribosomal DNA (rDNA) on chromosome XII. We compared rDNA from actively replicating wild type and *dna2-2* mutant cells by two-dimensional (2D) gel electrophoresis and monitored the disappearance of replication intermediates as cells progressed from S phase to nocodazole-induced G2/M arrest. In S phase, Dna2 helicase-defective cells showed a pattern of replication intermediates very similar to wild type (Fig. 6f). As expected, replication intermediate levels dropped significantly when cells accumulated in G2/M during nocodazole arrest. However, the resolution of replication intermediates, in particular of RFB-stalled and converged forks, was less efficient in *dna2-2* cells, leading to a ~2 and ~3-fold less prominent decrease compared to wild type, respectively. These results are in good agreement with previous observations of accumulating stalled and converged fork intermediates within the

rDNA of *dna2-2* cells[57], and corroborate the notion of an aberrant response to replication fork stalling in Dna2 helicase-defective cells. To see if Yen1 targets aberrant replication intermediates that persist in Dna2 helicase-defective cells, we expressed Yen1^{on} in asynchronous *dna2-2* cultures (Fig. 6g). Similar to staged S phase cells, 2D gel electrophoresis of exponentially growing *dna2-2* cells showed the expected rDNA replication intermediates and, in addition, a more prominent signal indicative of recombination intermediates (X-spike), consistent with an accumulation of G2/M cells with increased rates of HR[57]. Yen1^{on} did not affect the RFB signal, suggesting that replication forks arrested at the barrier are not immediately susceptible to Yen1 nuclease activity. In contrast, and consistent with the ability of Yen1 to resolve recombination intermediates, moderate constitutive Yen1^{on} expression markedly reduced the X-spike signal. Importantly, single fork intermediates (Y structures) and converged forks were also decreased upon Yen1^{on} expression, showing that Yen1, in addition to resolving four-way X-DNA, is able to remove replication intermediates that accumulate in Dna2 helicase-defective cells. These results suggest that Yen1 uniquely resolves persistent replication fork/converging fork structures to disentangle underreplicated nascent sister chromatids when Dna2 helicase-defective cells enter anaphase, thereby safeguarding chromosome segregation and enabling viable mitotic exit.

Discussion

Our analyses of the interplay between Dna2, HJ resolvase Yen1, and the DNA damage checkpoint allows us to define important functions of the Dna2 helicase activity and the Yen1 nuclease in the replication stress response. First, we find that Dna2 mutant R1253Q, encoded by *dna2-2*, is nuclease proficient and helicase dead (Fig. 1). We can therefore relate the negative genetic interaction between *DNA2* and *YEN1*[35] (Fig. 2) unambiguously to the helicase activity of Dna2, and we also show it involves the nuclease activity of Yen1 (Fig. 3b). Dna2 helicase-defective cells are sensitive to replication stress and exhibit hypersensitivity upon loss of Yen1 to drugs that impair

replication fork progression, such as MMS, CPT, and HU (Fig. 3a). This indicates that the molecular basis for the synthetic sick interaction between *DNA2* and *YEN1* is linked to replication fork stalling. While *dna2-2* cells are proficient in bulk DNA synthesis, even under replication stress conditions, post-replicative DNA damage checkpoint activation, within one cell cycle, indicates an inadequate response to replication fork stalling (Fig. 4a). Under these conditions, the G2/M DNA damage checkpoint impairs recovery by attenuating the activation of Yen1, which requires anaphase entry[54] (Fig. 5). Consistently, checkpoint disruption by deletion of *RAD9* promoted the survival of *dna2-2* cells after acute replication stress. In the absence of Yen1, however, cell viability remained low, and cells showed chromosomal bridges between the segregating masses of DNA (Fig. 5e). Importantly, mitotic failure is linked to aberrant replication intermediates, not HR intermediates, since the synthetic sick relationship between *DNA2* and *YEN1* is maintained in HR-deficient cells (Fig. 6e). In support, we show that replication intermediates, which accumulate in *dna2-2* cells[57] (Fig. 6f), are viable targets for Yen1 (Fig. 6g). We propose a model, where the Dna2 helicase activity represents a HR-independent replication stress response pathway that helps to ensure full replication of the genome. Replication intermediates that escape the attention of Dna2 persist and impair sister chromatid separation, unless they are resolved by Yen1. Thus, the actions of Yen1, which has so far only been known to target HR intermediates, allow viable chromosome segregation along a novel pathway, distinct from canonical HJ resolution (Fig. 7).

The precise constitution of the DNA structures that threaten chromosome segregation in Dna2 helicase-defective cells remains to be determined. However, the fact that Yen1 can detoxify them indicates that these DNA intermediates conform to the substrate spectrum of Yen1, which includes 5'-flaps and fully double-stranded DNA three-way and four-way junctions[36,45].

The ability of Yen1 to cut 5'-flaps is shared with all Rad2/XPF superfamily nucleases, including Rad27. This raises the formal possibility that Yen1 might support Rad27 in Okazaki

fragment processing, and that its role in safeguarding Dna2 helicase-defective cells might pertain to the proposed involvement of Dna2 in this process. In line with previous findings[58], we find no genetic indication that Yen1 might support Rad27 functions *in vivo*. Thus, deletion of *YEN1* did not aggravate the temperature-sensitive growth defect and MMS sensitivity associated with loss of Rad27, nor did overexpression of Yen1 or Yen1^{on} alleviate these phenotypes (Supplementary Fig. 7). In light of this, and our biochemical analyses showing that Dna2 R1253Q is fully proficient in removing RPA-covered 5'-flaps (Fig. 1f) that might become refractory to cleavage by Rad27 *in vivo*, we do not anticipate Okazaki fragment processing problems in *dna2-2* cells, or, should they exist, that Yen1 would be in a position to ameliorate such problems.

It is not immediately obvious how a Yen1 substrate might arise from a potential defect of *dna2-2* cells in DNA double-strand break repair. However, replication stress can lead to DNA breaks, so it might be conceivable that defects in *dna2-2 yen1*Δ cells reflect a functional overlap between Yen1 and the role of Dna2 in DNA end-resection. This seems unlikely, however, because of the functional redundancy that exists between the Exo1 and Dna2-dependent end-resection pathways[59]. Furthermore, we find that Dna2 R1253Q is proficient in degrading tailed DNA substrates (Fig. 1d,e), consistent with observations that plasmids containing *dna2-2*, but not a *dna2* nuclease-defective allele, could complement the DNA end-resection phenotype of Dna2-deficient cells *in vivo*[59]. Finally, *YEN1* exhibits no synthetic sick interaction with *SGS1*[44], the partner helicase of the Dna2 nuclease in DNA end-resection.

We thus favor the possibility that the capacity of Yen1 to target branched dsDNA intermediates is relevant for the protection of *dna2-2* cells, as indicated in Fig. 7b. This ability distinguishes Yen1, Mus81-Mms4, and Slx1-Slx4 from other structure-specific nucleases, and allows them to resolve HR-dependent HJs, but also analogs of replication forks, and presumably reversed fork intermediates, which are structurally equivalent to four-way HJs[37,39,60]. DNA intermediates that require detoxification by Yen1 arise in Dna2 helicase-defective cells in the

absence of Rad52 (Fig. 6e), and in the presence of Mus81-Mms4, which we find in the active, hyperphosphorylated[54] form in post-replicative *dna2-2* cells (Supplementary Fig. 8). Therefore, Yen1 appears not to be primarily required to remove HR intermediates in *dna2-2* cells, but instead for removing persistent replication intermediates, such as arrested forks or converged forks that fail to fuse (Fig. 6f,g).

Persistent replication intermediates can explain the chromosome segregation problems in Dna2 helicase-defective cells (Fig. 5e), and it is tempting to speculate that the Dna2 helicase activity may be involved in replication fork remodeling reactions that facilitate fork recovery. This would be mechanistically distinct, but conceptually similar, to the role of the Dna2 nuclease in replication restart at reversed forks through degradation of the regressed DNA branch[26-28]. A particular attractive possibility is that fork reversal might occur as a consequence of Dna2 helicase dysfunction. The resulting chicken-foot structure, which effectively contains a single-ended DNA double-strand break at the tip of the regressed DNA branch, could account for DNA damage checkpoint activation[61] in DNA2 helicase-defective cells (Figs. 2d and 4a). A four-way chicken-foot DNA intermediate would also be amenable to resolution by Yen1; perhaps more so than by Mus81-Mms4, which is greatly stimulated by pre-existing nicks within DNA four-way junctions, such as those present in HR-dependent joint molecules and maturing HJs[36,37,45]. Importantly, and regardless of their precise structural features, the intermediates resolved by Yen1 in Dna2 helicase-defective cells constitute a first DNA target that is uniquely processed by Yen1. This demonstrates greater complexity in the uses of HJ resolvases in cells, and could explain the evolutionary conservation of Yen1/GEN1.

Intriguingly, Dna2 and Yen1 are both subject to CDK1-regulated nucleocytoplasmic shuttling[48] (Fig. 7b). During S phase, phosphorylation of Yen1 mediates nuclear exclusion, whereas phospho-Dna2 accumulates inside the nucleus. Thus, Dna2 can access sites of impaired DNA replication, and, consistently, has been found to form discrete nuclear foci during HU-induced

replication arrest[62]. Dna2 helicase dysfunction gives rise to lesions that require Yen1 for resolution, but also triggers G2/M checkpoint activation, precluding dephosphorylation-dependent Yen1 activation and translocation to the nucleus upon anaphase entry (Fig. 5a). Paradoxical though it may seem, this likely reflects a trade-off between the need to protect chromosomes from the DNA de-branching activities of Yen1 during S phase, while exploiting its unique biochemical properties to remove persistent chromosomal DNA links in M phase. Indeed, Yen1^{on} expression in G2/M allows resolution of toxic DNA intermediates in *dna2-2* cells (Supplementary Fig. 6), but constitutive expression is associated with MMS sensitivity, and tight control over the activities of HJ resolvases has been shown to limit sister chromatid exchange and the associated risk of loss of heterozygosity[45,47,63-65]. Despite the risk of terminal G2/M arrest, Yen1 effectively maintains the viability of Dna2 helicase-defective cells in unperturbed conditions (Fig. 2a), indicating its late activation as an elegant failsafe mechanism that allows Yen1 to act indiscriminately on DNA structures that resemble normal replication intermediates, identified as aberrant only by their presence at the wrong time in the cell cycle. In future, it will be interesting to see whether Yen1 represents a more general surveillance nuclease for aberrant replication intermediates that persist into anaphase. Strong G2/M checkpoint signaling and terminal arrest at the G2/M boundary might have precluded the detection of Yen1 functions downstream of repair and replication factors other than *DNA2* in large-scale screening efforts thus far.

Loss and overexpression of DNA2 has been observed in human cancers and cancer cell lines[13,66,67], while haploinsufficiency promotes cancer formation in heterozygous *DNA2*-knockout mice[10]. This suggests a complex role in cancer, where genome instability caused by impaired DNA2 function may drive tumorigenesis, whereas upregulation of *DNA2* may help cancer cells to survive continuous DNA replication stress. Interestingly, a homozygous mutation in *DNA2* has recently been identified in patients with Seckel syndrome[68], a disease associated with a compromised response to replication fork stalling on the cellular level[69]. Depletion of DNA2 in mammalian cells recapitulates many of the phenotypes seen in Dna2-defective yeast, including

sensitivity to replication stress, elevated DNA damage, chromosome instability, and G2/M cell cycle delay[10-13], indicating functional conservation. Our results implicate the elusive Dna2 helicase in replication fork recovery. Yen1 provides a downstream survival pathway, along which toxic DNA intermediates that arise when the Dna2 helicase activity fails to respond adequately to replication fork stalling are resolved, protecting cells from mitotic catastrophe. Similar two-tiered mechanisms may contribute to the etiology of human pathologies involving *DNA2*.

Methods

Recombinant proteins. Wild type Dna2, Dna2 R1253Q, Dna2 E675A, and Dna2 K1080E were expressed from a modified p*GAL*:*DNA2* vector, adding N-terminal FLAG and HA tags and a C-terminal 6 x His tag, and purified as described previously[32,43]. RPA protein was expressed and purified as described[70].

Nuclease, helicase and ATPase assays. Experiments were carried out and analyzed as described[71,72]. 15 µl reactions contained 25 mM Tris-acetate (pH 7.5), 2 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, 0.1 mg/ml BSA, 1 mM phosphoenolpyruvate, 16 U/ml pyruvate kinase, 1 nM DNA substrate, 16.8 nM RPA, and Dna2 proteins as indicated. Nuclease assays were incubated at 30 °C for 30 min. For analysis by denaturing polyacrylamide electrophoresis, samples were heat-denatured in formamide. DNA substrates were assembled using oligonucleotides X12-3 and X12-4SC, and PC 92 and X12-4SC for the 19 and 30 nt 5'-tailed DNA substrates, respectively[32,43]; the 5'-flapped DNA substrate consisted of oligonucleotides X12–4NC, Flap 19 X12–4C, and 292, as described[32]. Where indicated, oligonucleotides were ^{32}P-labeled at the 5'-end using [γ-^{32}P] ATP and T4 polynucleotide kinase (New England Biolabs). Unincorporated nucleotides were removed using MicroSpin G25 columns (GE Healthcare) before annealing the respective DNA substrates.

Yeast strains and plasmids. *S. cerevisiae* strains (Supplementary Table 1) were derived from BY4741[73] using standard methods. The *dna2-2* allele was generated using pop-in/pop-out mutagenesis[74], and the DNA damage sensitivity of the resulting strain could be complemented with plasmid-borne wild type *DNA2* cloned into vector pAG416GPD-ccdB (p*DNA2*) (Supplementary Fig. 2b). For constitutive expression, *YEN1* was cloned into vector pAG416GPD-ccdB or pAG416GPD-ccdB-EGFP[75], and site-directed mutagenesis was performed to generate a catalytically inactive form of Yen1 bearing the mutations E193A and E195A. Yellow fluorescent protein (YFP)-tagged Rad52 was expressed from its endogenous promoter using centromeric plasmid pWJ1213[76]. If not stated otherwise, all strains were cultured at 30 °C using YPAD media. *YEN1*^{on} was cloned into vector pAG416GPD-ccdB, or pYES-DEST52 (Invitrogen) for expression from a *GAL1* promoter in YPLG medium with 2% (w/v) galactose and 1% (w/v) raffinose. Antibodies used to monitor the expression of tagged proteins were Abcam mouse monoclonal anti-V5 antibody ab27671, and Sigma mouse monoclonal anti-Myc antibody 9E10. Santa Cruz Biotechnology goat polyclonal anti-Mcm2 antibody yN-19 was routinely used to ensure gel lanes were equally loaded for total protein.

Viability, drop assays and survival assays. Doubling times were determined as described[77] and averaged over at least three independent experiments. For microscopic determination of cell cycle stage (budding index), an average of 400 cells per strain and replicate were scored. Plating efficiency as a measure of strain viability was determined by colony outgrowth after plating a defined number of cells. The number of colonies formed after 3–4 days at 30 °C was divided by the number of cells plated as quantified in hemocytometer counts. For drop assays, exponentially growing cells were normalized to 10^7 cells/ml, and 2 µl drops of 10-fold serial dilutions were spotted onto the appropriate medium with or without MMS, HU, or CPT. If not stated otherwise, plates were incubated for 3–4 days at 30 °C. For liquid survival assays, overnight cultures were diluted to OD_{600} = 0.1–0.2 and grown for 4 h, then synchronized with α-factor in G1 and released

into YPAD containing 50 mM HU for 120 min. Relevant dilutions were plated onto YPAD plates and colonies were counted after 3–4 days.

Mitotic time-courses. For time-course experiments, cells were grown exponentially (OD_{600} = 0.4–0.6) and synchronized by addition of α-factor (routinely > 95% unbudded cells for wild type, ≥ 90% for Dna2 helicase-defective strains). Cells were then harvested, washed, and released into YPAD containing 50 mM HU for 2 h. After HU wash-out, cells were cultured in drug-free medium. Aliquots for flow cytometry, Western blot analysis, and microscopy were withdrawn at regular intervals. Where indicated, α-factor or nocodazole (15 μg/ml) was added during and/or after treatment.

Analysis of Rad53-phosphorylation. TCA-precipitated proteins were separated by SDS-PAGE using precast gels (Invitrogen), and blotted onto PVDF membranes using a Bio-Rad Turbo blot system. Rad53 protein was detected using a custom-made mouse monoclonal antibody[78].

Flow cytometry. Cells were fixed overnight in 70% ethanol at 4 °C with rotation and processed as described[79]. Cells were then washed and resuspended using 50 mM Na-citrate (pH 7). After brief sonication, RNase A was added (0.25 mg/ml), and cells were incubated overnight at 37 °C, washed, and resuspended in 50 mM Na-citrate (pH 7) containing 16 μg/ml propidium iodide. Measurements of DNA content were done using a BD LSR II flow cytometer (Becton Dickinson) operated with BD FACSDiva software. Data was processed with FlowJo (TreeStar).

Microscopy. DIC images were obtained using a Zeiss Axio Imager Z1 with a Plan-Apochromat 63x/1.4 DIC oil objective (Zeiss) and an AxioCam camera controlled by ZEN Blue 2012 software. To analyze nuclear DNA and chromosome segregation, cells were fixed with 70% ethanol for 5 min at room temperature and stained with DAPI (50 ng/ml). For Yen1-EGFP and Rad52-YFP analyses, cells were fixed in 4% paraformaldehyde for 3 min at room temperature and stained with DAPI. Confocal images were collected using a Zeiss Axio Imager M1/Yokogawa CSU-X1 scanhead multipoint confocal microscope with a Plan-Neofluar 100x/1.45 oil objective and EM-CCD Cascade

II camera (Photometrics) controlled by Metamorph 7.7.2 software (Molecular Devices), or a Rolera Thunder Back Illuminated EM-CCD camera (Q Imaging) controlled by VisiView software (Visitron Systems). Stacks of > 20 optical slices separated by 200 nm were collected, and images of two-dimensional projections were prepared with ImageJ software (Fiji).

Analysis of rDNA replication by neutral-neutral two-dimensional gel electrophoresis. Synchronized or exponentially growing cells were harvested by centrifugation, and genomic DNA was purified using G-20 columns (Qiagen) before digestion with *Bgl*II. For S phase samples, aliquots were withdrawn every 10 min for 60 min upon release from α-factor-induced G1 arrest, and pooled before preparing genomic DNA. Ethanol-purified DNA digests (2.5 µg) were subjected to 2D gel analysis as described[80], with minor modifications. The first dimension gel (0.4% agarose in TBE) was run at 1 V/cm at room temperature for 16 h. The second dimension gel (1.5% in TBE containing 0.3 g/ml ethidium bromide) was run at 5V/cm at 4 °C in circulating TBE buffer for 5 h. The DNA was then blotted onto Hybond XL membrane (Amersham) by capillary transfer in 0.4 N NaOH. After UV-crosslinking, the membrane was blocked with ssDNA, probed for rDNA, and washed according to instructions by the manufacturer. A DNA template for the Southern probe was prepared by PCR from genomic DNA using primers 5'-GCCATTTACAAAAACATAACG and 5'-GGGCCTAGTTTAGAGAGAAGT[57]. The radiolabeled probe was then synthesized in the presence of [α-^{32}P] dCTP and [α-^{32}P] dATP using Klenow fragment polymerization (New England Biolabs/Bioconcept). Radioactive Southern blots were imaged using a phosphorimager screen (Kodak) and a Typhoon^{TM} 9400 system (GE Healthcare), and quantified using ImageQuant TL v2005 software as described[81]. In brief, the signal intensities for individual image objects were normalized to the intensity of the 1N spot after background correction. The fold change was calculated by dividing the normalized signal intensity of each intermediate in G2 phase by the corresponding signal in S phase. For the experiment with Yen1^{on} expression in *dna2-2* cells, the normalized signal intensity for each scrutinized DNA intermediate in the strain harboring the Yen1^{on} construct was divided by the corresponding signal in the strain with empty vector.

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Author contributions

G.Ö. and U.R. planned and analyzed the experiments. G.Ö. performed the experiments with help from D.K. B.F. supported the two-dimensional gel analyses, and G.A.F. imaging and microscopy. M.L. and P.C. purified and analyzed Dna2 *in vitro*. U.R. wrote the paper.

Figure Legends

Figure 1 | Biochemical analysis of Dna2 variant R1253Q. (**a**) Domain structure of *S. cerevisiae* nuclease-helicase Dna2. *Above*, single amino acid changes within the helicase domain of Dna2 that result in MMS sensitivity, including R1253Q, encoded by *dna2-2*. *Below*, position of mutations E675A and K1080E, which have been shown to inactivate the Dna2 nuclease or helicase activity, respectively. (**b**) Dna2 variant R1253Q carrying 6 x His and FLAG tags was expressed in *S. cerevisiae* and purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA)-agarose and anti-FLAG affinity gel. Fractions were analyzed by polyacrylamide gel electrophoresis followed by Coomassie blue staining. (**c**) Kinetics of ATP hydrolysis by wild type (wt) Dna2 and indicated variants (all 4 nM) in the presence of a 5'-tailed DNA substrate (1 μM nucleotides). Error bars indicate s.e.m. (n = 2). (**d**) Processing of 5'-tailed DNA by Dna2. The panel shows a representative 10% polyacrylamide gel with reaction products after incubation of the DNA substrate with the indicated DNA2 variants and RPA (16.8 nM). *, position of the ^{32}P-label on the DNA. Heat, heat-denatured DNA substrate. (**e**) Quantification of experiments such as those shown in panel **d**. Error bars indicate s.e.m. (n = 2). (**f**) 5'-flap cleavage by Dna2 variants (all 2 nM) in the presence of RPA (30 nM). Reaction products were separated on a 20% polyacrylamide denaturing urea gel. Cleavage at the base of the flap produces a radiolabeled fragment of 32 nt.

Figure 2 | Characterization of Dna2 helicase-defective cells in the presence or absence of Yen1 and a functional DNA damage checkpoint. (**a**) Cell viability of the indicated strains assessed by colony outgrowth, relative to wild type. Error bars indicate s.e.m. (*n* = 3). PE, plating efficiency. (**b**) Microscopic analysis of the indicated strains growing exponentially in rich medium. *Left*, representative images (DIC) showing morphological changes associated with Dna2 helicase dysfunction and loss of Yen1. Average doubling times are given (*n* = 3). Scale bar, 5 μm. *Center*, distribution of G1, S, and G2/M cells ± s.e.m. (*n* = 4). *Right*, DNA segregation in G2/M cells as determined by DAPI-staining. An average of 200 cells were scored per strain. (**c**) Flow cytometric analysis of the indicated strains synchronized in G1 using α-factor and released into YPAD. The position of cells with 1N and 2N DNA content is indicated. Topmost tracks are asynchronous cultures overlaid with the outline of the wild type profile, showing that Dna2 helicase-defective strains accumulate cells with a 2N content over time. (**d**) Western blot analysis showing chronic low-level DNA damage checkpoint activation in Dna2 helicase-defective cells as indicated by Rad53 phosphorylation (Rad53-P). (**e**) Effect of DNA damage checkpoint disruption by deletion of *RAD9* on the distribution of G1, S, and G2/M cells in exponentially growing cultures of the indicated strains ± s.e.m. (*n* = 3). (**f**) Cell viability ± s.e.m. (*n* ≥ 3) of the indicated strains assessed by colony outgrowth, relative to a *rad9*Δ control.

Figure 3 | Dna2 helicase dysfunction and loss of Yen1 synergistically sensitize cells to exogenous replication stress. (**a**) Drop assays to determine the drug-sensitivity of the indicated strains were done by spotting normalized 10-fold serial dilutions of exponentially growing cells onto YPAD plates containing the indicated amounts of MMS, CPT, or HU. (**b**) Analysis of the effects of Yen1 overexpression. Cells of the indicated strains were transformed with empty vector, or derivatives encoding wild type or nuclease-deficient ($Yen1^{n.d.}$) versions of Yen1, and plated on YPAD medium in the presence or absence of HU.

Figure 4 | Dna2 helicase-defective cells are defective in the response to acute replication stress and require Yen1 for subsequent growth. (**a**) Mitotic time-courses were performed as indicated. Cells, synchronized in G1, were released into acute replication stress in medium containing 50 mM HU for 2 h, followed by drug wash-out and incubation in drug-free medium with α-factor. Checkpoint activation and the progression of DNA replication were monitored by Western blot analysis of Rad53 phosphorylation (Rad53-P) and flow cytometry (1N and 2N DNA content indicated). As, asynchronous; S, synchronous; o/n, overnight. (**b**) Quantification of single-nucleated G1 cells and double-nucleated G2/M cells in the indicated overnight yeast cultures shown in panel **a**. 200 cells per strain were analyzed by microscopic inspection. (**c**) Viability of the indicated strains after acute replication stress ± s.e.m. (*n* = 3), relative to wild type. Cells were plated on drug-free YPAD medium and colony formation was quantified. PE, plating efficiency.

Figure 5 | Post-replicative chromosomal links in Dna2 helicase-defective cells and mitotic resolution by Yen1. (**a**) Subcellular localization of Yen1 in *dna2-2* cells exposed to acute replication stress. Mitotic time-courses were performed as indicated with wild type and *dna2-2* cells expressing Yen1-EGFP. Samples were analyzed for nuclear and cytoplasmic localization of Yen1-EGFP following α-factor arrest, 1 h after release into HU-containing medium, and 2 and 4 h after drug wash-out. (**b**) Quantitative view of Yen1-EGFP localization as determined in experiments such as those shown in panel **a** (≥ 100 cells scored for G1 and S phase per strain; ≥ 200 cells 2h and 4 h after drug wash-out). (**c**) Mitotic time-course experiment with DNA damage checkpoint-disrupted strains, performed as indicated and analyzed as described for Fig. 4a. (**d**) Relative distribution of cells in early anaphase (elongated nucleus), late anaphase (double-nucleated), and post-cytokinesis (single-nucleated), as determined by microscopic inspection of samples from panel **c**, 60 min after HU wash-out (≥ 100 cells scored per strain). (**e**) Representative microscopic images showing an early anaphase cell with elongated nucleus spanning the bud neck (i), and late anaphase cells with chromosomal DNA bridges between the segregated masses of nuclear DNA (ii-v), a phenotype exclusively observed in *dna2-2 yen1*Δ *rad9*Δ cells. Cells treated as

described for panel **c**. Scale bar, 5 µm. (**d**) Cell viability ± s.e.m. ($n \geq 3$) of the indicated strains, treated as in panel **c**, assessed by colony outgrowth on YPAD, relative to *rad9*Δ. PE, plating efficiency.

Figure 6 | Yen1 uniquely resolves toxic DNA intermediates in Dna2 helicase-defective cells along a pathway distinct from canonical HJ resolution. (**a**) Synergistic defects in the resistance to exogenous replication stress in homologous recombination-deficient *dna2-2 rad52*Δ cells. Drop assay performed as described for Fig. 3a. (**b**) Spontaneous Rad52-YFP foci in wild type and *dna2-2* cells in different cell cycle stages, determined by microscopic analysis (≥ 180 cells scored per strain). (**c**) Genetic interactions of *dna2-2* with the HJ resolvases *YEN1*, *MUS81-MMS4*, and *SLX1-SLX4*. Drop assays performed as described for Fig. 3a. (**d**) Cell viability under replication stress conditions. The indicated strains were plated on medium with different amounts of HU and colony formation was quantified. Error bars indicate s.e.m. (n = 3). PE, plating efficiency. (**e**) Interaction between *dna2-2*, *RAD52* and *YEN1*. Cells of the indicated genotypes and containing a plasmid expressing wild type *DNA2* (p*DNA2*) were grown under uracil selection to ensure retention of the p*DNA2*, or on medium containing 5-FOA to counter-select against the plasmid. Failure to grow on 5-FOA is indicative of an inviable genotype. (**f**) Analysis of replication and recombination intermediates of an rDNA region in wild type and *dna2-2* cells traversing S phase into nocodazole-induced G2/M arrest. Genomic DNA was digested with *Bgl*II and subjected to 2D gel electrophoresis. The fragment probed by Southern hybridization contained the rDNA autonomously replicating sequence (ARS), the 5S transcriptional unit, and the RFB in its center, as indicated. DNA structures chosen for quantification included RFB-arrested forks (1), Y-arc structures containing a replication fork at varying positions outside the RFB (2,3), the X-spike indicative of four-way branched DNAs containing Holliday junctions or hemicatenanes (4), and forks converging at the RFB (5), as shown schematically. Representative autoradiographies are marked for RFB-stalled and converged replication fork intermediates, which were resolved less efficiently in *dna2-2* cells compared to wild type following S phase. Three independent

experiments were quantified. (**g**) 2 D analysis as in panel **f**, but using exponentially growing *dna2-2* cells expressing or not Yen1^{on}. Intermediates that were reduced upon Yen1^{on} expression are indicated. Three independent experiments were quantified. Error bars indicate s.e.m. in **f** and **g**.

Figure 7 | Model for a two-tiered response to replication stress by Dna2 and Yen1. (**a**) Canonical role of Yen1 in the resolution of HR intermediates that arise during HR-mediated recovery of stalled replication forks. Mus81-Mms4 is activated by hyperphosphorylation in G2/M, prior to activation and nuclear import of Yen1 upon anaphase onset. Thus, HR intermediates are predominantly cleaved by Mus81-Mms4, with Yen1 acting as a catchall in M phase to remove persistent recombination structures in time for chromosome segregation. (**b**) Parallel to HR, the Dna2 helicase is tending to stalled replication forks. Replication intermediates that escape the attention of Dna2 give rise to toxic structures that are sensed by the DNA damage checkpoint, but which are refractory to processing by Mus81-Mms4. On anaphase entry, Yen1 is activated and uniquely resolves persistent replication intermediates, averting mitotic catastrophe. See text for details.

Supplementary Information

Replication intermediates that escape Dna2 activity are processed by Holliday junction resolvase Yen1

Gizem Ölmezer, Maryna Levikova, Dominique Klein, Benoît Falquet, Gabriele Alessandro Fontana, Petr Cejka & Ulrich Rass

Supplementary Figure 1 | Apparent ATP turnover number of Dna2 proteins. Apparent k_{cat} values were calculated from the initial rate of ATP hydrolysis in experiments such as those shown in Fig. 1 c, containing a 5'-tailed DNA substrate, but with 3 nM Dna2 variants. Error bars indicate s.e.m. (n = 2).

Supplementary Figure 2 | Assessment of Dna2 helicase-defective *dna2-2* cells. (**a**) Western blot analysis showing that the R1253Q mutation within the helicase domain of Dna2 does not alter protein expression or protein stability. Whole cell extracts were prepared from logarithmically growing cultures and resolved on a NuPAGE 7% Tris-acetate gel (Life Technologies). The upper part of the membrane was probed for wild type and mutant Dna2 tagged with 13 x Myc at the C-terminus and expressed from the endogenous *DNA2* locus. The lower part of the membrane was probed for Mcm2, which served as loading control. The positions of size-markers are indicated. (**b**) Drop assay, performed as described for Fig. 3, showing that *dna2-2* cells are sensitive to MMS, and that expression of *DNA2* from a low-copy number plasmid with a GPD promoter restores resistance. (**c**) Drop assay showing that *dna2-2 yen1*Δ cells are viable at elevated temperature.

Supplementary Figure 3 | Unscheduled post-replicative checkpoint activation in Dna2 helicase-defective cells is not discernible in unperturbed conditions. The indicated strains were synchronized in G1, released into YPAD medium without HU, and monitored by Western blot analysis for phosphorylation of Rad53 over a period of 4 h. Under these conditions, cells routinely completed bulk DNA synthesis within 60 min of α-factor release (see Fig. 2c). S, synchronous; C,

control samples showing Rad53 phosphorylation (Rad53-P) after exposure of the respective strains to HU.

Supplementary Figure 4 | Green fluorescent protein-tagged Yen1-EGFP is functional. (**a**) Drop assay, performed as described for Fig. 3, showing that Yen1-EGFP suppresses the severe HU sensitivity of *dna2-2 yen1*Δ cells, which demonstrates that the tag does not interfere with Yen1 function. (**b**) Biphasic checkpoint activation in response to acute replication stress is maintained in Dna2 helicase-defective cells expressing Yen1-EGFP. Mitotic time-course experiments performed as described for Fig. 4. Checkpoint activation and replication progression were monitored by analyzing Rad53 phosphorylation (Rad53-P) and DNA content (1N and 2N indicated), respectively. S, synchronous; o/n, overnight.

Supplementary Figure 5 | Constitutively active Yen1^{on} suppresses post-replicative DNA damage checkpoint activation in Dna2 helicase-defective cells recovering from acute replication stress. (**a**) Yen1^{on} is mutated at all CDK consensus sites (serine to alanine substitutions), as indicated. This eliminates CDK-dependent control, allowing the active enzyme into the nucleus at any cell cycle stage. (**b**) Mitotic time-courses with *dna2-2 yen1*Δ cells harboring an empty vector control or a vector for the expression of YEN1^{on} under control of a galactose-inducible promoter. Cells were synchronized in G1, released into acute replication stress in the presence of 50 mM HU for 2 h, and then shifted to medium containing galactose to induce Yen1^{on} expression, and nocodazole to block cells in G2. Checkpoint activation and replication progression were monitored by assessing Rad53 phosphorylation (Rad53-P) and DNA content (1N and 2N indicated), respectively. Insets show the expression of Yen1^{on}, detected using an anti-V5 antibody.

Supplementary Figure 6 | Disruption of the DNA damage checkpoint does not suppress the sensitivity of Dna2 helicase-defective cells to chronic replication stress. Drop assays of the indicated strains on plates containing increasing amounts of HU, performed as described for Fig. 3.

Supplementary Figure 7 | Genetic analysis of the relationship between Yen1 and Rad27. (**a**) A deletion of *YEN1* does not aggravate the temperature or MMS sensitivity of *rad27*Δ cells. (**b**) Constitutive or (**c**) galactose-induced expression of Yen1 or Yen1^{on} does not alleviate the temperature or MMS sensitivity of *rad27*Δ cells. Note how Yen1^{on} expression even attenuates growth in *rad27*Δ mutants under all conditions, whereas the wild type is inhibited only in the presence of MMS. Drop assays were performed as described for Fig. 3 and plates were imaged after 2 to 3 days.

Supplementary Figure 8 | Mus81-Mms4 is hyperphosphorylated in Dna2 helicase-defective cells after replication stress. Mitotic time-courses with wild type and *dna2-2* cells with an endogenously 13 x Myc-tagged version of Mms4, performed as described for Fig. 4. Phosphorylation of Rad53 (Rad53-P) indicates checkpoint activation. Western blot analysis with an anti-Myc antibody reveals an upshift in the Mms4 signal caused by G2/M-specific hyperphosphorylation (Mms4-P). Hyperphosphorylation of Mms4 is transient in the wild type, but persistent in *dna2-2* cells, which recover slowly from acute HU treatment and delay at the G2/M boundary (see also Fig. 4a). As, asynchronous; S, synchronous; o/n, overnight.

Supplementary Table 1 *S. cerevisiae* strains used in this study.

Strain	**Relevant genotype**	**Source**
BY4741 (wild type)	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	GE Healthcare
clone ID 174	BY4741 *yen1*Δ::*KanMX4*	GE Healthcare
clone ID 540	BY4741 *rad52*Δ::*KanMX4*	GE Healthcare
clone ID 3368	BY4741 *slx1*Δ::*KanMX4*	GE Healthcare
YRL31	BY4741 *DNA2-13myc:: KanMX4*	this study
YRL33	BY4741 *dna2-2-13myc:: KanMX4*	this study
YRL96	BY4741 *dna2-2*	this study
YRL97	BY4741 *dna2-2 yen1*Δ::*KanMX4*	this study
YRL98	BY4741 *dna2-2 mus81*Δ::*URA3*	this study
YRL99	BY4741 *dna2-2 slx1*Δ::*KanMX4*	this study

YRL129	BY4741 *dna2-2 rad52*Δ::*URA3*	this study
YRL133	BY4741 *rad9*Δ::*URA3*	this study
YRL134	BY4741 *yen1*Δ::*KanMX4 rad9*Δ::*URA3*	this study
YRL136	BY4741 *dna2-2 rad9*Δ::*URA3*	this study
YRL138	BY4741 *dna2-2 yen1*Δ::*KanMX4 rad9*Δ::*URA3*	this study
YRL241	BY4741 *MMS4-13myc::URA3*	this study
YRL243	BY4741 *dna2-2 MMS4-13myc::URA3*	this study
YRL249	BY4741 *rad27*Δ::*HIS3*	this study
YRL250	BY4741 *rad27*Δ::*HIS3 yen1*Δ::*KanMX4*	this study
YRL268	BY4741 *dna2-2 yen1*Δ::*KanMX4 rad52*Δ::*HIS3* p*DNA2* (*URA3*)	this study
YRL272	BY4741 *dna2-2 rad52*Δ::*HIS3* p*DNA2* (*URA3*)	this study
YWL169	BY4741 *mus81*Δ::*HIS3*	ref. 1

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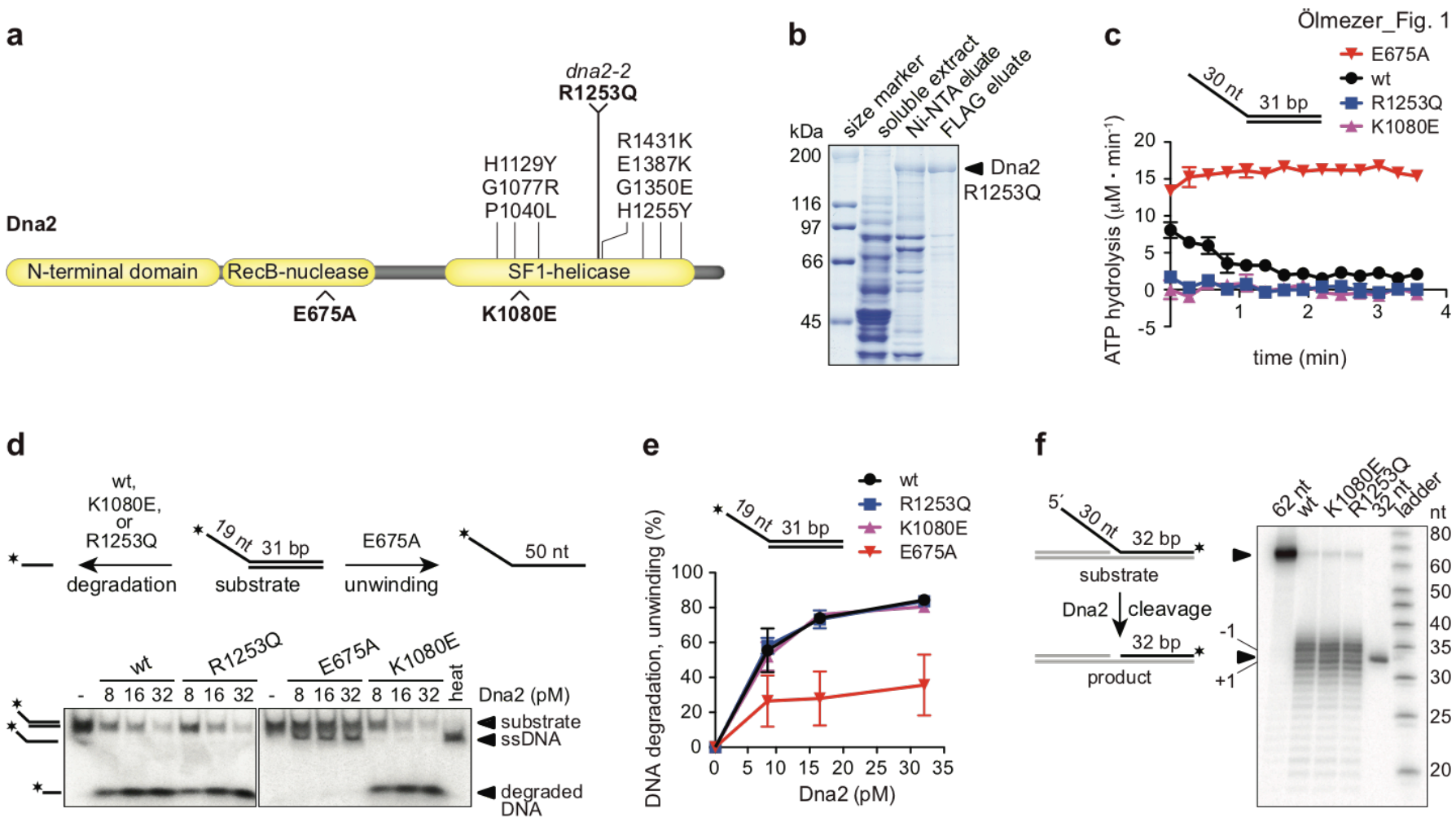
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DNA degradation, unwinding (%)
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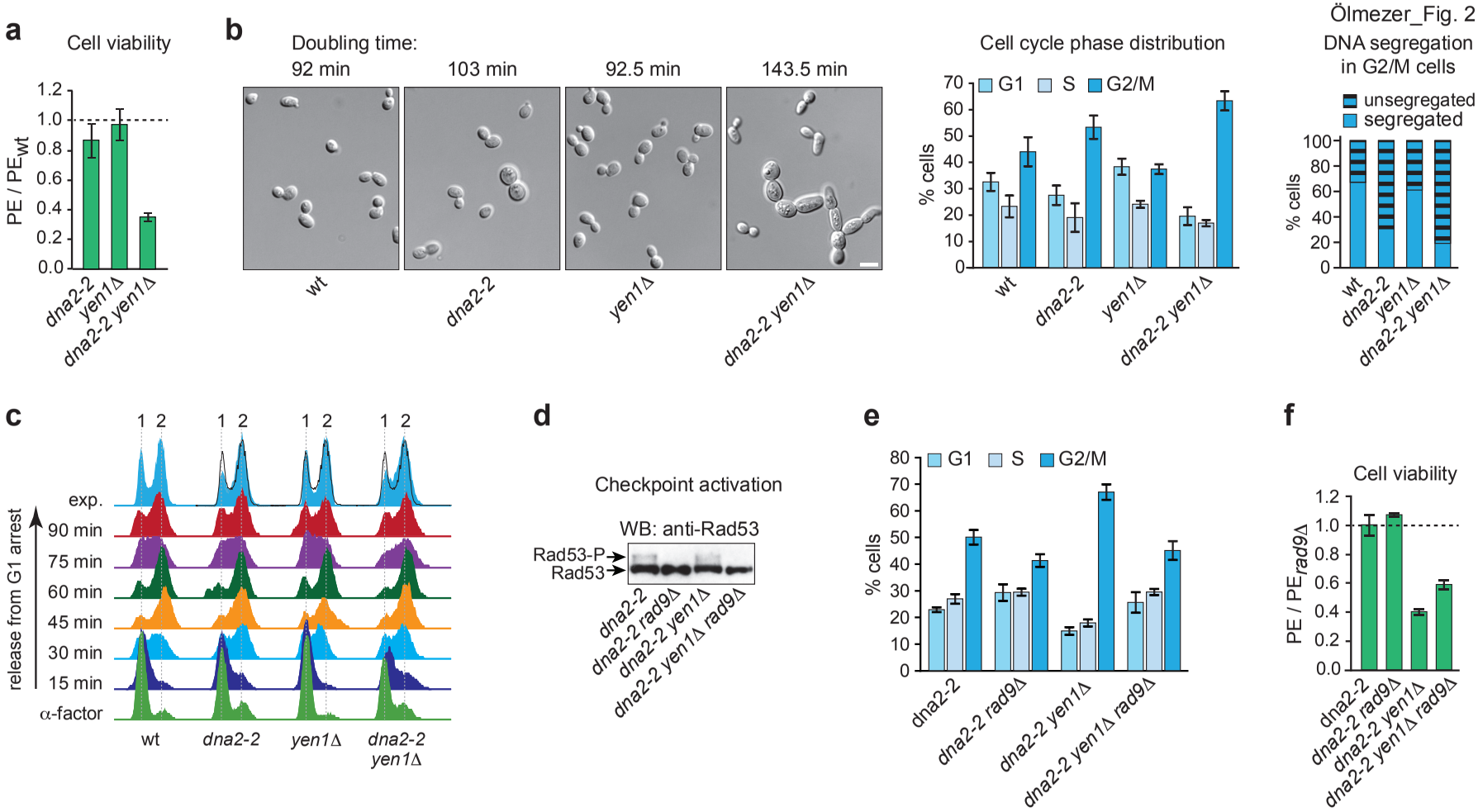
Ölmezer_Fig. 2
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1.0
0.8
0.6
0.4
0.2
0.0
dna2-2
yen1Δ
dna2-2 yen1Δ
b
Doubling time:
92 min
103 min
92.5 min
143.5 min
wt
dna2-2
yen1Δ
dna2-2 yen1Δ
Cell cycle phase distribution
% cells
G1
S
G2/M
DNA segregation
in G2/M cells
unsegregated
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c
release from G1 arrest
exp.
90 min
75 min
60 min
45 min
30 min
15 min
α-factor
wt
dna2-2
yen1Δ
dna2-2
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Checkpoint activation
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Rad53-P
Rad53
dna2-2
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e
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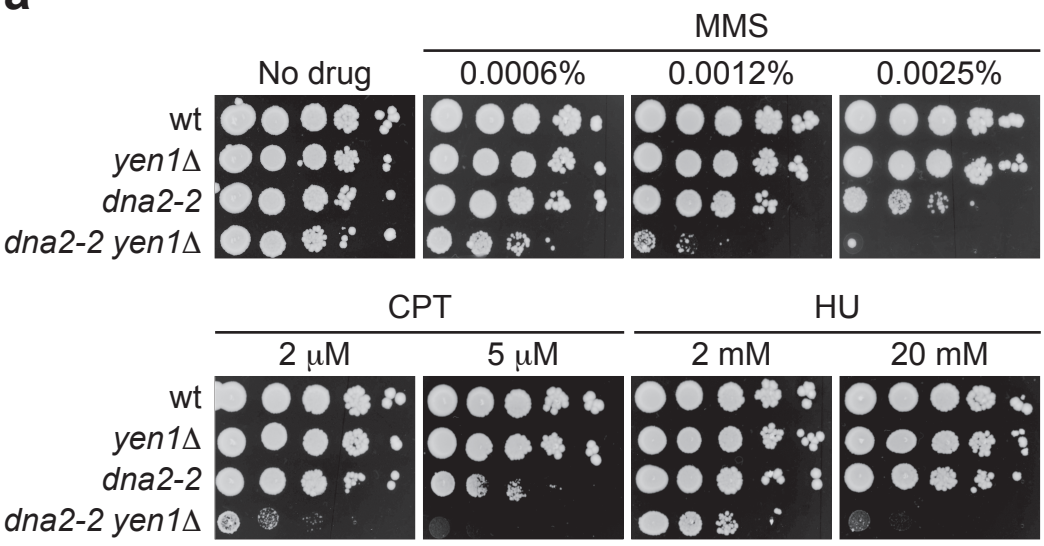
Cell viability
PE / PE$_{rad9Δ}$

a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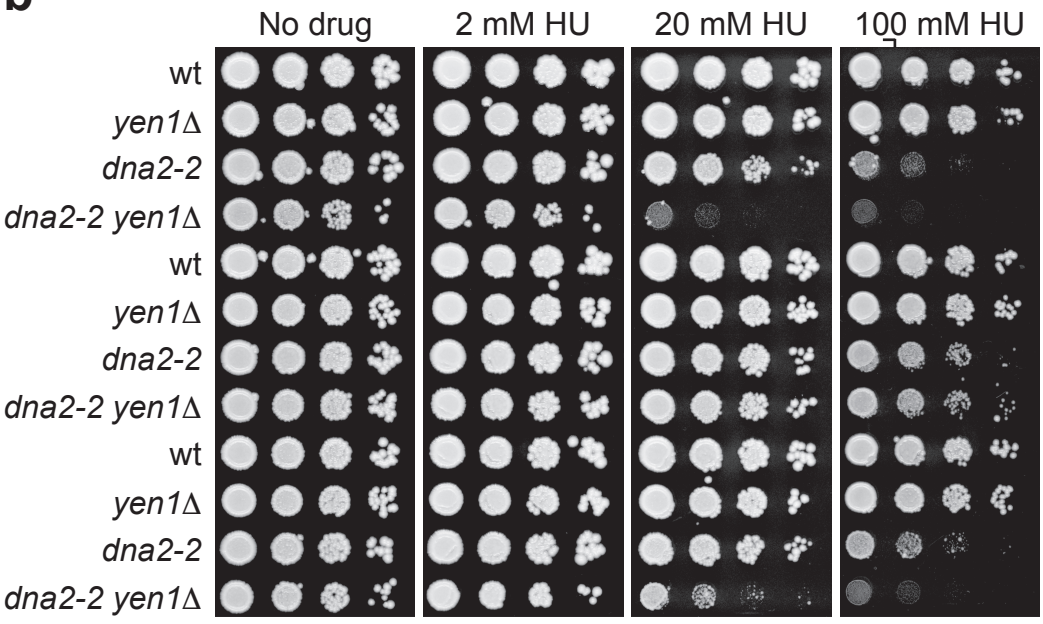
b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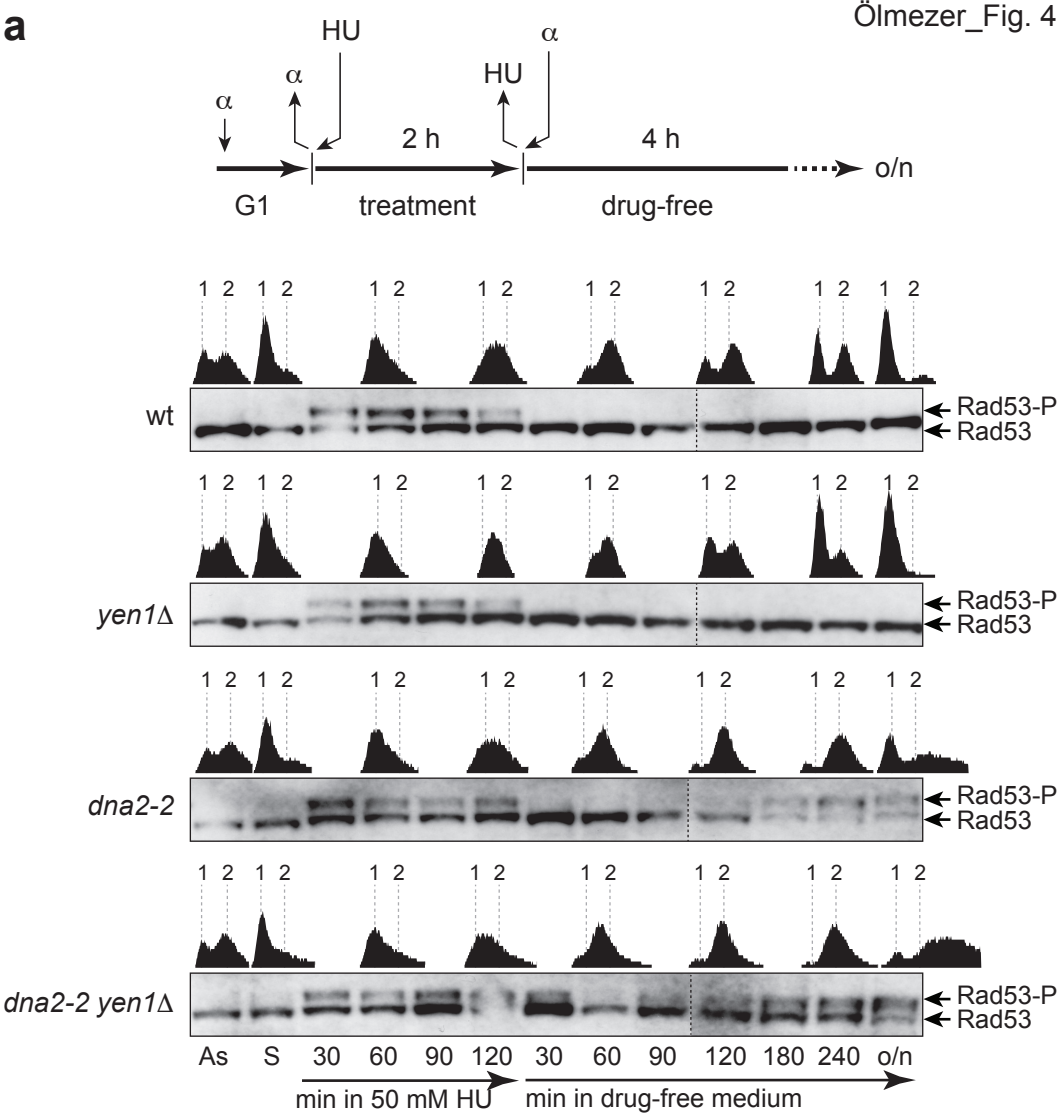
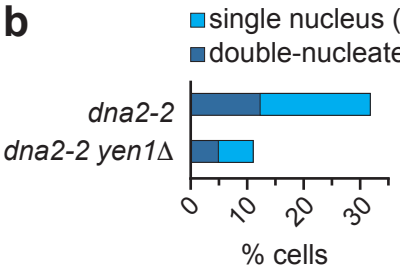
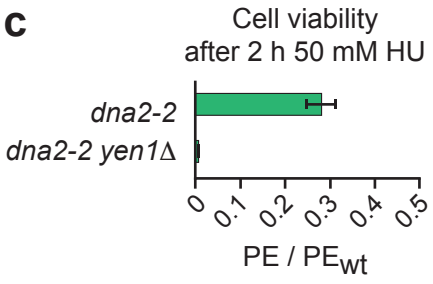
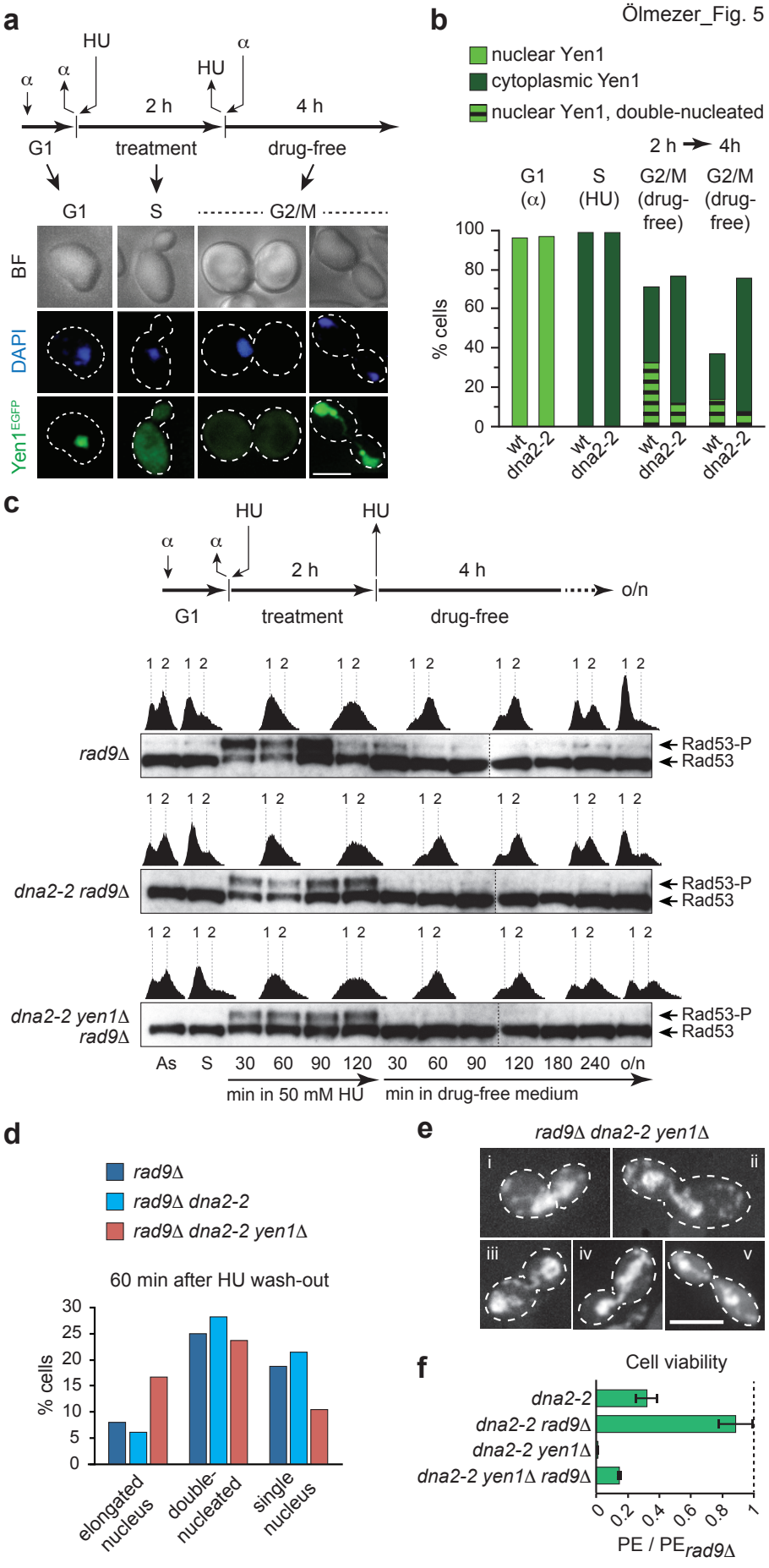
Ölmezer_Fig. 4

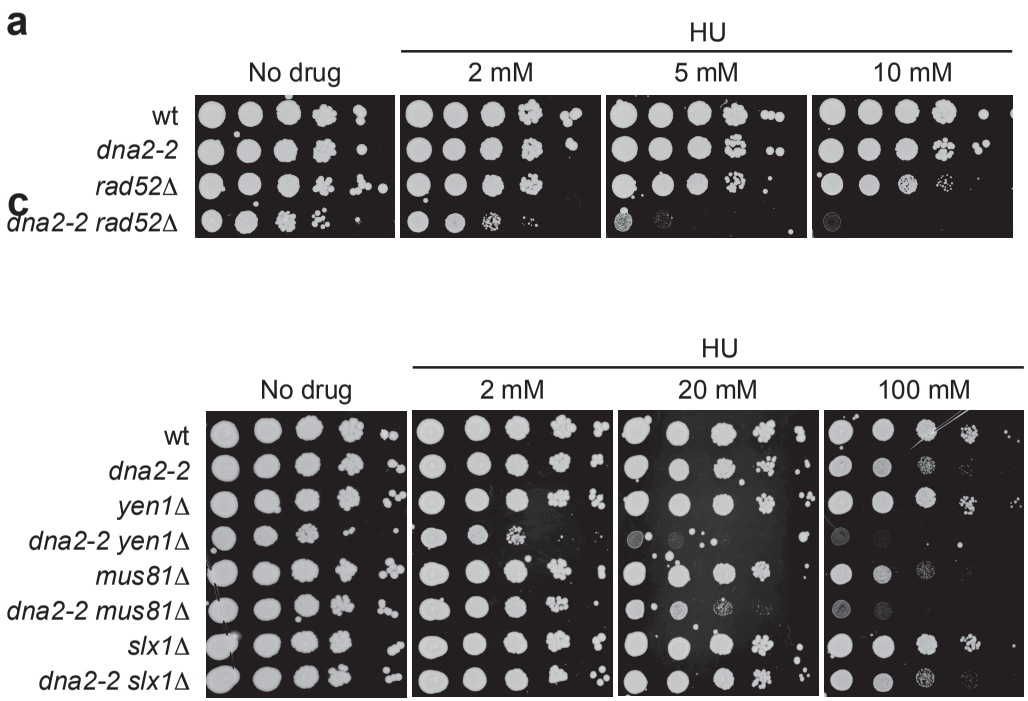






Ölmezer_Fig. 5

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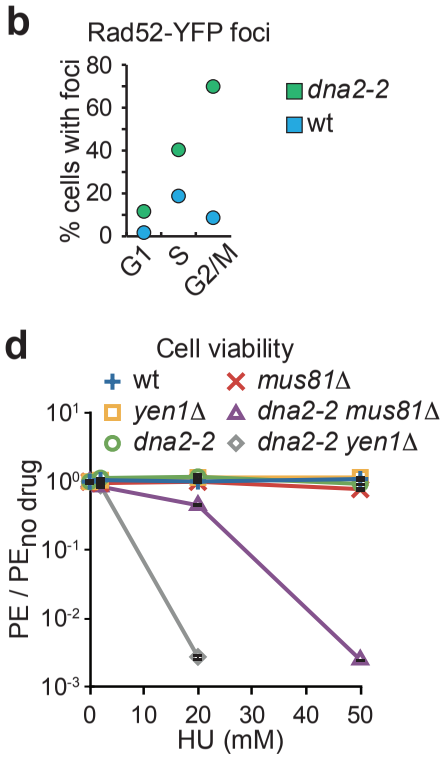
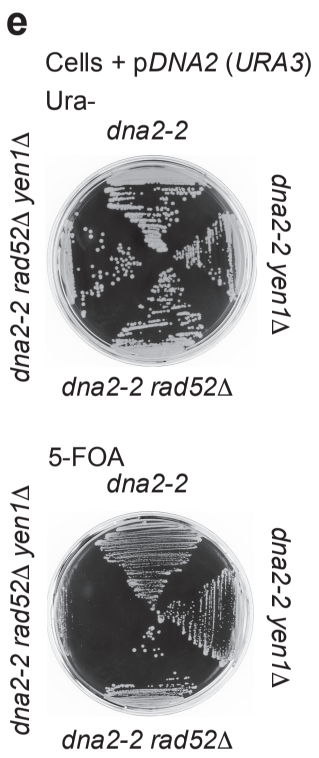
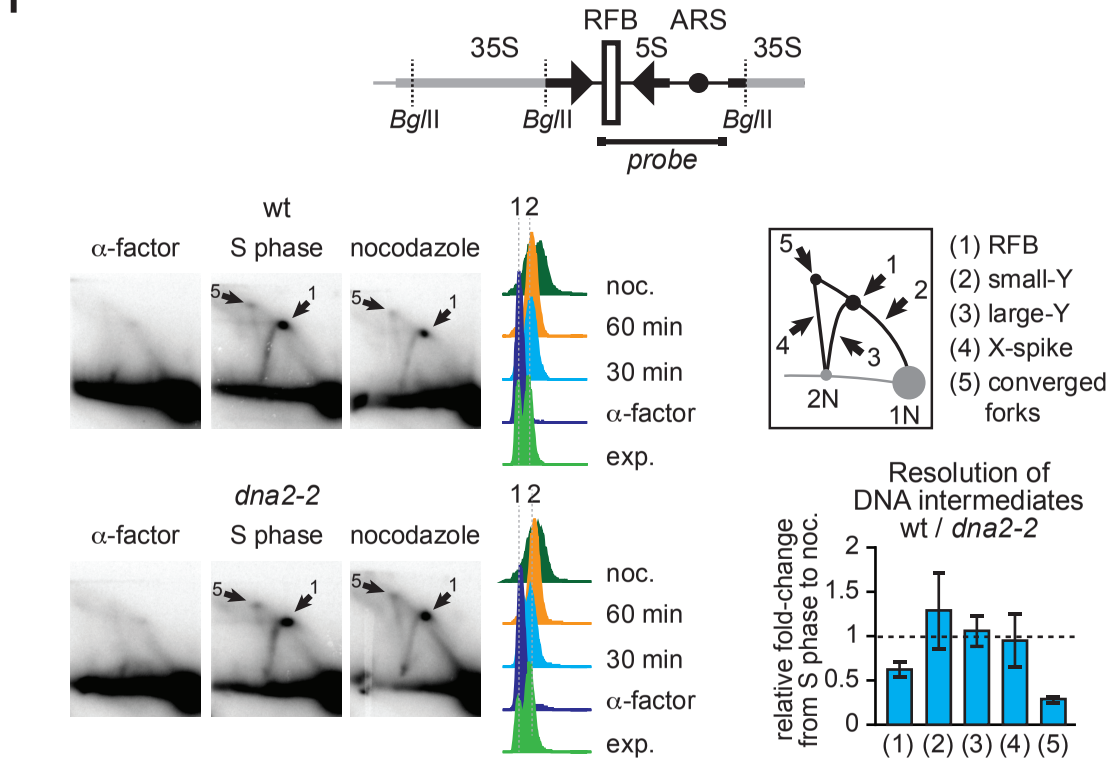
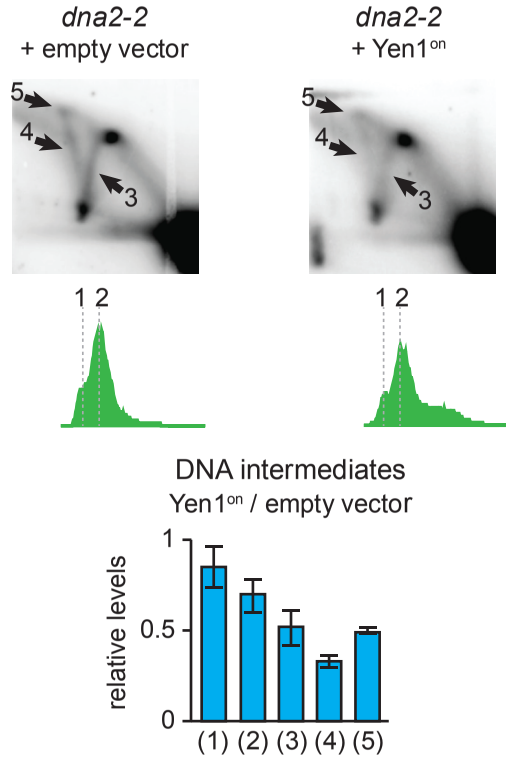
wt
dna2-2
rad52Δ
dna2-2 rad52Δ
c
HU
No drug
2 mM
20 mM
100 mM
wt
dna2-2
yen1Δ
dna2-2 yen1Δ
mus81Δ
dna2-2 mus81Δ
slx1Δ
dna2-2 slx1Δ


b
Rad52-YFP foci
% cells with foci
80
60
40
20
0
G1
S
G2/M
dna2-2
wt
d
Cell viability
wt
mus81Δ
yen1Δ
dna2-2 mus81Δ
dna2-2
dna2-2 yen1Δ
PE / PEno drug
10¹
10⁰
10⁻¹
10⁻²
10⁻³
0
10
20
30
40
50
HU (mM)

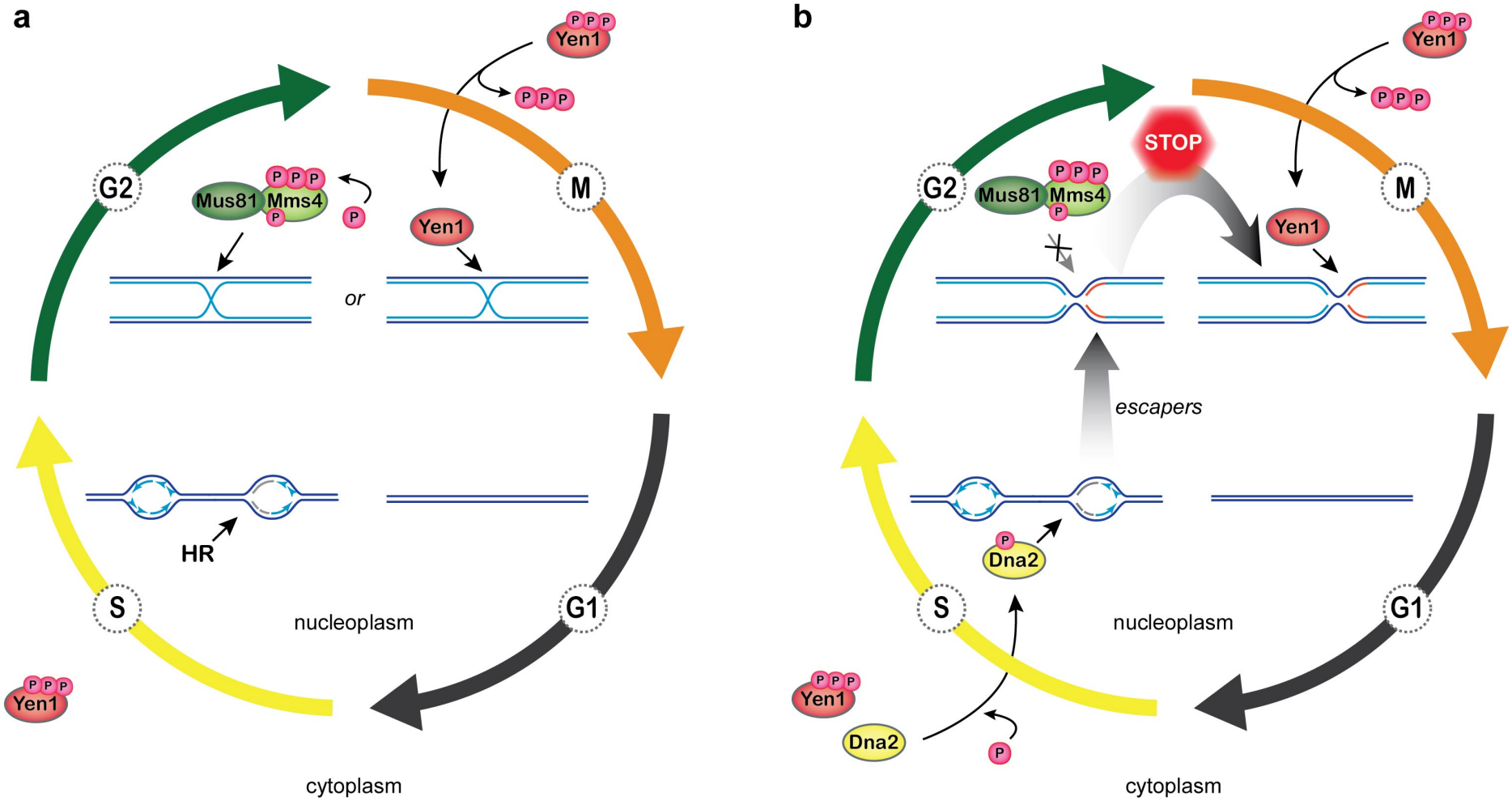

e
Cells + pDNA2 (URA3)
Ura-
dna2-2
dna2-2 rad52Δ yen1Δ
dna2-2 yen1Δ
dna2-2 rad52Δ
5-FOA
dna2-2
dna2-2 rad52Δ yen1Δ
dna2-2 yen1Δ
dna2-2 rad52Δ

Ölmezer_Fig. 6

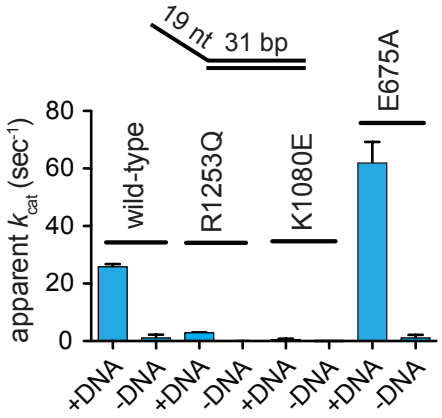


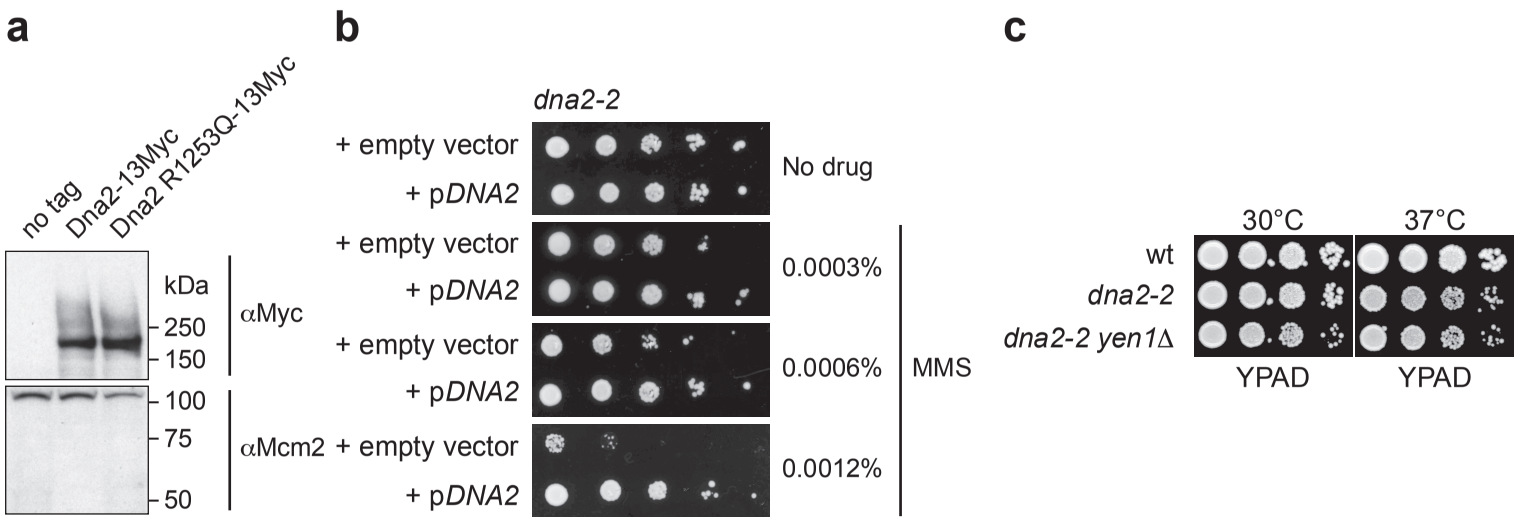


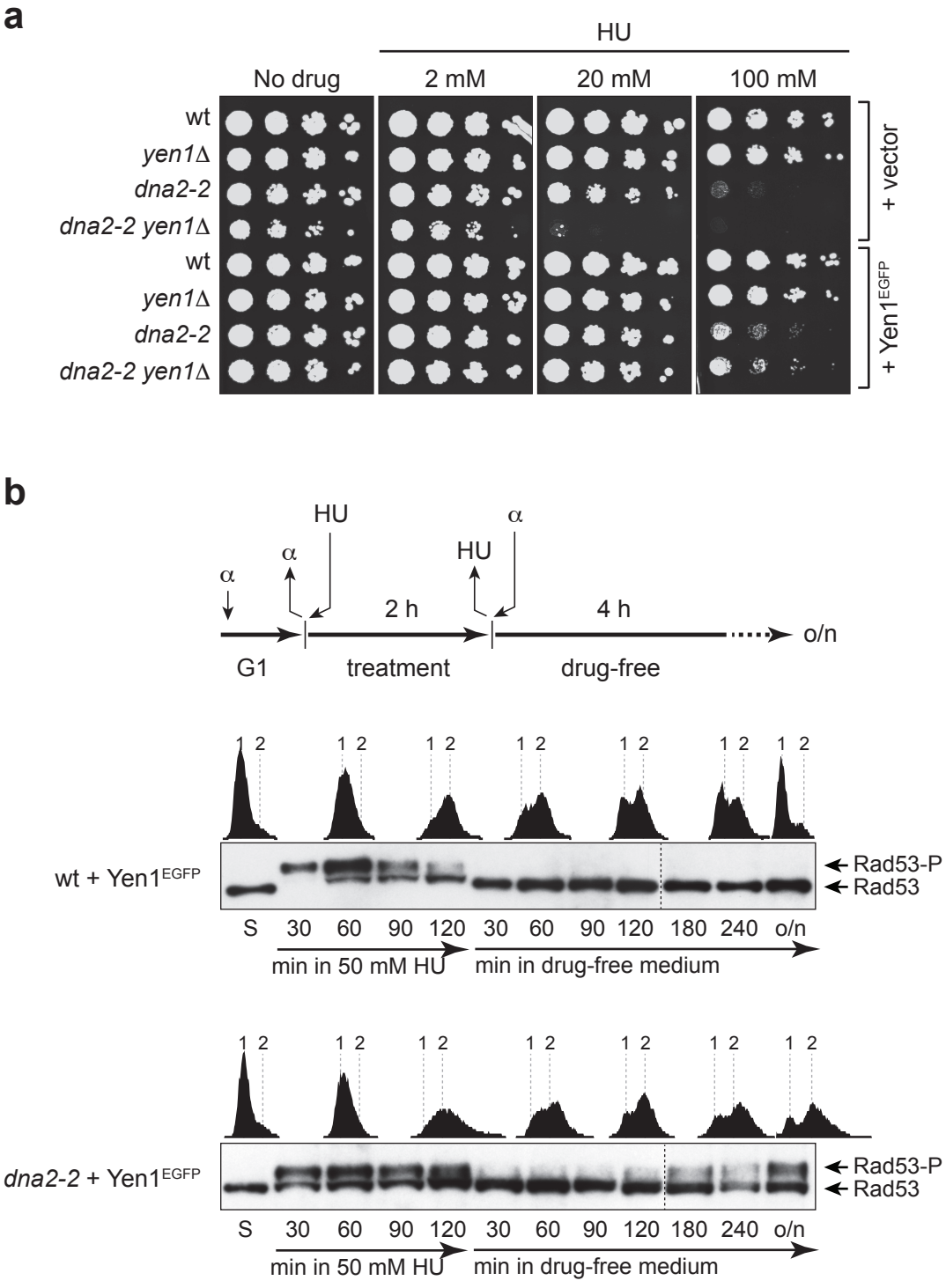
Ölmezer_Fig. 7

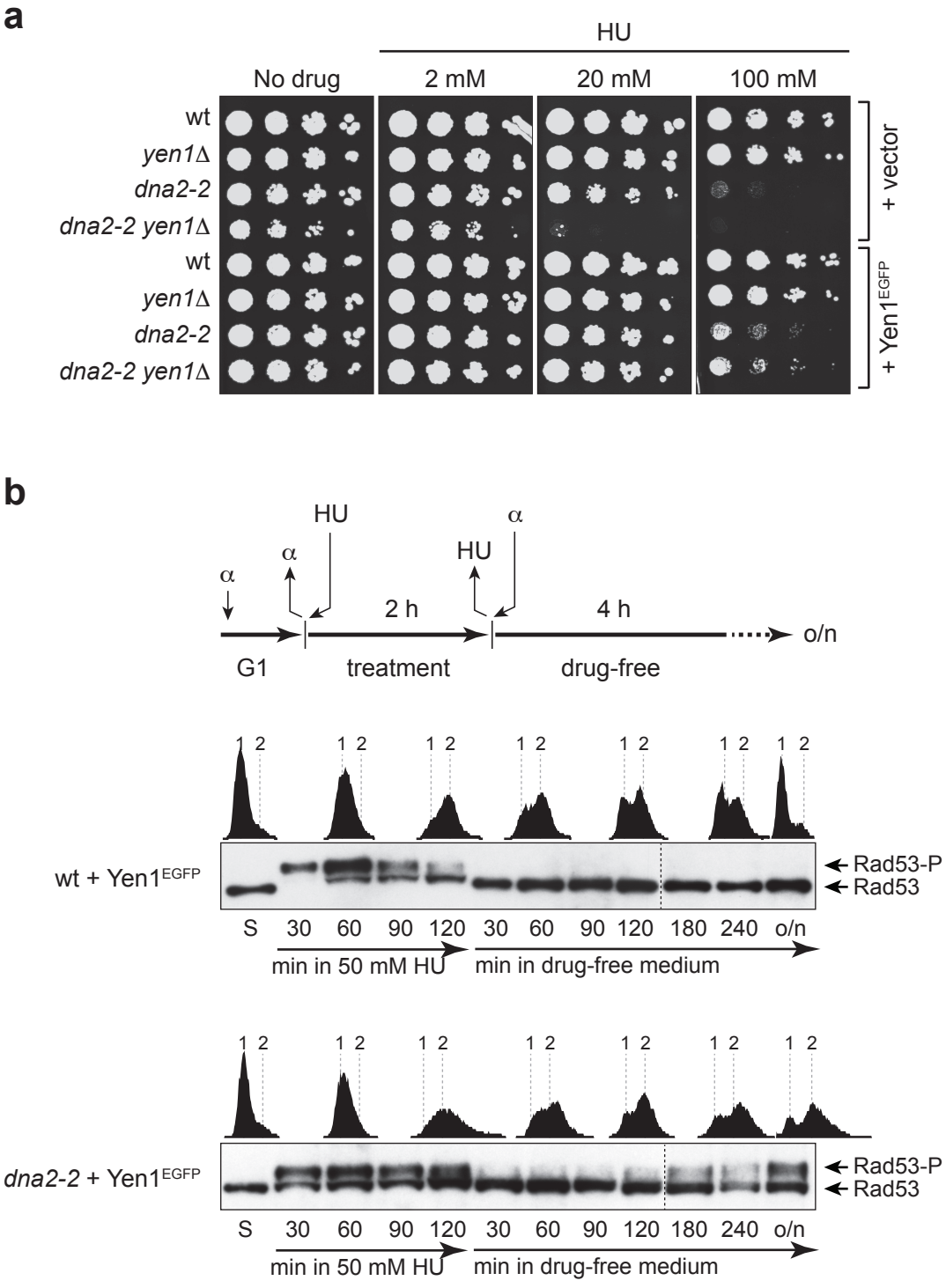


Ölmezer_Supplementary Fig. 1

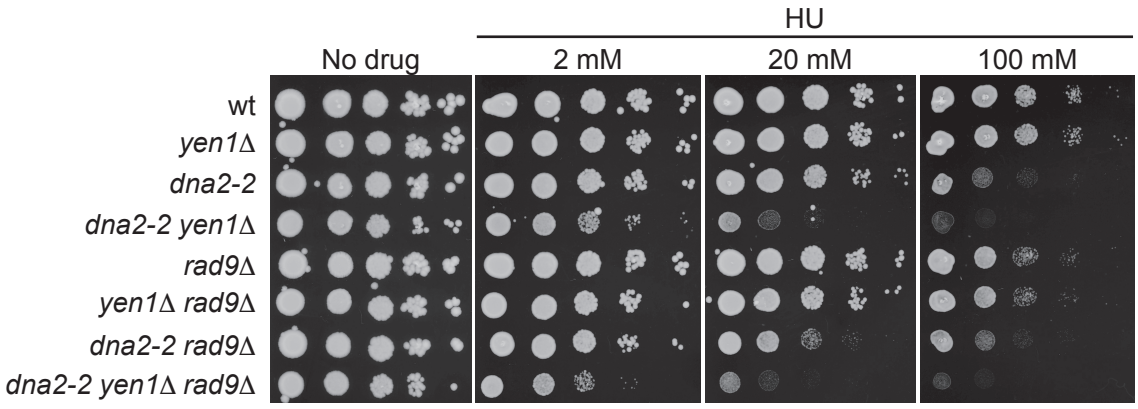



a
no tag
Dna2-13Myc
Dna2 R1253Q-13Myc
kDa
250
150
100
75
50
αMyc
αMcm2
b
dna2-2
+ empty vector
+ pDNA2
No drug
0.0003%
0.0006%
0.0012%
MMS
c
30°C
37°C
wt
dna2-2
dna2-2 yen1Δ
YPAD
YPAD

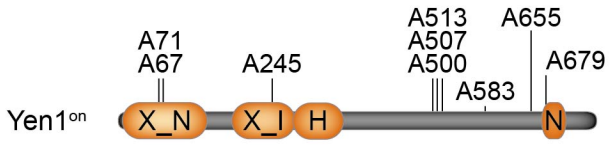

a
HU
No drug
2 mM
20 mM
100 mM
wt
yen1Δ
dna2-2
dna2-2 yen1Δ
wt
yen1Δ
dna2-2
dna2-2 yen1Δ
+ vector
+ Yen1EGFP
b
α
α
HU
α
HU
2 h
4 h
o/n
G1
treatment
drug-free
1 2
wt + Yen1EGFP
Rad53-P
Rad53
S 30 60 90 120 30 60 90 120 180 240 o/n
min in 50 mM HU
min in drug-free medium
dna2-2 + Yen1EGFP
Rad53-P
Rad53
S 30 60 90 120 30 60 90 120 180 240 o/n
min in 50 mM HU
min in drug-free medium


a
HU
No drug
2 mM
20 mM
100 mM
wt
yen1Δ
dna2-2
dna2-2 yen1Δ
wt
yen1Δ
dna2-2
dna2-2 yen1Δ
+ vector
+ Yen1EGFP
b
α
α
HU
α
HU
2 h
4 h
o/n
G1
treatment
drug-free
1 2
wt + Yen1EGFP
Rad53-P
Rad53
S 30 60 90 120 30 60 90 120 180 240 o/n
min in 50 mM HU
min in drug-free medium
dna2-2 + Yen1EGFP
Rad53-P
Rad53
S 30 60 90 120 30 60 90 120 180 240 o/n
min in 50 mM HU
min in drug-free medium

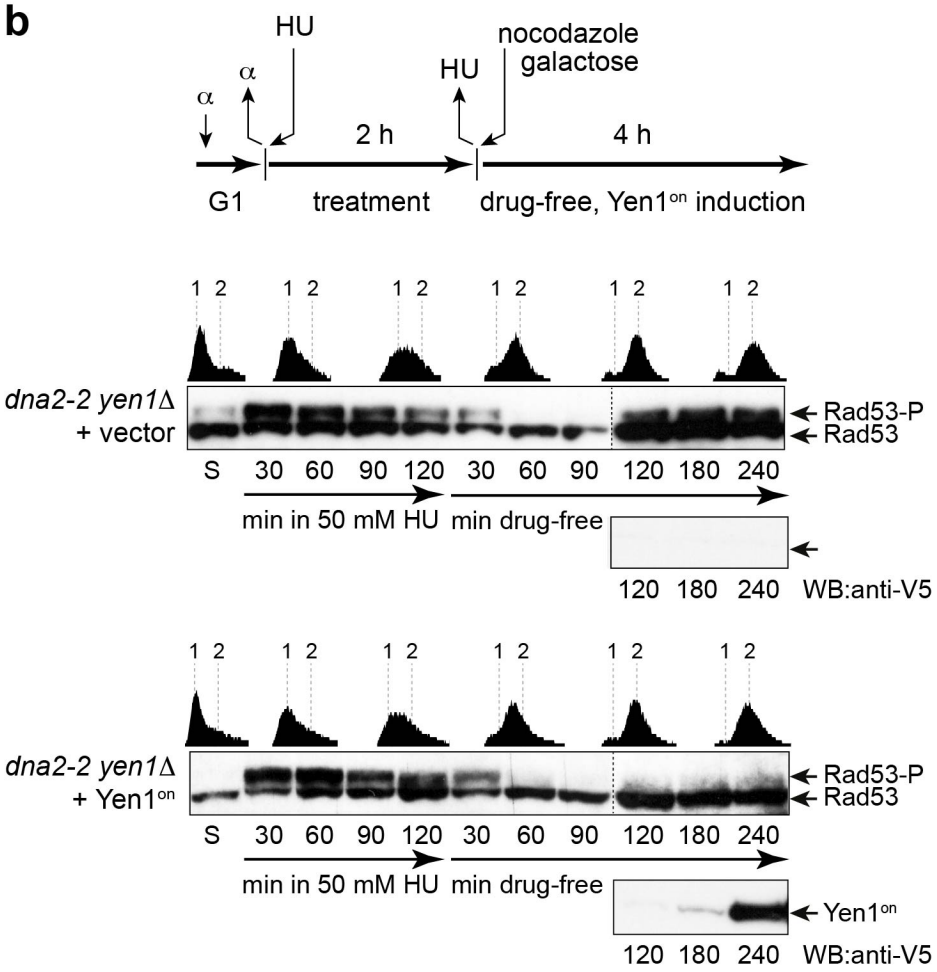
Ölmezer Supplementary Fig. 5

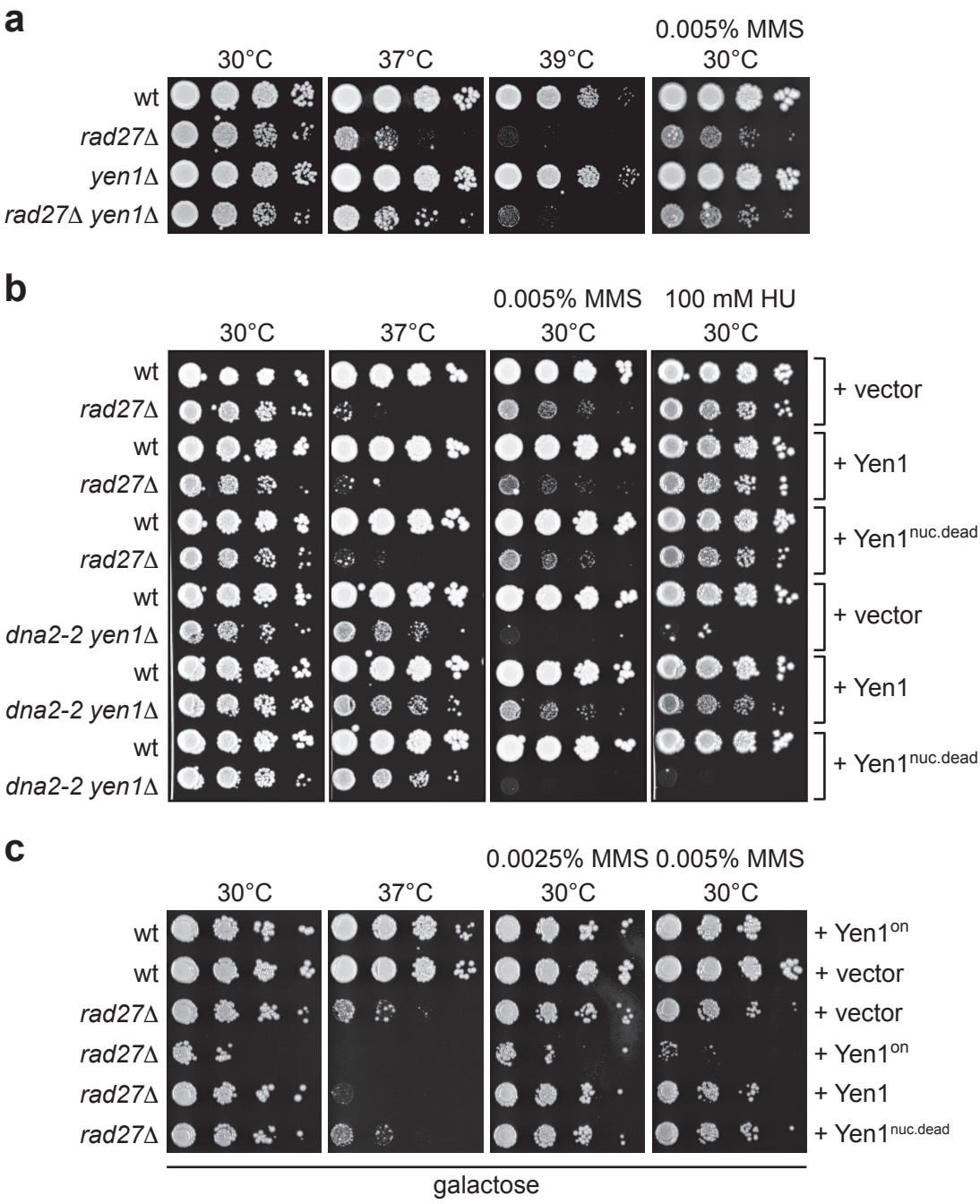


a

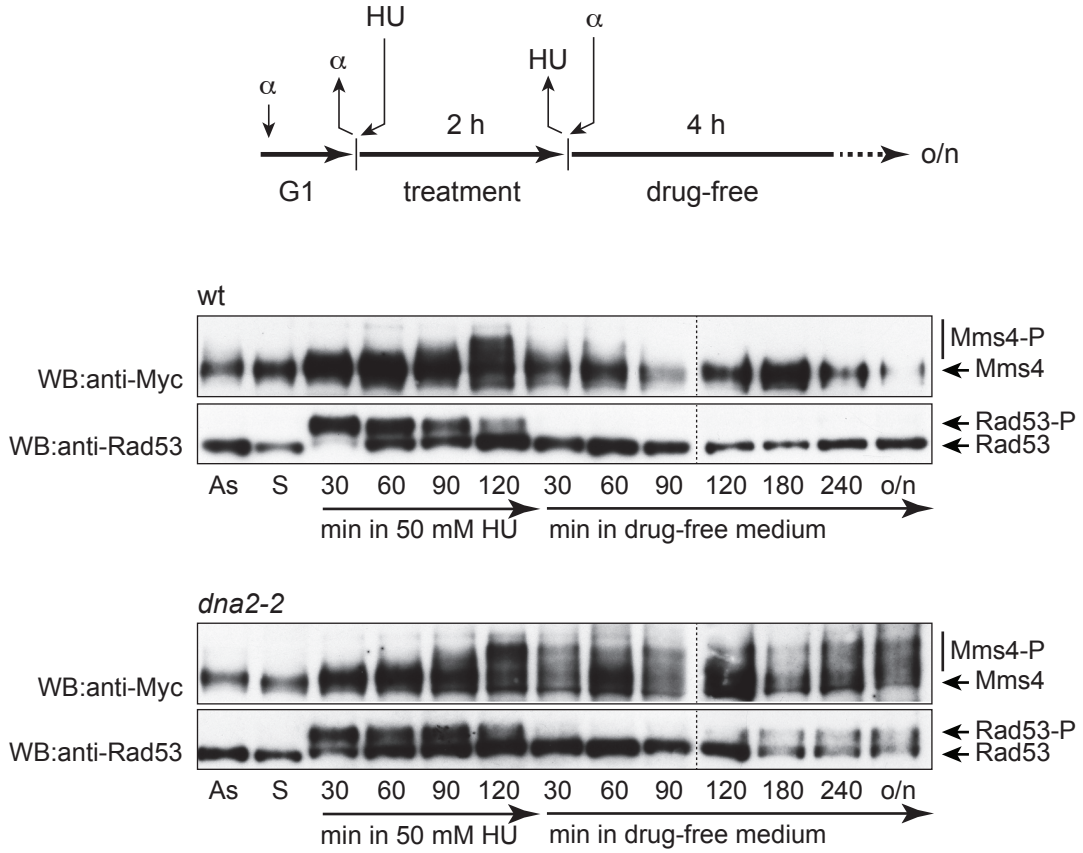

A71
A67
A245
A513
A507
A500
A583
A655
A679
Yen1on
X_N
X_I
H
N

b


HU
α
α
HU
nocodazole
galactose
2 h
4 h
G1
treatment
drug-free, Yen1on induction
1 2
dna2-2 yen1Δ
+ vector
Rad53-P
Rad53
S 30 60 90 120 30 60 90 120 180 240
min in 50 mM HU
min drug-free
120 180 240 WB:anti-V5
dna2-2 yen1Δ
+ Yen1on
Rad53-P
Rad53
S 30 60 90 120 30 60 90 120 180 240
min in 50 mM HU
min drug-free
Yen1on
120 180 240 WB:anti-V5


a
30°C
37°C
39°C
0.005% MMS
30°C
wt
rad27Δ
yen1Δ
rad27Δ yen1Δ
b
30°C
37°C
0.005% MMS
30°C
100 mM HU
30°C
wt
rad27Δ
wt
rad27Δ
wt
rad27Δ
wt
dna2-2 yen1Δ
wt
dna2-2 yen1Δ
wt
dna2-2 yen1Δ
+ vector
+ Yen1
+ Yen1nuc.dead
+ vector
+ Yen1
+ Yen1nuc.dead
c
30°C
37°C
0.0025% MMS
30°C
0.005% MMS
30°C
wt
wt
rad27Δ
rad27Δ
rad27Δ
rad27Δ
+ Yen1on
+ vector
+ vector
+ Yen1on
+ Yen1
+ Yen1nuc.dead
galactose

Ölmezer_Supplementary Fig. 8



4 Chapter 4: Additional Results

4.1 Functional overlap between Mus81 and Yen1 is due to Yen1's nuclease activity

YEN1 deletion does not lead to an obvious phenotype; however, *mus81 yen1* double mutants are extremely sensitive to replication stress (Blanco et al., 2010; Ho et al., 2010). This synthetic sick relationship indicates a partially overlapping role in replication-associated repair processes for both nucleases. Yen1's nuclease activity resides in the N-terminus, while the large C-terminal domain does not contain any recognizable domain (Ip et al., 2008; Rass et al., 2010). We wished to determine whether the C-terminus contributes to any function of Yen1 in compensating the absence of Mus81. Therefore, we constructed a catalytically dead version of Yen1 with mutations in the XPG-I domain (Yen1 E193A/E195A), which was expressed from the genomic locus both in the wild type and the *mus81* background, and analyzed these strains. We found that the genomic mutation of the nuclease active site had the same effect as a full deletion of *YEN1* (**Fig. 4-1**). We conclude that Yen1 compensates for loss of Mus81 purely by providing structure-specific nuclease activity. Furthermore, we assessed whether nucleocytoplasmic shuttling of Yen1 is required for backing up Mus81 function. We substituted S679 with alanine at the NLS-overlapping CDK consensus site, which was previously shown to promote nuclear localization throughout the cell cycle (Kosugi et al., 2009). Yen1 S679A, however, behaved as wild type Yen1 in terms of DNA damage resistance (**Fig. 4-1**). This is in good agreement with findings that cell cycle-related transition between the nucleus and the cytoplasm is imposed by several phosphorylation sites, rather than one, in the C-terminus of Yen1 (Blanco et al., 2014; Eissler et al., 2014).

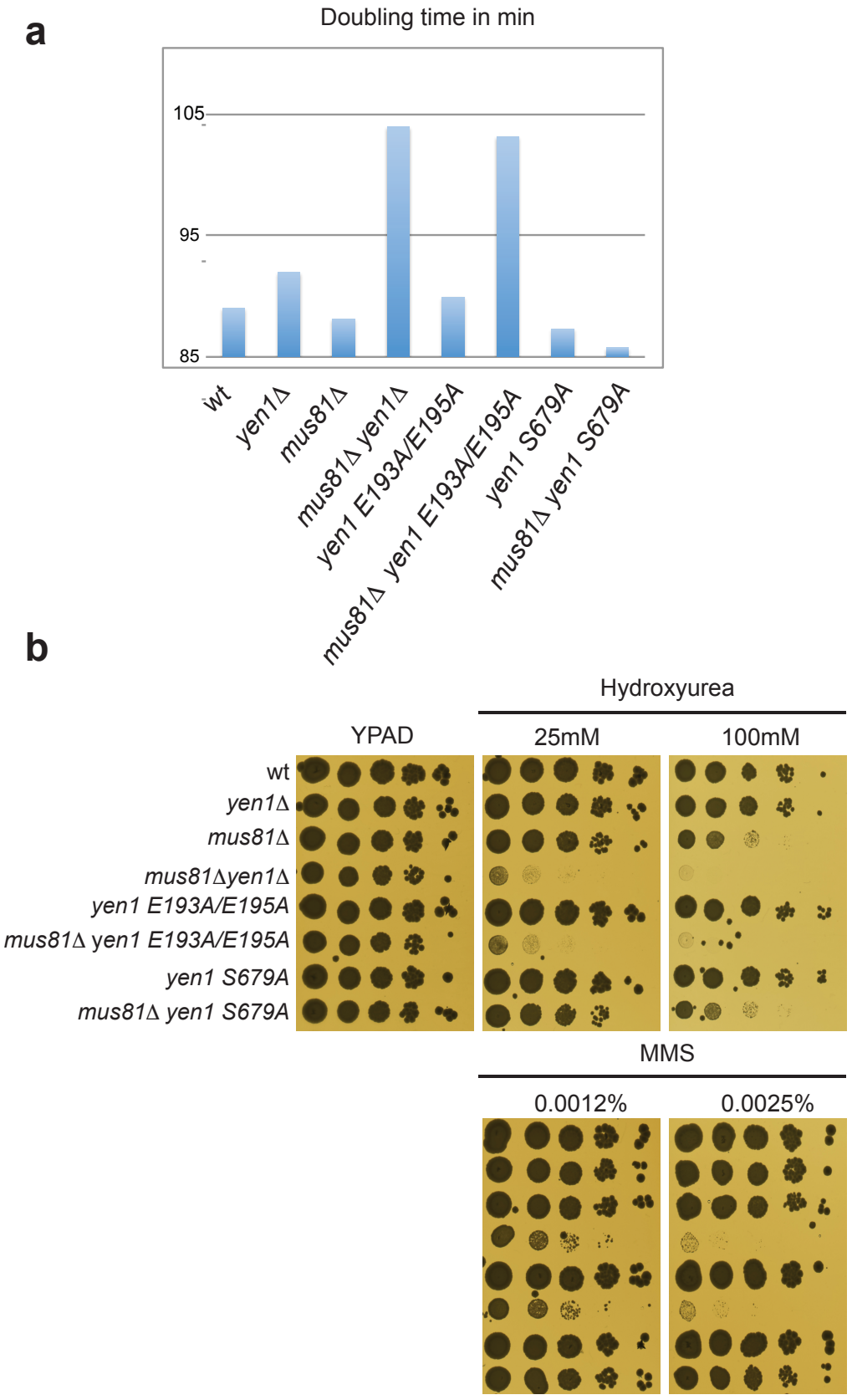


Figure 4-1 (a) Doubling times of *yen1* mutants compared to the parental strains with and without intact *MUS81*. (b) Drop assays to detect DNA damage sensitivity of the indicated strains were done by spotting normalized 10-fold serial dilutions of exponentially growing cells onto YPAD plates containing the indicated amounts of MMS, CPT, or HU.

4.2 Low temperature does not suppress the sensitivity of *dna2-2* and *dna2-2 yen1* cells to replication stress-inducing drugs

Growth at lower temperatures, which presumably slows down replication, did not suppress the dependence of *dna2-2* cells on Yen1 for growth, both, in the presence or absence of MMS and HU (**Fig. 4-2, Fig. 4-3**).

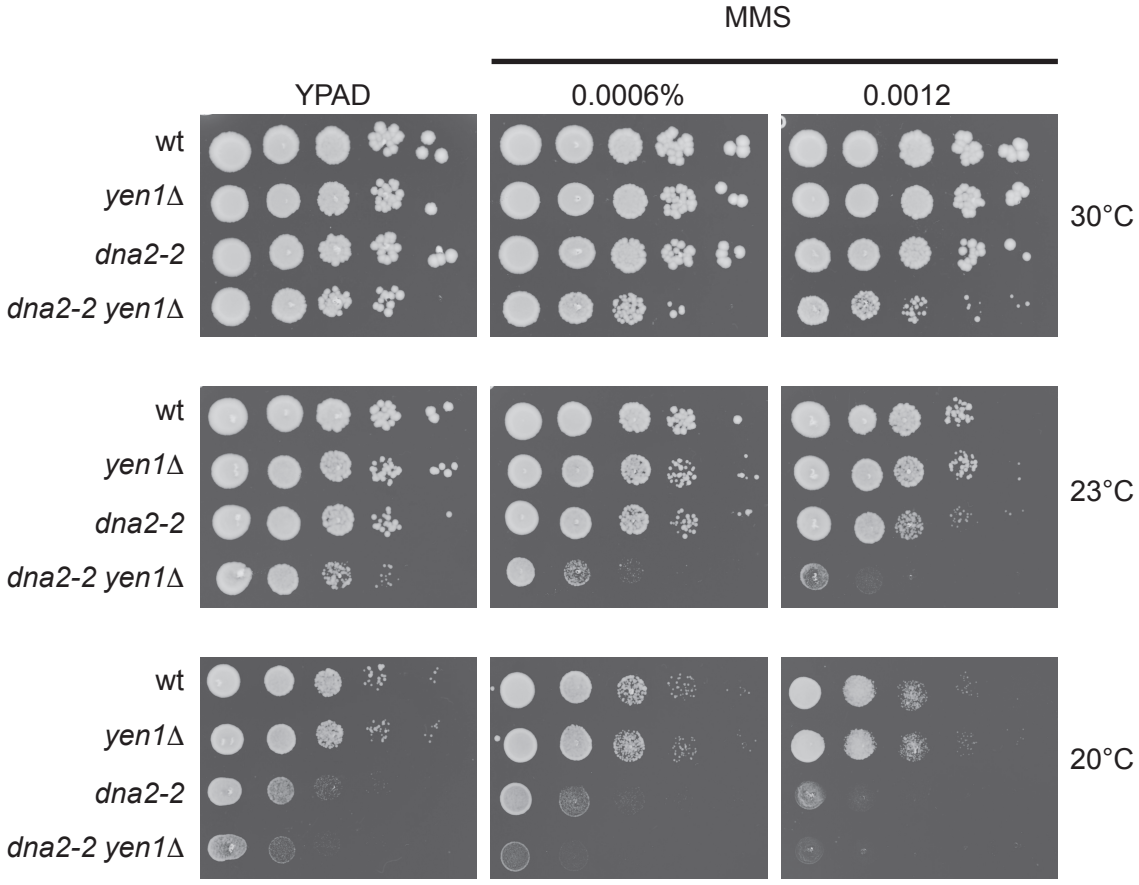


Figure 4-2 Growth defects of *dna2-2* cells and the synthetic sick interaction between *dna2-2* and *YEN1* is manifest when cells are grown at lower temperature in presence of MMS. Drop assays were performed as described for Fig. 4-1.

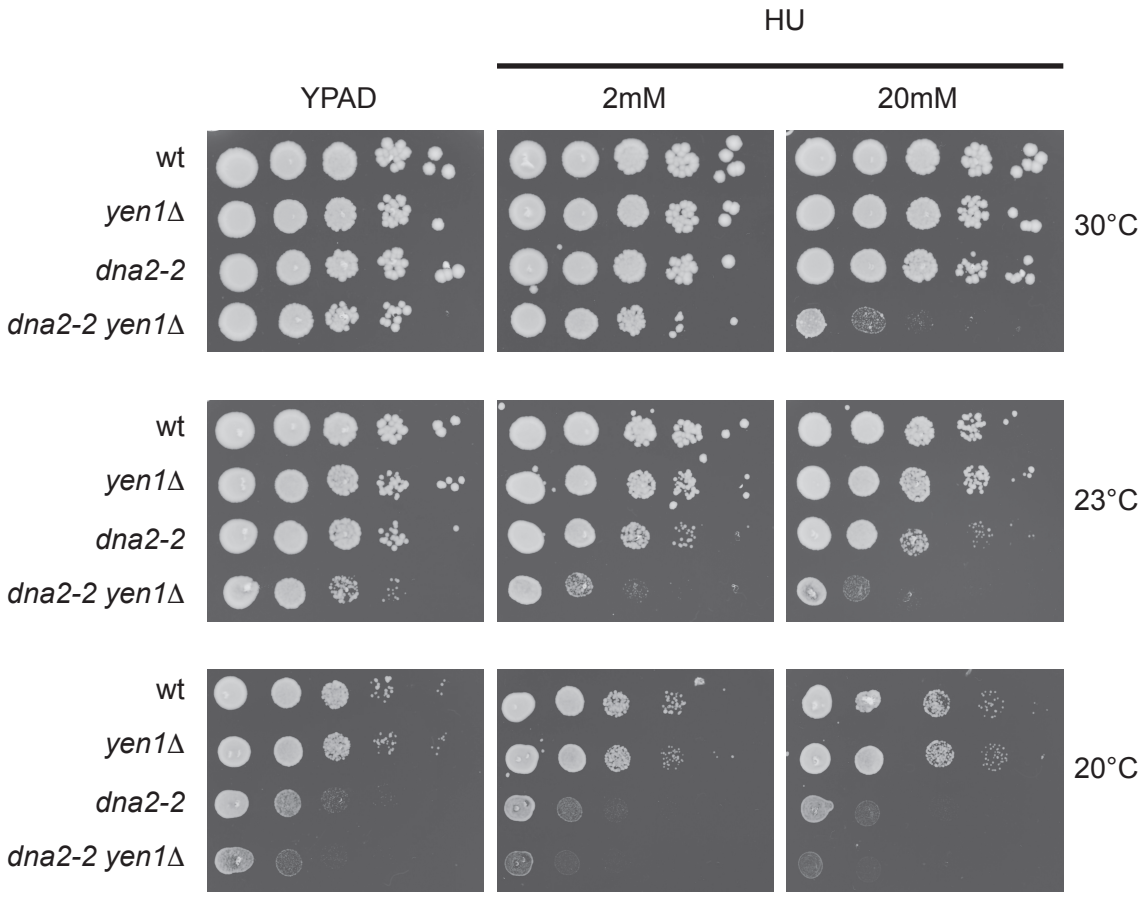


Figure 4-3 Growth defects of *dna2-2* cells and the synthetic sick interaction between *dna2-2* and *YEN1* is manifest when cells are grown at lower temperature in presence of HU. Drop assays were performed as described for Fig. 4-1.

4.3 Dna2 helicase-defective cells accumulate post-replicative chromosomal DNA links

As assessed by 2D gel electrophoresis, Dna2 helicase-defective cells show reduced resolution of RFB-stalled forks and converged fork structures as cells progressed from S phase into nocodazole-induced G2/M arrest (**Fig. 3-6g**). This raised the question whether fork-stalling events are restricted to rDNA. To directly assess the accumulation of unresolved chromatid entanglements across the genome, we analyzed the electrophoretic mobility of chromosomes from wild type, *yen1*, *dna2-2*, and *dna2-2 yen1* by pulsed-field gel electrophoresis (PFGE) after globally induced RF arrest. Cells were synchronized in G1 and then released into medium containing 200 mM HU for 2 hours. Under these conditions all strains retained the ability to restart and complete bulk DNA synthesis upon removal of the drug, and cell division occurred 180 min post treatment in the wild

type, concomitant with DNA damage checkpoint activation in *dna2-2* and *dna2-2 yen1* cells (**Fig. 4-4**). As expected, chromosomes from G1 arrested cells migrated into the PFGE gel, but failed to enter the gel after cells had been released into S phase, due to the presence of arrested RFs (**Fig. 4-5**). Cells were then recovered in YPAD medium in the presence of nocodazole to allow only a single round of replication and no further cell cycle progression, and genomic DNA was subjected to PFGE after 240 min, a time at which all cells had reached G2 as indicated by a 2N DNA content determined by flow cytometry. Next, we quantified the chromosomal material resolved for G1 cells and G2 cells after recovery from HU. Compared to the wild type and *yen1* strains, which doubled their DNA content efficiently, PFGE-resolved chromosomal DNA is significantly lower for both *dna2-2* and *dna2-2 yen1* strains (average fold-change of 1.6 and 2, respectively). This reduction was observed across all the chromosomes. These results indicate that Dna2 helicase dysfunction leads a global replication defect – independently of presence or absence of Yen1- that leads to accumulation of post-replicative inter-sister chromatid DNA links.

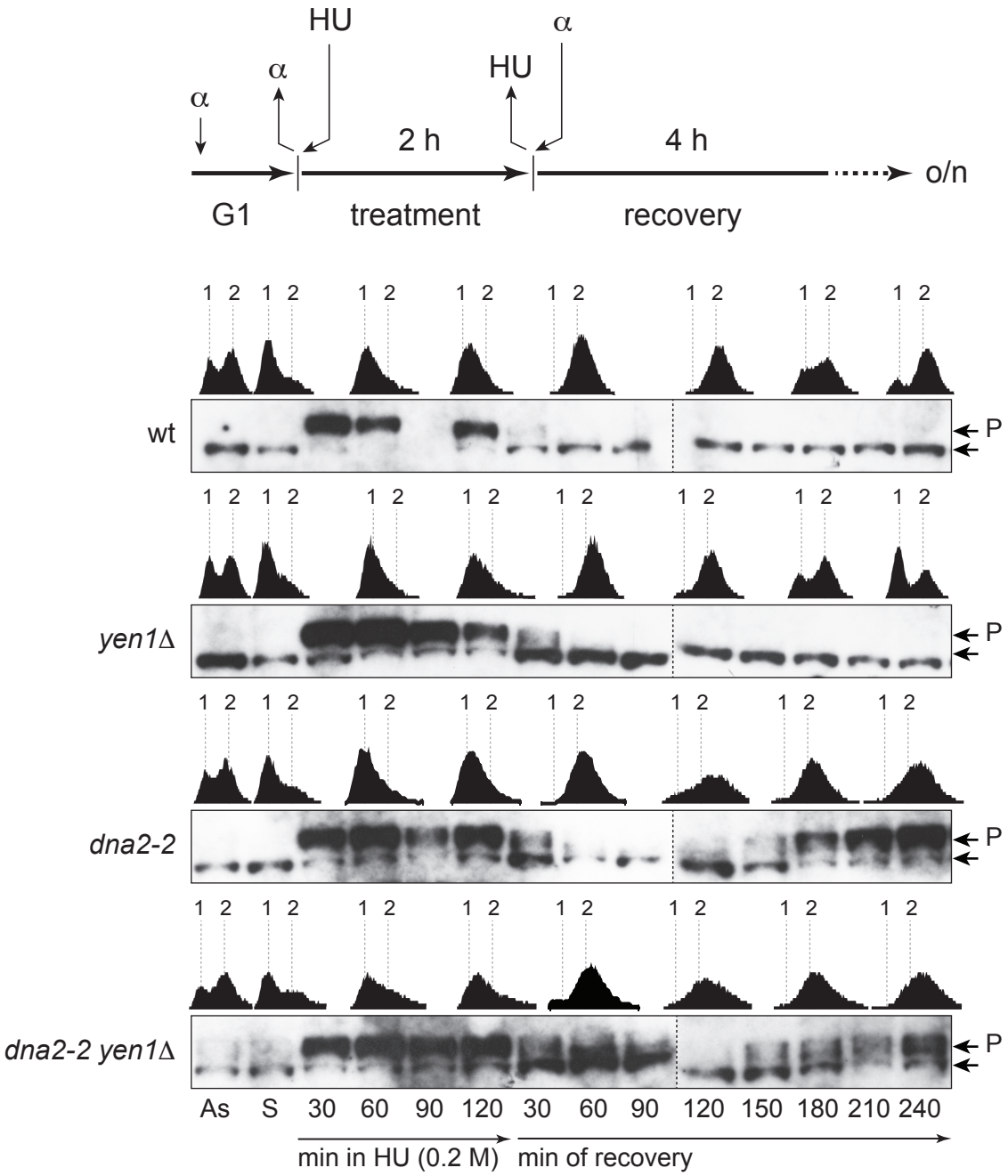


Figure 4-4 Mitotic time-course after global RF arrest. Cells were synchronized in G1 and released into S phase in the presence of 200 mM HU for 2 h and recovered in YPAD. The experiment was carried out and analyzed as described in Chap. 4.6.3. Checkpoint activation and the progression of DNA replication were monitored by Western blot analysis of Rad53 phosphorylation (Rad53-P) and flow cytometry (1N and 2N DNA content indicated). As, asynchronous; S, synchronous.

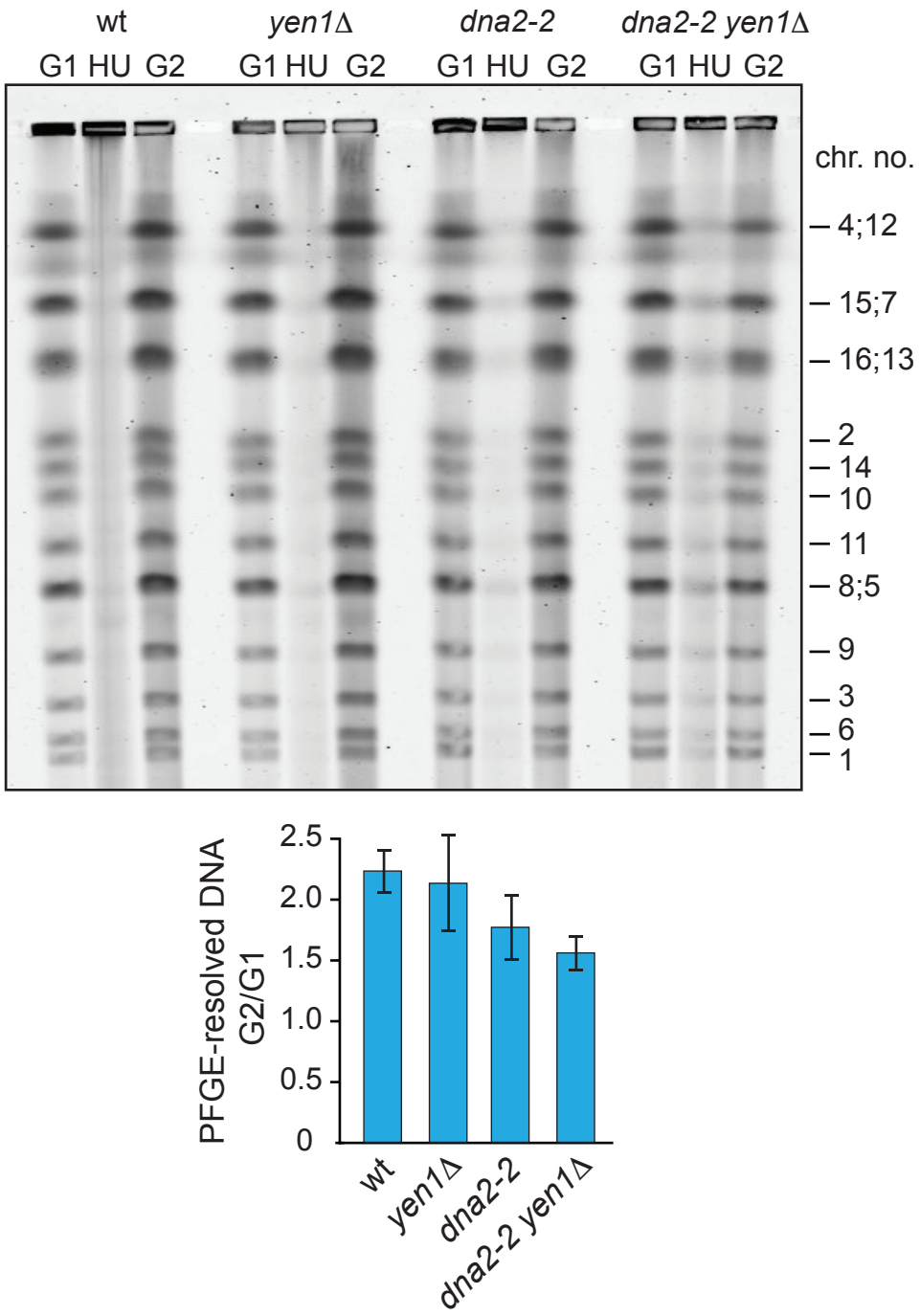


Figure 4-5 Representative PFGE analysis of cells treated as in Fig. 4.4, except for the addition of nocodazole to allow only one round of DNA replication; Quantification of gel-resolved chromosomal DNA bands. For experimental details, see Chap. 4.6.4.

4.4 *FOB1* deletion does not suppress *dna2-2* defects

Fob1 binds and enforces replication stalling at RFB, avoiding the collision between transcription and replication within the rDNA repeats (Sanchez et al., 1998). Previously, it was reported that *dna2-2* cells accumulate RFB-stalled forks and converging forks in

logarithmically growing cultures due to Fob1, leading to DSB formation and MMS sensitivity (Weitao, 2003). Therefore, functions of Dna2 helicase in fork recovery appeared limited to the rDNA. Our 2D gel analysis confirmed the persistence of the same DNA intermediates; namely stalled forks at RFB, and converging forks. However, in our hands, *FOB1* deletion, despite slightly improving growth (**Fig. 4-6a**) – an effect seen in all mutants as well as wild type cells – failed to suppress the MMS and HU sensitivity of *dna2-2* cells (**Fig. 4-6b**). Thus, the role of the Dna2 helicase activity in recovering paused RFs is not be limited to the rDNA and is important throughout the genome, as also implied by the PFGE analysis presented above (see **Fig. 4-5**).

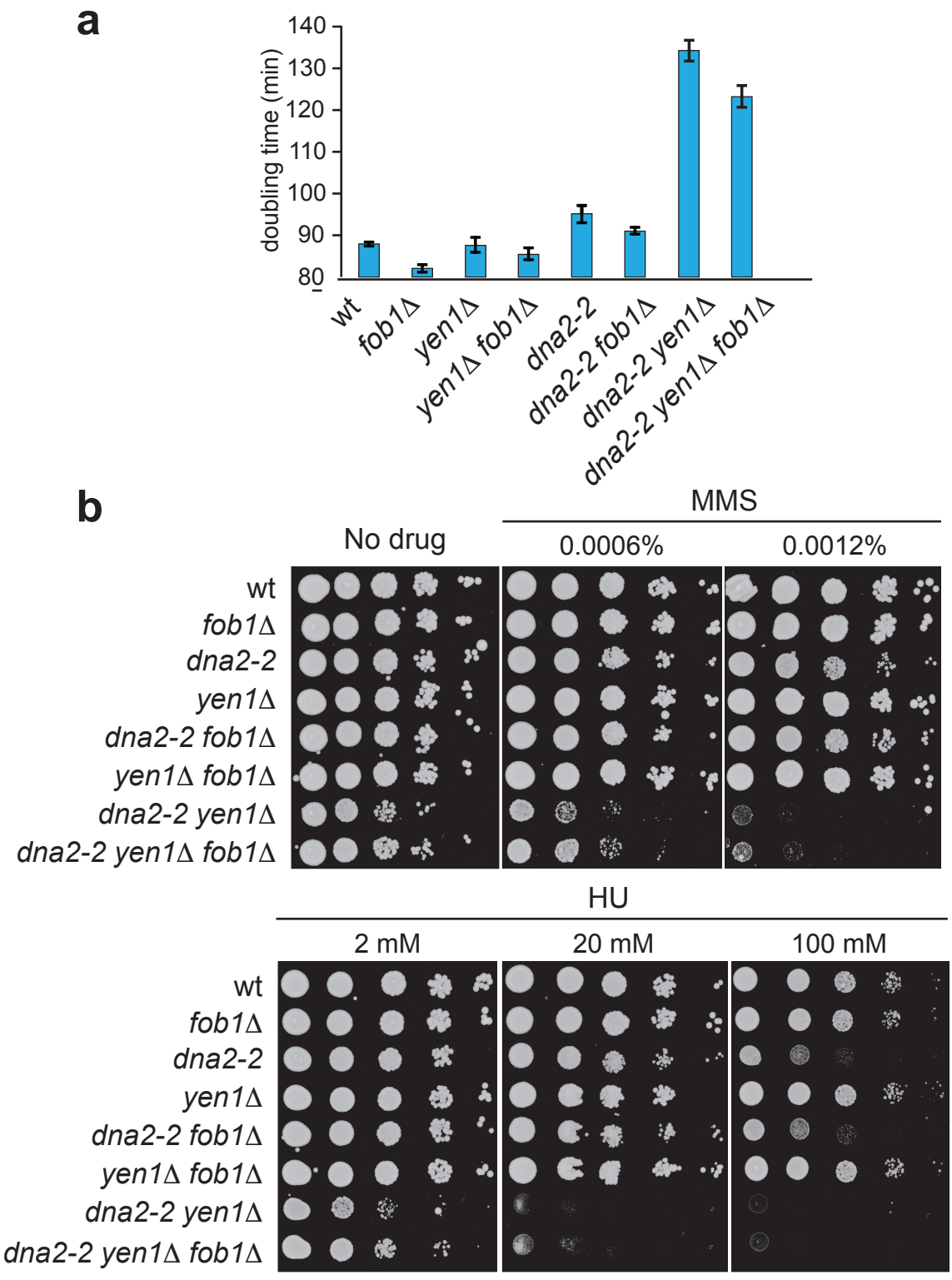


Figure 4-6 (a) Average doubling times of *FOB1*-deleted strains compared to the corresponding parental strains. (b) Drop assays to determine the effect of a *FOB1* deletion on the drug-sensitivity of the indicated strains. Drop assays were performed as described for Fig. 4-1.

4.5 Suppression of the MMS sensitivity of *dna2-2* mutants by Pif1 helicase mutants

We made the observation that *dna2-2* strains give rise to spontaneous suppressor mutants when grown in MMS. These suppressor mutants subsequently grow at MMS concentrations that are lethal to *dna2-2*. To identify the responsible mutation, we sequenced *DNA2* (making sure suppression was not due to reversion or compensatory mutations in Dna2) and genes that have previously implicated in suppressing *dna2* defects (Budd et al., 2006; 2005; Formosa and Nittis, 1999; Stith et al., 2008). To our surprise, all suppressor mutations analyzed mapped to the *PIF1* helicase (**Fig. 4-7**). Even though the extent of suppression changes between mutants, many restored wild type resistance levels in presence of 0.01% MMS (**Fig. 4-8**). This suggests that Pif1, at least in part, is responsible for the toxicity that arises in *dna2-2* cells under replication stress conditions.

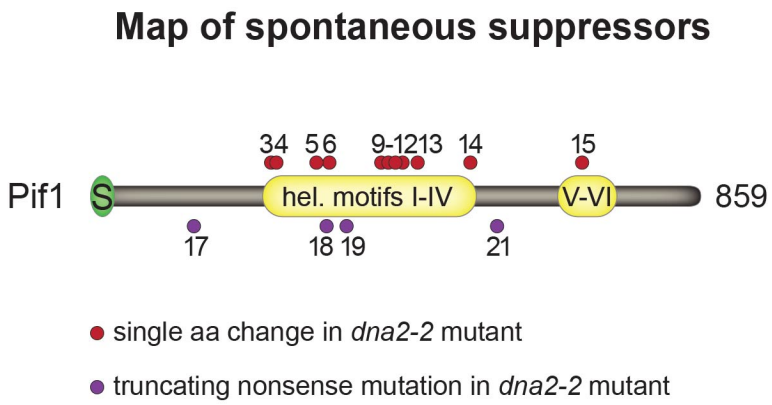


Figure 4-7 Map of Pif1 mutations found in *dna2-2* cells with suppressed MMS sensitivity

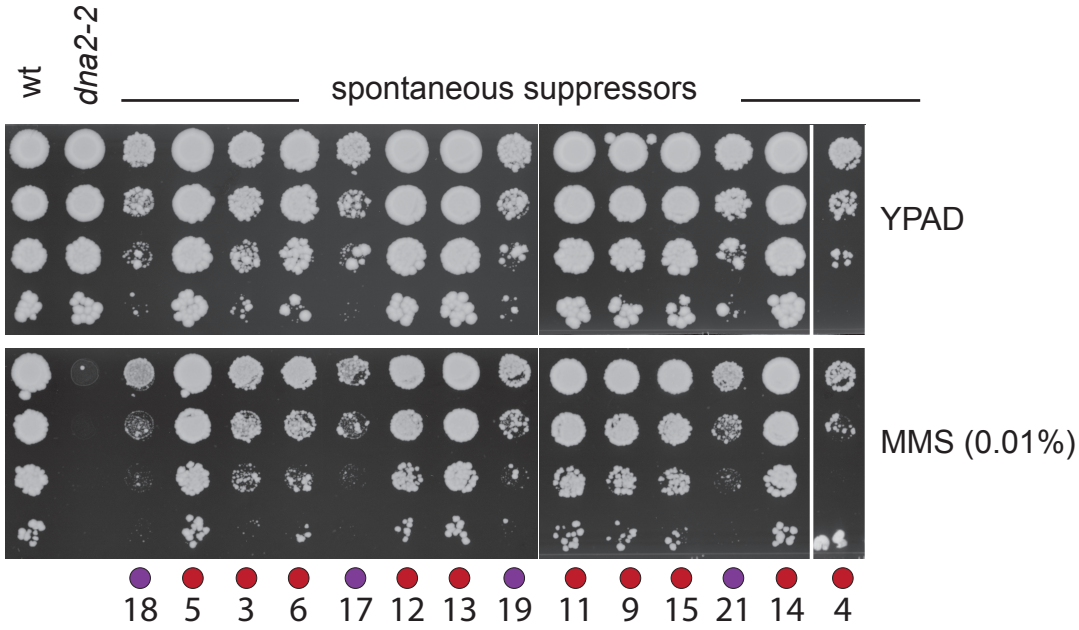


Figure 4-8 Spontaneous *dna2*-2 MMS sensitivity suppressor mutants. Serial dilutions of exponentially growing cells were spotted on to plates with 0.01% MMS.

Pif1 is a 5′ to 3′ helicase implicated in multiple pathways promoting genome stability. These roles include telomere elongation (Ivessa et al., 2000; Zhou et al., 2000), replication through problematic DNA structures (Paeschke et al., 2013; 2011), maintenance of fork pausing at natural pause sites (Ivessa et al., 2000). Recently, Pif1 has been implicated in fork reversal (Rossi et al., 2015). This is intriguing, because it points to a potential interplay of Pif1 and Dna2 at stalled forks, whose activities at the fork may have to be finely balanced. A detailed analysis of this relationship between Dna2 and Pif1 is ongoing.

4.6 Materials and Methods

4.6.1 Yeast strains

The list of *S. cerevisiae* strains used is presented in **Table 4.7**.

4.6.2 Doubling time and drop assays

Doubling times were determined as described (Ölmezer et al., manuscript in revision). For drop assays, exponentially growing cells were normalized to 10^7 cells/ml, and 2 µl drops of 10-fold serial dilutions were spotted onto the appropriate medium with or without MMS or HU. If not stated otherwise, plates were incubated for 3–4 days at 30 °C.

4.6.3 Mitotic time-course experiment

Cells were grown exponentially (OD_{600} = 0.4–0.6) and synchronized by addition of α-factor. Then, cells were released in medium containing 200 mM HU for 2 h, followed by drug wash-out and incubation in drug-free medium. Aliquots for flow cytometry and Western blot analysis were withdrawn at regular intervals. FACS and analysis of Rad53-phosphorylation with Western blot were performed as described (Ölmezer et al., manuscript in revision).

4.6.4 Analysis of chromosomal replication by pulsed-field gel electrophoresis

Cells were grown exponentially (OD_{600} = 0.4–0.6), synchronized in G1 by addition of α-factor, and released into YPAD medium containing 200 mM HU and nocodazole to allow cells entering S phase only one round of replication. Aliquots containing 5 x 10^7 cells were removed before replication (α-factor arrest), after HU treatment for 2 h, and post replication (4 h after release from HU), and resuspended in a buffer containing 50 mM NaPO4 (pH 7), 50 mM EDTA, and 1 mM dithiothreitol. PFGE analysis was done using a CHEF-DR II system (Bio-Rad). CleanCut agarose (1%)-embedded genomic DNA plugs were prepared as described by the manufacturer. For cell lysis, plugs were incubated in the presence of 0.4 mg/ml Zymolyase (20T, Seikagaku) for 1 h at 37°C, and treated with 1 mg/ml proteinase K (Eurobio) in 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 1% sarkosyl at 50°C overnight. Agarose plugs were embedded in a 1% agarose gel prepared with 0.5 x TBE buffer and electrophoresis was performed at 6 V/cm with switch times of 60 s for 15

h, then 90 s for 7 h, at 14°C. Ethidium bromide stained chromosomal DNA was imaged with a TyphoonTM 9400 system (GE) and quantified using ImageQuant TLv2005 software (GE).

4.6.5 Spontaneous suppressor collection

Suppressor colonies were collected after growth on plates containing 0.005% MMS for 4-5 days at 30°C. After a round of plating on YPAD plates, genomic DNA was isolated and the *PIF1* allele of each candidate were PCR-amplified and sequenced. For drop assays, serial dilutions of exponentially growing cells were spotted on to plates with 0.01% MMS.

4.7 Table of strains

Strain	**Relevant genotype**	**Source**
BY4741 (wild type)	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	GE Healthcare
clone ID 174	BY4741 *yen1Δ::KanMX4*	GE Healthcare
YRL96	BY4741 *dna2-2*	this study
YRL97	BY4741 *dna2-2 yen1Δ::KanMX4*	this study
YWL169	BY4741 *mus81Δ::HIS3*	{Blanco:2010iw}
YWL170	BY4741 *yen1Δ::KanMX4 mus81Δ::HIS3*	{Blanco:2010iw}
YRL82	BY4741 *yen1 E193A/E195A*	this study
YRL83	BY4741 *yen1 E193A/E195A mus81Δ::HIS3*	this study
YRL80	BY4741 *yen1 S679A*	this study
YRL81	BY4741 *yen1 S679A mus81Δ::HIS3*	this study

YRL101	BY4741 *fob1*Δ::*URA3*	this study
YRL102	BY4741 *yen1*Δ::*KanMX4 fob1*Δ::*URA3*	this study
YRL103	BY4741 *dna2-2 fob1*Δ::*URA3*	this study
YRL104	BY4741 *dna2-2 yen1*Δ::*KanMX4 fob1*Δ::*URA3*	this study

5 Chapter 5: Discussion and Future Perspectives

Yen1 (GEN1) is one of three conserved eukaryotic HJ resolving enzymes that act redundantly in the nucleolytic processing of late HR intermediates. The other HJ resolvases, which can associate to form a multi-nuclease complex (Princz et al., 2015), are Mus81-Mms4 (MUS81-EME1/EME2) and Slx1-Slx4 (SLX1-FANCP). These nucleases are temporally activity-controlled, and in yeast, Mus81-Mms4 activity peaks prior to Yen1 activity (West et al., 2015). Similarly, human MUS81-EME1 resolves HJs in G2/M, while GEN1 is strictly cytoplasmic, gaining access to the nuclear DNA only after nuclear envelope breakdown. Thus, nucleolytic HJ resolution is hierarchically organized, and Mus81/MUS81-dependent resolvases provide the major activity (Sarbajna and West, 2014). In yeast, HJ resolution defects associated with loss of *YEN1* are only apparent in the absence of Mus81-Mms4.

Here, we asked whether Yen1 might have roles that are distinct from the function shared with Mus81-Mms4. Our strategy was to investigate the molecular underpinnings of reported synthetic sick interactions of *YEN1*, which, at present, are limited to *PBY1* and *DNA2*. We falsified the purported *YEN1-PBY1* interaction, and in the process, unexpectedly, expanded the genetic interactome of *MUS81-MMS4*. The *YEN1-DNA2* interaction was verified, and our analyses have revealed a non-canonical function of Yen1. Importantly, by understanding the interplay between Yen1, Dna2, and the DNA damage checkpoint, we were able to define a key role for the Dna2 helicase, whose function has remained elusive, in the completion of DNA replication. These findings might help understand human pathologies associated with *DNA2*, such as cancer and Seckel syndrome.

Our starting point was the negative genetic interaction between *YEN1* and a novel factor, *PBY1*, whose chemical-genetic interaction, revealed in large-scale screens, suggested a role for Pby1 in DNA repair. We have reported that the *pby1* deletion strain in the widely used yeast knockout library carries a deletion mutation within the *MMS4* gene, not *PBY1*, explaining why the strain scored repeatedly in genome instability screens. We also showed that deletion of *PBY1* causes no synthetic sick phenotype in *yen1* mutant

cells. On the other hand, we demonstrated that the large body of data that has been generated with the Genome Deletion Project's BY4741 *pby1* strain identifies novel genetic interactions for the *MUS81-MMS4* HJ resolvase (Ölmezer et al., 2015). The molecular function of Pby1 remains to be determined at this point, and a suggested function associated with P-bodies is still controversial (Sweet et al., 2007). However, removal of the uncertainty that the spurious implication of Pby1 in DNA repair has caused, might expedite the elucidation of a P-body role, which has recently gained new support though protein localization studies showing Pby1 associated with cytoplasmic P-bodies (Tkach et al., 2012).

Next, we investigated the *YEN1-DNA2* interaction (Ölmezer et al., manuscript in revision). We found that cells harboring a helicase-dead version of Dna2, which is proficient in processing tailed DNA substrates and cleaving RPA-covered 5'-flaps, suffer from an inadequate response to replication stress. Moreover, they depend on Yen1 for survival. A further complication arises from the fact that Dna2 helicase-deficiency causes post-replicative DNA damage checkpoint signaling and a cell cycle delay at G2/M. This inhibits Yen1, whose activation/nuclear accumulation requires anaphase entry. We show that the nucleocytoplasmic shuttling pattern of Yen1 is maintained in *dna2-2* mutant cells. Thus, Yen1 remains largely excluded from the nucleus during replication stress-induced G2/M arrest. This provides an explanation for the slow recovery of Dna2 helicase-defective cells from replicative stress. Eliminating the DNA damage checkpoint by deletion of *RAD9* allows Dna2 helicase-defective cells to proceed into mitosis without delay after acute replication stress. Strikingly, this restored full cell viability in a strictly Yen1-dependent manner. In the absence of Yen1, however, cells exhibited chromosomal bridges connecting the two masses of segregating DNA and poor survival. Importantly, the aberrant DNA intermediates that Yen1 uniquely removes are not HR intermediates, given that *YEN1* exhibits synthetic sickness with *dna2-2* in the absence of *RAD52* and HR. Furthermore, Dna2 helicase-defective cells accumulate replication and recombination intermediates, and the levels of either were reduced upon expression of the constitutively active form of Yen1 ($Yen1^{on}$). Together, these data suggest that Yen1 safeguards chromosome segregation in Dna2 helicase-defective cells by removing unresolved replication, and not HR intermediates.

Could fork stalling in *dna2-2* cells be linked with the checkpoint function of Dna2 (Friedel et al., 2009; Kumar and Burgers, 2013)?. This seems unlikely given that we observe a normal S-phase checkpoint response in HU-treated *dna2-2* cells (Ölmezer et al., manuscript in revision). Having said this, the N-terminus of Dna2, which provides a redundant means of Mec1 activation, deserves further attention. Thus, it harbors several Cdk1 target sites and NLS motifs (Chen et al., 2011; Kosugi et al., 2009), and removing it causes temperature sensitivity (Bae et al., 2001b; Liu et al., 2000). It would therefore be interesting to explore the involvement of the N-terminus and proper Dna2 localization in the replication stress response role of Dna2 described herein.

Biochemical data have pointed to an Okazaki fragment maturation role for the Dna2 nuclease activity, specifically in processing occasional long 5′ flaps. This idea is mainly based on a negative genetic interaction with the major Okazaki fragment processing nuclease, Rad27 (Bae et al., 2001a; Budd and Campbell, 1997). It raises the possibility that the replication problems in *dna2-2* cells might be due the accumulation of 5′ flaps, which are also within the substrate spectrum of Yen1 (Blanco et al., 2010; Ip et al., 2008). However, our biochemical analyses and previous studies demonstrate that helicase-dead Dna2 variant R1253Q is fully proficient in flap cleavage and end resection activities (Ölmezer et al., manuscript in revision) (Zhu et al., 2008). This does not rule out an involvement of the helicase domain of Dna2 in potential actions of the Dna2 nuclease in Okazaki fragment processing, but indicates that the crosstalk between Yen1 and the Dna2 helicase activity is highly unrelated to Okazaki fragment processing. Consistently, we find no indication of a genetic interaction between *RAD27* and *YEN1* (Ölmezer et al., manuscript in revision). Moreover, even if Yen1 acted on Okazaki fragment-derived 5′ flaps in *dna2-2* cells, activation of Yen1 in anaphase would be ill-timed to support lagging strand synthesis.

We propose that the Dna2 helicase acts in parallel to Rad52-mediated HR to ameliorate the consequences of replication stress, for the first time defining a physiological function for the Dna2 helicase. This replication function was unmasked by removing the checkpoint and the compensatory actions of Yen1, which revealed that the Dna2 helicase activity is required to suppresses the formation of dead-end replication

intermediates, facilitating sister chromatid disjunction at mitosis. Our work throws up a number of specific questions regarding the actions of the Dna2 helicase at stalled forks, such as for example the execution point of its activities. This can be addressed by restricting the expression of Dna2 to certain cell cycle phases, using cell cycle-specific promoters, auxin-controlled degradation systems, or acute depletion. Another important issue is the relationship between the Dna2 helicase and the nuclease activity. The nuclease has been implicated in replication restart (Thangavel et al., 2015), and it could be that the nuclease and helicase cooperate in the RF recovery.

We show that the ability to protect Dna2-helicase defective cells is not shared between Yen1 and Mus81-Mms4. This is unprecedented, defining a unique requirement for Yen1 in a replication stress setting. In targeting dead-end replication intermediates, Yen1 safeguards chromosome segregation. This is different from canonical HJ resolution (directed at HR intermediates), which demonstrates greater complexity in the uses of HJ resolvases in cells. It will be interesting to explore the downstream steps in Yen1-dependent repair in this new context, and how general a requirement for Yen1 in resolving replication intermediates in mitosis is. It is conceivable that a requirement of Yen1 downstream of factors other than Dna2 has escaped detection in screens due to strong checkpoint activation, precluding the compensatory actions of Yen1. This could be circumvented in future screening efforts by eliminating the checkpoint. More immediately, however, the challenge lies in elucidating the exact nature of the toxic DNA structures that accumulate in *dna2-2* cells and the pathways that give rise to them.

Our findings have thrown a light on Dna2 at stalled RFs, providing a starting point to interrogate the rich, and often unexplained, genetic interactome of *DNA2* with the aim to further elucidate the cellular response to replication stress. To give one example, Dna2 helicase mutants were initially identified in a screen for mutants synthetically lethal with a *CTF4* deletion (Formosa and Nittis, 1999). *CTF4* is nonessential, but deletions cause similar defects to the ones observed in *dna2-2* mutants: elevated levels of chromosome loss and recombination, sensitivity to MMS, and a checkpoint-dependent G2/M delay (Formosa and Nittis, 1998). Furthermore, deletion of *RAD9* rescues the G2/M delay of *ctf4* mutants, yet, chronic replication stress causes a severe reduction in viability –

another phenotype shared between *dna2-2* and *ctf4* (Tanaka et al., 2009). Ctf4 (AND-1 in human) acts within the replisome, as a bridging factor between two subunits of DNA Pol α - primase (the catalytic subunit Pol1 and Pri-m4) and MCM (Gambus et al., 2009; Simon et al., 2014; Tanaka et al., 2009). Interestingly, human DNA2 was shown to bind to AND-1, although there is currently no evidence for a Dna2-Ctf4 interaction in yeast (Duxin et al., 2012). It is tempting to speculate that the Dna2 helicase may modulate the assembly or stability of replisome components upon recruitment by Ctf4. As a matter of fact, further studies identified negative genetic interactions between *dna2-2* and a number of genes whose absence destabilizes the replication machinery and compromises RF restart after stalling. These comprise several components of the replication progression complex (reviewed in (Errico and Costanzo, 2012)), including *mrc1*, *tof1*, and *csm3* (Budd et al., 2005), *spt16* {Formosa:2001jv} and *pob3* {Schlesinger:2000wc} of the FACT complex, *mcm10* (Araki et al., 2003), as well as *mms1* and *mms22* (Budd et al., 2005; Vaisica et al., 2011) of the $Cul4^{Ddb1}$-like $Rtt101^{Mms1/Mms22}$ E3 ubiquitin ligase, and *ctf18* (Crabbé et al., 2010; Formosa and Nittis, 1999) of the alternative clamp loader complex RFC^{CTF18}. These synthetic interactions of *dna2-2* might indicate a redundant or complementary function for Dna2 in promoting the processivity of DNA replication. Alternatively, they may hint at an important role of Dna2 in stabilizing and/or processing stalled or collapsed forks. Interestingly, increased replication fork reversal has recently been described in *ctf4* mutant cells (Fumasoni et al., 2015). It will be interesting to see whether Dna2 may be involved in protecting or restarting reversed forks in a *ctf4* context, much in the same way we envisage the Dna2 helicase activity to act in cells exposed to exogenous replication stress.

In chapter 4.5, we provide evidence that inactivating mutations in *PIF1* frequently arise upon MMS exposure of *dna2-2* cells, suppressing their DNA damage sensitivity. Pif1 is a 5′-3′ helicase found in almost all eukaryotes (reviewed in (Bochman et al., 2010)). There are two *PIF1*-like genes encoded in *S.cerevisiae*: Pif1 and Rrm3. Although Pif1 and Rrm3 share the same substrates, their effects are different and sometimes even opposing; yet, both are important for successful genome duplication. For example, at the rDNA, while Rrm3 promotes fork progression through the RFB, Pif1 maintains stalling at RFB (Ivessa et al., 2000). Although *pif1 rrm3* double mutants are viable (Ivessa et al., 2000),

rrm3 dna2-2 is lethal. Interestingly, lethality of *dna2-2 rrm3* could not be suppressed by deletion of *RAD51*, implying that their role is independent of recombination (Budd et al., 2005). On the other hand, Pif1 is a known suppressor of *dna2* lethality (Budd et al., 2006). Noteworthy, Pif1 appears to be regulated by the intra-S phase checkpoint. It has been reported that phosphorylation by Rad53 kinase inhibits Pif1 activity at stalled forks, while dephosphorylation when the checkpoint is turned off leads to re-activation (Rossi et al., 2015). It will be important to explore whether this re-activation might cause the toxicity related to Pif1 in *dna2-2* cells after acute HU treatment (Ölmezer et al., manuscript in revision). It is certainly tempting to speculate that a re-activated Pif1 helicase might mediate an unwanted fork-remodeling pathway, generating an intermediate that requires Yen1 for resolution.

It is intriguing that *dna2-2* was shown to be synthetically lethal with other non-essential helicases, such as Sgs1 and Rrm3 (Budd et al., 2006; 2005; Hoopes et al., 2002; Weitao, 2003). Sgs1 has also been implicated in RF protection and recovery (Cobb et al., 2003; 2005). In view of the role of the Dna2 helicase described herein, it appears that cells use a large and finely balanced network of helicases and nucleases to solve the problem of RF arrest. Further investigation of Dna2 and its genetic interactions provides an attractive starting point to unravel this important interaction network. Our understanding on human DNA2 is relatively limited at the moment; yet, the RF protection problems associated with Dna2 helicase dysfunction in yeast are likely to be relevant for some of the severe phenotypes previously described in DNA2-depleted cell lines and human diseases associated with DNA2, including cancer (Dominguez-Valentin et al., 2013; Shaheen et al., 2014; Strauss et al., 2014). Given that replication stress is viewed as a common driver in tumorigenesis, further work might eventually translate our acquired insight into effective therapies.

Yen1 nuclease uniquely removes remaining post-replicative inter-chromosomal DNA links in *dna2-2* cells, promoting viable chromosome segregation and genome stability. More generally, Yen1 could be a surveillance factor for underreplicated DNA, which is not sensed by the checkpoint. The consequence of Yen1-cleavage would either be mitotic repair synthesis or the mitotic transmission of ssDNA-gaps or DNA breaks. It is

interesting that the transmission of DNA damage in mitosis seems to be more common than previously thought (Lukas et al., 2011; Ying et al., 2013). Active breakage of replication intermediates at mitosis could ensure timely chromosomal segregation and, thus, maintain the genome integrity. It will be intriguing to address what kind of repair pathways are involved downstream of Yen1.

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