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Understanding the differential consequences of checkpoint adaptation following DNA damage in repair-proficient and repair-deficient cells

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List of Publications

Results obtained during this PhD thesis contributed to the following publication:

Klermund J., Bender K., and Luke B. (2014). „High nutrient levels and TORC1 activity reduce cell viability following prolonged telomere dysfunction and cell cycle arrest.“ Cell Reports 9(1):324-35

List of Abbreviations

α	anti
αF	α-Factor
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer
8-oxo-G	8-oxoguanine
A	Adenine
ALT	Alternative lengthening of telomeres
ATP	Adenosine triphosphate
BER	Base excision repair
BIR	Break-induced replication
bp	Base pair
C	Cytosine
cdc	*cell division cycle*
CIN	Chromosomal instability
CPT	Camptothecin
Cvt	Cytosol-to-vacuole targeting
DDC	DNA damage checkpoint
ddH_2O	Double-distilled water
DDR	DNA damage response
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DSB	Double strand break
DSBR	Double-strand break repair
dsDNA	Double-strand DNA
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
ESR	Environmental stress response
FEAR	Cdc Fourteen Early Anaphase Release
G	Guanine
g	Gram
Gal	Galactose
GAP	GTPase activating protein
GCR	Gross-chromosomal rearrangements
gDNA	Genomic DNA
GEF	Guanine nucleotide exchange factor
GFP	Green-fluorescent protein
GO	Gene ontology
GTP	Guanosine triphosphate
GTPase	GTP-hydrolyzing enzyme
h	Hour
HCl	Hydrogen chloride
HDAC	Histone deacetylase
HDR	Homology-directed repair
HF	High Fidelity
HJ	Holliday junction
HML	Hidden Mat Left (mating type cassette left)

HMR	Hidden Mat Right (mating type cassette right)
HO cut	Homothallic switch (HO) endonuclease-induced cut
HR	Homologous recombination
HYG	Hygromycin
IP	Immunopurification
KAN	Kanamycin
kb	Kilobases
L	Liter
LiAc	Lithium acetate
M	Molar
MEN	Mitotic exit network
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MMEJ	Microhomology-mediated end joining
MMR	Mismatch repair
MMS	Methyl methanesulfonate
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAT	Nourseothricin
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
nM	Nanomolar
nm	Nanometer
Noc	Nocodazole
nt	Nucleotide
OD	Optical density
PAR	Poly-(ADP-ribose)
PCR	Polymerase chain reaction
PE (lipid)	Phosphatidylethanolamine
PEG	Polyethylenglycol
Raff	Raffinose
Rapa	Rapamycin
RiBi	Ribosome biogenesis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
rRNA	ribosomal RNA
S. cerevisiae	*Saccharomyces cerevisiae*
SAC	Spindle assembly checkpoint
SAM	S-adenosyl methionine
SC/ SD	Synthetic complete/ Synthetic dropout
SDS	Sodium dodecyl sulfate
SDSA	Synthesis-dependent strand annealing
SPB	Spindle pole body
SSB	Single-strand break
ssDNA	Single-stranded DNA

List of Abbreviations

T	Thymine
Top1-cc	Top1- cleavable complex
TORC1	Target of rapamycin complex 1
tRNA	transfer RNA
Trp	Tryptophan
Ura	Uracil
UV	Ultraviolet
w/v	Weight per volume
wt	Wild type
YPD	Yeast extract peptone dextrose

Summary

When cells experience DNA damage, they halt the cell cycle before sister chromatid separation has begun in response to the activation of the DNA damage checkpoint. This surveillance mechanism provides time to repair the damage and only when repair has been successful the cell cycle is resumed. Therefore, cell cycle arrest and damage repair are important processes to ensure the stability of the genome and the faithful transfer of genetic information to daughter cells. However, if repair is not possible, cells can override the DNA damage checkpoint and terminate the cell cycle arrest by a mechanism called (checkpoint) adaptation. Although many proteins have been shown to be involved in the adaptation process, its molecular mechanisms still remain elusive. Especially the critical determinants initiating checkpoint adaptation have not been fully identified. Understanding this pathway is of particular interest since checkpoint adaptation is a driving force of genome instability and has detrimental consequences including cell death and various genomic aberrations. Interestingly, the concept of checkpoint adaptation is not only found in unicellular eukaryotes like yeast but also in multicellular organisms. Especially during cancerogenesis, checkpoint adaptation is thought to contribute to genome instability. We could previously show that inhibition of the highly conserved TOR nutrient signalling pathway either by genetic or pharmacologic means prevents checkpoint adaptation in *Saccharomyces cerevisiae*. These observations suggests that nutrient signalling pathways involving TOR signalling node play an important role in response to DNA damage.

We set out to further investigate the link between nutrient signalling, checkpoint adaptation and genome stability. We show that prevention of adaptation can be achieved by modulating the Tap42-PP2A axis downstream of TOR signalling. We found that pharmacological inhibition of TOR by rapamycin affects protein levels of Cdc5, a major factor promoting adaptation. Using *RAD52*-deficient yeast cells to mimic the DNA repair defect observed in many human cancers, we confirmed previous results showing that preventing adaptation sensitizes these cells to genotoxins. However, if adaptation is allowed to occur, repair-deficient cells acquire genotoxin resistance and display an aneuploid karyotype. Gene expression profiling revealed that resistant repair-defective yeast cells exhibit common aneuploidy-associated phenotypes. Although resistant aneuploid cells are still checkpoint-competent, they can proliferate in the presence of persistent DNA damage. This underlines the role of checkpoint adaptation in the acquisition of genotoxin resistance. Taken together, our results highlight an intriguing relationship between the DNA damage response and genome stability, which appears to be associated with checkpoint adaptation and nutrient signalling pathways. Furthermore, using an easily tractable model organism such as budding yeast, we provide insights into the relationship between fundamental and highly conserved cellular processes that could be useful for drug development and disease treatment in humans as well.

Zusammenfassung

Wenn die DNA einer Zelle beschädigt wird, stoppt die Zelle im Zellzyklus bevor die Trennung der Schwesterchromatiden begonnen hat, als Reaktion auf die Aktivierung des „DNA-Schaden-Checkpoints“, kurz Checkpoint. Dieser Kontrollmechanismus stellt Zeit zur Reparatur zur Verfügung und nur nach erfolgreicher Reparatur kann der Zellzyklus wieder aufgenommen werden. Das Anhalten des Zellzyklus und die Reparatur der beschädigten DNA sind daher wichtige Prozesse, um die Stabilität des Genoms und den zuverlässigen Transfer der genetischen Information an die Tochterzelle zu gewährleisten. Ist eine Reparatur jedoch unmöglich, können sich Zellen über den Checkpoint hinwegsetzen und das Anhalten des Zellzyklus durch einen Mechanismus beenden, der als Adaptation bezeichnet wird. Obwohl nachweislich viele Proteine in den Adaptationsprozess involviert sind, bleibt der molekulare Mechanismus immer noch schwer fassbar. Besonders die kritischen Faktoren, welche die Adaptation in Gang setzen, wurden noch nicht vollständig identifiziert. Das Verständnis dieses Signalwegs ist von besonderem Interesse, da Adaptation eine treibende Kraft für Genominstabilität darstellt und schädliche Auswirkungen hat, einschließlich Zelltod und verschiedene genetische Abweichungen. Interessanterweise findet man das Konzept der Adaptation nicht nur in einzelligen Eukaryoten wie der Hefe, sondern auch in vielzelligen Organismen. Besonders während der Karzinogenese trägt Adaptation vermutlich zur Instabilität des Genoms bei. Wir konnten bereits nachweisen, dass die Hemmung des hoch konservierten TOR-Nährstoff-Signalwegs, entweder durch genetische oder pharmakologische Mittel, Adaptation in *Saccharomyces cerevisiae* verhindert. Diese Beobachtungen lassen vermuten, dass Nährstoff-Signalwege einschließlich des Knotenpunkts um den TOR-Signalweg eine wichtige Rolle bei der Antwort auf DNA-Schäden spielen.

Das Ziel dieser Dissertation war es, die Verbindung zwischen Nährstoff-Signalwegen, Adaptation und Genominstabilität genauer zu untersuchen. Wir weisen nach, dass Adaptation verhindert werden kann durch das Anpassen der Tap42-PP2A-Achse, die dem TOR-Signalweg nachgeschaltet ist. Wir haben herausgefunden, dass die pharmakologische Hemmung von TOR durch Rapamycin den Proteinspiegel von Cdc5 beeinflusst, einem wichtigen Faktor, der Adaptation vorantreibt. Indem wir Hefezellen benutzen, denen das Gen *RAD52* fehlt um den Reparaturdefekt vieler Krebsarten nachzuahmen, konnten wir frühere Ergebnisse bestätigen, die zeigen, dass diese Zellen durch das Verhindern von Adaptation anfälliger sind für Genotoxine. Wenn Adaptation jedoch stattfinden kann, erwerben diese Reparatur-defekten Zellen Resistenzen gegen Genotoxine und weisen einen aneuploiden Karyotyp auf. Genexpressionsprofile dieser resistenten und Reparatur-defekten Hefezellen zeigen allgemeine Phänotypen, die mit Aneuploidie assoziiert werden. Obwohl resistente aneuploide Zellen immer noch kompetent sind bezüglich des Checkpoints, können sie in der Anwesenheit anhaltender DNA-Schäden proliferieren. Dies unterstreicht die Rolle von Adaptation beim Erwerb von Resistenzen gegenüber Genotoxinen. Zusammenfassend zeigen unsere Ergebnisse einen verblüffenden Zusammenhang zwischen der Reaktion auf DNA-Schäden und der Genomstabilität, welcher in Verbindung mit Adaptation und Nährstoff-Signalwegen zu stehen scheint. Indem wir Bäckerhefe als leicht lenkbaren Modellorganismus benutzen, können wir Einblicke in den Zusammenhang grundlegender und hochkonservierter zellulärer Prozesse gewinnen, die auch bei der Entwicklung von Medikamenten und der Behandlung von Krankheiten des Menschen nützlich sein können

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1 Introduction

Every day, the integrity of the genome is challenged by a variety of agents that could potentially cause harm to the genetic material by introducing a wide range of modifications or aberrations. In order to ensure that the transfer of genetic information to their progeny can occur as reliably as possible, cells are equipped with a variety of mechanisms and pathways to detect and repair damage to their DNA.
Amongst the myriad of DNA lesions, double-stranded breaks (DSBs) are thought to be the most lethal due to their ability to induce chromosomal aberrations (Natarajan et al., 1980) including deletions, inversions, duplications and translocations.

1.1 The response to genome-wide DNA damage

At every critical step during the cell cycle, surveillance mechanisms called checkpoints are in place to ensure that cell cycle progression will only occur if all necessary requirements are fulfilled, e.g. sufficient cell size for the transition from G1 to S phase or complete replication of the genome for the transition from S to G2. Accordingly, when cells experience DNA damage, an evolutionary conserved response is initiated which leads to the activation of a DNA damage checkpoint. Interestingly, cells respond differently depending on the timing of damage occurrence with respect to the replication of the genome and the type of lesion. Although the set of proteins involved in the checkpoint response remains similar, the checkpoint responses seem to differ in terms of outcomes depending on whether or not the cell has already completed DNA replication. In the thesis presented here, I will focus on the role and influence of activating the DNA damage checkpoint (DDC) in the G_2/M phase of the cell cycle. Activation of the DDC will ultimately arrest the cell cycle in G_2/M at the metaphase to anaphase transition prior to the separation of sister chromatids (reviewed in (Melo and Toczyski, 2002)). To prevent the transmission of damaged DNA to the daughter cell, a signalling cascade comprising sensors and mediators is turned on to ensure signal amplification and transmission to the effectors of the DNA damage checkpoint. Activation of the DDC leads to an arrest of the cell cycle to provide time to repair the damage. When the damage is repaired, the initial checkpoint trigger is abolished which leads to the inactivation of the checkpoint signalling cascade and eventually allows cell cycle progression.

1.1.1 Checkpoint sensors and effectors

After a DSB has occurred, the damage site is processed by nucleases that resect the DNA to create single-stranded DNA (ssDNA). This serves as the primary trigger for the activation of the DNA damage checkpoint. The MRX complex, consisting of Mre11, Rad50 and Xrs2, together with the nuclease Sae2, is responsible for the initial short-range resection occurring after a DSB (Mimitou and Symington, 2008; Zhu et al., 2008). In a second step, the MRX complex stimulates the recruitment of the Sgs1 helicase as well as Exo1, a nuclease with 5’ to 3’ processing activity that facilitates long-range resection around the DNA lesion site (Shim et al., 2010). The nucleolytic processing of one strand (in 5’ to 3’ direction) in either direction from the DSB creates stretches of ssDNA which are then rapidly bound by the heterotrimeric

RPA (Replication Protein A) complex (Alani et al., 1992). Binding of RPA serves as a signalling platform for the recruitment of a plethora of checkpoint proteins and kinases. One of the most upstream factors is Mec1 (Nakada et al., 2005), which is the homolog of human ATR. Mec1 is a member of the phosphoinositide (PI)-3-kinase (PI3K)-related kinase (PIKK) superfamily (Kato and Ogawa, 1994) and collaborates with Ddc2 (Nakada et al., 2005; Paciotti et al., 2000), the yeast homolog of human ATRIP. In addition to Mec1-Ddc2, the PCNA-like checkpoint clamp composed of Mec3, Rad17 and Ddc1 is loaded onto the damage site at the transition of the single-stranded to the double-stranded DNA (Kondo et al., 1999; Majka and Burgers, 2003). Loading of the clamp, which is referred to as the 9-1-1 clamp named after the human components Rad9, Hus1 and Rad1, is aided by the clamp loader containing Rad24 and four small subunits of the Replication Factor C (RFC) members (Rfc2, 3, 4 and 5) (Green et al., 2000; Majka and Burgers, 2003; Venclovas and Thelen, 2000). When the 9-1-1 component Ddc1 was artificially co-localized with the Mec1-Ddc2 dimer in the absence of damage by tethering them to DNA in close proximity, this was sufficient to activate the DNA damage checkpoint (Bonilla et al., 2008). This suggests that the 9-1-1 checkpoint clamp is involved in Mec1 activation and establishment of the checkpoint signalling cascade. The recruitment of the checkpoint adaptor (or mediator) protein Rad9 in response to DNA damage seems to be regulated via different pathways. Firstly, Rad9 can localize to damage sites by interacting with histones that have been modified in response to damage. However, this mechanism seems more critical for checkpoint activation during the G1 phase of the cell cycle (Hammet et al., 2007). In a second pathway, Rad9 can interact with a second checkpoint mediator, Dpb11 (human TopBP1) (Puddu et al., 2008). Dpb11 gets recruited via Ddc1 (Wang and Elledge, 2002). The Rad9 phosphorylation sites that allow Dpb11 interaction were initially found to be cell-cycle regulated (Pfander and Diffley, 2011). Later on, also damage-induced Rad9 phosphorylation events were observed to facilitate the Dpb11- Rad9 interaction (di Cicco et al., 2017). However, the exact order of events as well as the possible involvement of additional factors have not been fully elucidated.

Once Mec1 is activated, it targets the checkpoint mediator Rad9 for phosphorylation at multiple sites (Schwartz et al., 2002) allowing the recruitment and subsequent activation of Rad53 (human CHK2) (Gilbert et al., 2001; Sweeney et al., 2005). Full Rad53 activity requires multiple autophosphorylation events as well as *trans* phosphorylations in a Mec1-dependent manner (Ma et al., 2006; Sanchez et al., 1996; Sun et al., 1998). The activation steps of Rad53 rely on the FHA (forkhead-associated) domains present in Rad53 that serve as phosphopeptide binding platforms (Schwartz et al., 2003). In contrast to the high level of conservation regarding checkpoint protein orthologues amongst eukaryotes, no single orthologue for Rad9 has been identified in humans so far. Rather, a set of proteins has been characterized as checkpoint mediators, including MDC1, 53BP1 and BRCA1 (reviewed in (Zhou and Elledge, 2000)).

In addition to Rad53, a second effector checkpoint kinase named Chk1 (human CHK1) has been characterized in yeast (Liu et al., 2000). Rad53 and Chk1 have been shown to achieve a DNA damage-induced cell cycle arrest by distinct targets (Sanchez et al., 1999) (See section 1.1.2). However, Rad53 is considered the central checkpoint kinase to halt the cell cycle by phosphorylating multiple downstream targets (See section 1.1.2, see Figure 1 for a schematic overview of the initial steps in DDC activation).

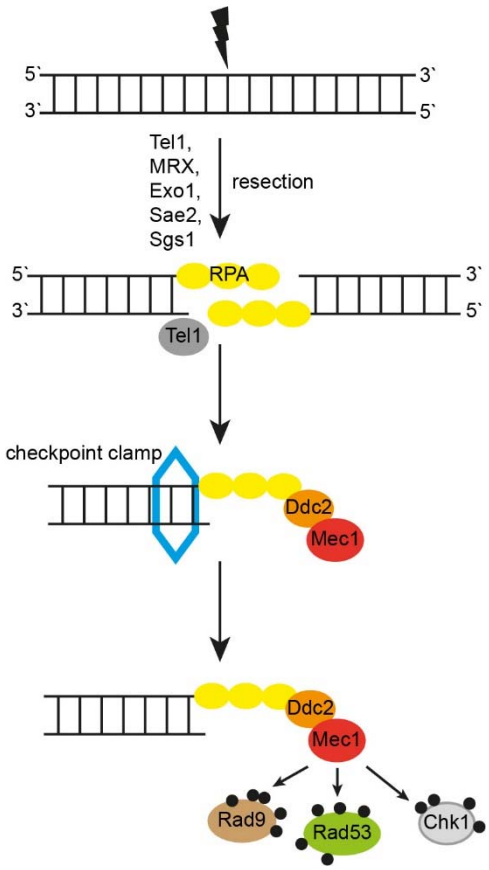


Figure 1. Schematic overview of the initial steps in DDC activation. Black dots represent critical phosphorylation events. For simplicity, some proteins (e.g. the clamp loader) are omitted.

The human orthologue of Mec1, ATR, seems to play a minor role in the response to DSBs. In contrast, the main responder in mammalian cells is ATM which corresponds to Tel1 in budding yeast. Tel1 associates with the MRX complex in an Xrs2-dependent fashion (Nakada et al., 2003) and thereby contributes to the production of ssDNA by enhancing resection (Mantiero et al., 2007) which in turn allows the recruitment of Mec1 via RPA binding to ssDNA. Tel1 can also activate the DNA damage checkpoint in the absence of Mec1, however, Tel1 activity seems to be triggered mostly by several DSBs and is decreased when resection of the lesion is initiated (Mantiero et al., 2007) suggesting that Tel1 might be activated in response to blunt or minimally resected damage sites. Accordingly, Mec1-regulated resection has been shown to decrease Tel1-mediated checkpoint signalling (Clerici et al., 2014). Consequently, resection could serve as a tool to switch from Tel1- to Mec1-mediated checkpoint signalling (Mantiero et al., 2007). A similar swap from ATM- to ATR-mediated DSB signalling has been proposed to occur in human cells in order to coordinate the respective events sparked by both checkpoint kinases (Shiotani and Zou, 2009).

Furthermore, the core histone H2A is phosphorylated at a serine residue at position 129 in response to DNA damage in a Mec1- and Tel1-dependent manner (Downs et al., 2000). Modification of H2A leads to local chromatin de-compaction enabling the recruitment and action of repair proteins (Downs et al., 2000; Redon et al., 2003). Consistently with the high conservation of the DNA damage response (DDR) in eukaryotic cells, phosphorylation of

H2AX, a H2A variant, at serine 139 (dubbed γH2AX) has also been described in mammalian cells (Rogakou et al., 1998) where it plays a similarly crucial role in facilitating DNA repair (Paull et al., 2000) and is therefore widely used as a read-out for the presence of DNA damage.

1.1.2 Checkpoint targets to halt the cell cycle

As mentioned above, there are two checkpoint kinases, Chk1 and Rad53, which act as the effectors of the DNA damage checkpoint. In order to achieve cell cycle arrest in response to DNA damage, both Chk1 and Rad53 elicit downstream signalling pathways to different checkpoint targets (Sanchez et al., 1999). Chk1 has been shown to prevent degradation of securin, known as Pds1 in budding yeast. In unperturbed conditions, Pds1 is ubiquitinated by a central cell cycle regulatory E3 ubiquitin ligase termed anaphase promoting complex (APC) (Cohen-Fix et al., 1996; King et al., 1995) in association with its specificity-promoting co-activator Cdc20 (APC^{Cdc20}) (Visintin et al., 1997). The APC plays important roles in cell cycle regulation depending on its binding to either Cdc20 or another co-activator, Cdh1. APC^{Cdh1} is responsible for mitotic cyclin degradation including Clb2 (Schwab et al., 1997; Shirayama et al., 1998; Visintin et al., 1997). Subsequently, Pds1 is targeted for proteasomal degradation (Cohen-Fix et al., 1996) leading to the activation of the protease Esp1, also known as separase (Uhlmann et al., 1999). Esp1 is responsible for cleaving the Scc1 subunit of cohesin involved in sister chromatid cohesion (Uhlmann et al., 1999). During anaphase, Esp1-mediated cohesin cleavage allows the segregation of chromatids to the daughter cell and completion of mitosis (Ciosk et al., 1998; Michaelis et al., 1997). Upon DNA damage, Pds1 is phosphorylated in a Chk1-dependent manner at 9 putative phosphorylation sites (Cohen-Fix and Koshland, 1997; Sanchez et al., 1999; Wang et al., 2001), thereby possibly masking or counteracting its degradation signal for APC^{Cdc20} and ultimately leading to Pds1 stabilization (Wang et al., 2001). Rad53 has been implicated in Pds1 stabilization as well by phosphorylating it and thereby disrupting the Pds1-Cdc20 interaction (Agarwal et al., 2003). Therefore, Rad53-mediated phosphorylation prevents recognition of Pds1 by the co-activator Cdc20, while Chk1-dependent Pds1 phosphorylation has been suggested to render Pds1 a poor APC^{Cdc20} substrate. Consequently, Esp1 remains inhibited by Pds1 and sister chromatid separation cannot be initiated thereby ensuring mitotic arrest at the metaphase to anaphase transition after DDC activation. Importantly, Pds1 does not only function as an Esp1 inhibitor but is also required to ensure Esp1's nuclear localization in order to facilitate Esp1-mediated cohesin cleavage. Hence, a two-step model for the roles of Pds1 and Esp1 has been proposed: After phosphorylation by the main yeast Cyclin-dependent kinase (Cdk) Cdc28 (reviewed in (Mendenhall and Hodge, 1998)), Pds1 shuttles previously cytoplasmic Esp1 into the nucleus to allow Esp1-mediated proteolysis of cohesin (Agarwal and Cohen-Fix, 2002). After Pds1 degradation via the APC^{Cdc20}, Esp1 is re-localized from the nucleus to the spindle pole body (SPB), the yeast functional equivalent of the centrosome (reviewed in (Kilmartin, 2014)), to participate in sister chromatid separation, possibly directed by a remaining pool of Pds1 that had escaped bulk proteasomal degradation (Agarwal and Cohen-Fix, 2002; Jensen et al., 2001). Therefore, DDC-dependent phosphorylation of Pds1 upon damage might prevent the Cdc28-dependent phosphorylation observed in unperturbed conditions to ensure that Esp1 remains inhibited and

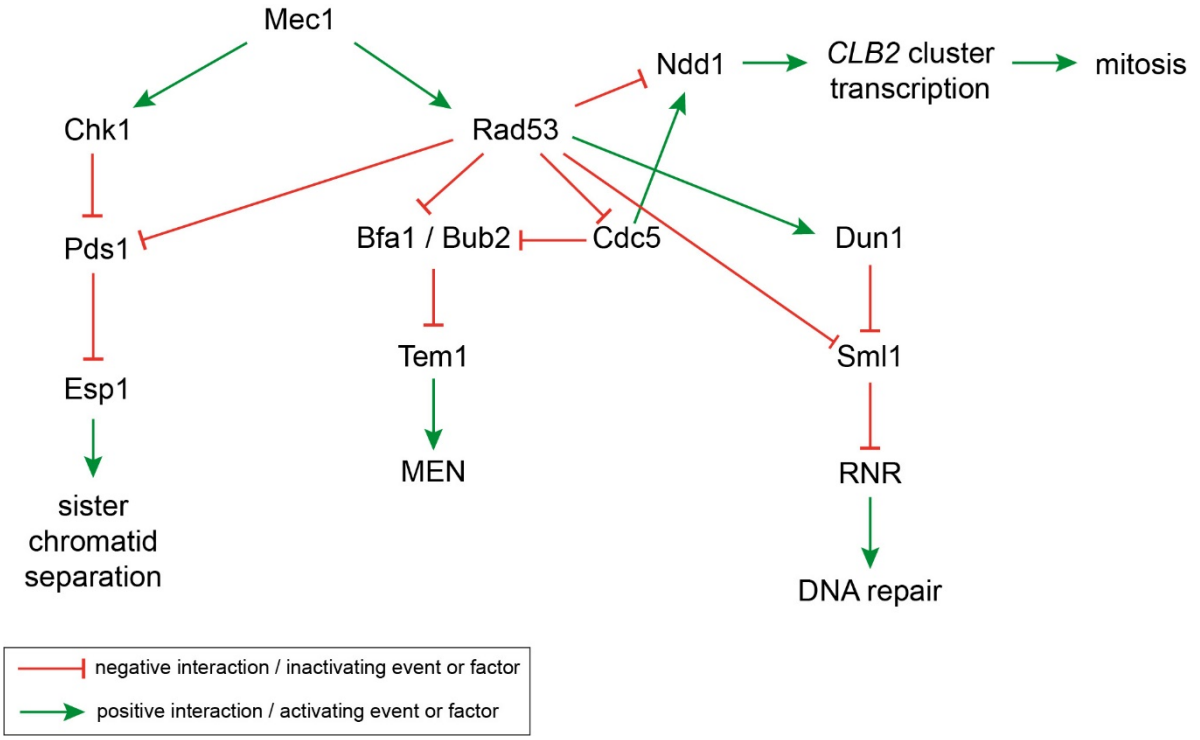


Figure 2. Simplified scheme of DDC effectors and targets leading to cell cycle arrest in response to DNA damage. RNR: ribonucleotide reductase complex.

is excluded from the nucleus. Thereby, a cell cycle arrest can be elicited and sister chromatid separation is prevented.

In addition to preventing sister chromatid separation via the Chk1-Pds1 pathway, Rad53 affects mitotic progression by regulating Cdc5. Upon DNA damage- induced activation of Rad53, Cdc5 is phosphorylated and thereby inhibited in a Rad53-dependent manner (Cheng et al., 1998; Zhang et al., 2009). In this condition, Cdc5 can no longer inhibit the two-component GTPase-activating protein (GAP) Bub2/Bfa1 caused by the Cdc5-dependent phosphorylation of Bfa1 (Hu et al., 2001). The Bub2/Bfa1 complex promotes GTP hydrolysis and thereby inhibits the small GTPase Tem1 which positively regulates the downstream Cdc15 kinase (Hu et al., 2001; Lee et al., 2001a; Shirayama et al., 1994). Being localized at the SPB, the combined activation of the protein kinases Cdc15, Dbf2 and Mob1 are thought to drive mitotic exit (Visintin and Amon, 2001). This is achieved by a complex regulatory pathway termed the Mitotic Exit Network (MEN) which ultimately releases the Cdc14 phosphatase from the nucleolus to the cytoplasm where it can remove Cdc28-mediated phosphorylations on multiple proteins thereby driving cells from mitosis into the subsequent G1 phase of the cell cycle (Jaspersen et al., 1999; Shou et al., 1999; Visintin et al., 1999). In summary, DDC activation leads to the inhibition of Cdc5 and thereby prevents activation of MEN resulting in a damage-induced cell cycle arrest. In addition to the Cdc5-mediated inhibition of Bfa1, Rad53 has been proposed to positively modulate Bub2/Bfa1 interaction with Tem1 thereby keeping Tem1 inactivated and preventing the onset of MEN (Liang and Wang, 2007).

Taken together, at least two Rad53-mediated pathways have been proposed to ensure a cell cycle arrest induced by DNA damage both independent and dependent on Cdc5.

1.1.3 Transcriptional response to DNA damage

In addition to halting the cell cycle, DNA damage also elicits a transcriptional response. The protein kinase Dun1 has been identified as one of the central transcriptional regulators after DNA damage. Rad53 activation leads to the phosphorylation and subsequent activation of Dun1 accompanied by Dun1 autophosphorylation events (Bashkirov et al., 2003; Zhou and Elledge, 1993). Subsequently, Dun1 phosphorylates the DNA binding protein Crt1 preventing its binding to consensus DNA binding motifs. Consequently, gene expression from these loci is allowed to occur, and this has been shown to include the redundant *RNR* genes (Huang et al., 1998). These genes encode the subunits of the ribonucleotide reductase (RNR) complex responsible for converting ribonucleotides (NTPs) into deoxyribonucleotides (dNTPs) and thereby maintaining dNTP pools within the cell (Lammers and Follmann, 1984). To facilitate dNTP pool homeostasis and to account for the increased demand due to DNA repair mechanisms, the RNR inhibitor Sml1 (Zhao et al., 1998) has been shown to be targeted and degraded after DNA damage in a DDC-dependent manner also involving Dun1 (Zhao et al., 2001; Zhao and Rothstein, 2002). Appropriate activity of the RNR complex seems indispensable for cell viability in response to damage since deletion of the RNR inhibitor *SML1* or overexpression of RNR components can rescue lethality of both *RAD53* and *MEC1* deletions (Desany et al., 1998; Zhao et al., 1998) which is probably due to the higher demand for dNTPs required for DNA repair processes following DNA damage.
In a second pathway parallel to the Dun1-mediated transcriptional response, DDC activation has been shown to downregulate the *CLB2* transcription cluster including *CLB2* itself, as well as *CDC5*, *CDC20* and many other genes required for the execution of mitosis (Gasch et al., 2001). Transcription of the *CLB2* cluster is mediated by the transcription factors Mcm1 and the forkhead transcription factors, Fkh1 and Fkh2 (Kumar et al., 2000; Zhu et al., 2000). Fkh2 recruits the positive regulator Ndd1 to promoters of the *CLB2* cluster (Koranda et al., 2000) via an interaction of Fkh2's forkhead-associated domain (FHA) and a phosphorylation in Ndd1 (Reynolds et al., 2003). Clb2-Cdc28 and Cdc5 kinase activity have been shown to facilitate the Fkh2-Ndd1 interaction (Darieva et al., 2006; Reynolds et al., 2003) thereby promoting a positive feedback-loop that leads to high transcription of the *CLB2* cluster. In response to DNA damage, Ndd1 is phosphorylated directly by Rad53. This prevents Ndd1 association with promoters of the *CLB2* transcriptional cluster via the Rad53-Dun1 axis (Edenberg et al., 2014).
In addition, gene expression profiling performed after exposure of yeast to different environmental stresses has revealed a common environmental stress response (ESR) (Gasch et al., 2000). Interestingly, the checkpoint kinase Mec1 was shown to regulate the ESR in response to the DNA alkylating agent methylmethanosulfate (MMS) and ionizing radiation (Gasch et al., 2001) showing that DNA damage influences the transcriptome in a DDC-dependent manner.
In summary, checkpoint activation in response to DNA damage (i) halts the cell cycle by targeting proteins involved in mitotic progression such as Pds1, Cdc5 and Bfa1, (ii) ensures DNA repair capacity by upregulating the RNR complex, and (iii) elicits a transcriptional response involving the *CLB2* transcriptional cluster to reinforce the checkpoint arrest and to facilitate a more general stress response (summarized in Figure 2).

1.2 Types of DNA damage

Single-stranded (SSB) and double-stranded breaks (DSB) are thought to be the most common types of DNA damage with an estimated frequency of 55,000 or 25 lesions, respectively, per cell every day (reviewed in (Tubbs and Nussenzweig, 2017)). However, SSBs have a propensity to be converted into DSBs which, in turn, are considered the most deleterious type of damage since no complementary strand is left to enable template-based repair processes. This suggests DSBs are potentially lethal damage. Consequently, cells have developed multiple pathways to ensure appropriate and timely repair of the different types of DNA damage.

1.2.1 Endogenous sources of DNA damage

Spontaneous DNA damage occurring from endogenous sources can contribute to evolution to a certain extent. However, it also imposes a major threat to genome stability by introducing unwanted or harmful mutations. It has been recognized a long time ago that due to its chemical properties, DNA can engage in multiple chemical reactions affecting its stability and characteristics. Since then, these chemical reactions have been identified as sources of endogenous DNA damage and there are different pathways dedicated to deal with the distinct outcomes and consequences.

One source of endogenous damage is given by the spontaneous hydrolysis of DNA. Direct conversion of the four bases into another base is a common type of hydrolytic damage occurring in the aqueous cellular environment. For example, cytosine can undergo deamination and is thereby converted to uracil. Consequently, this will lead to a base pairing mismatch since uracil is now base-paired to guanine. In the subsequent round of DNA replication, the base incorporated opposite of the uracil will be adenine while the uracil itself will be replaced by thymidine thereby introducing a point mutation (reviewed in (Marnett and Plastaras, 2001)). This has been estimated to occur approximately 190 times a day per cell (reviewed in (Tubbs and Nussenzweig, 2017)).

Spontaneous depurination or depyrimidination is another type of hydrolytic DNA damage causing the occurrence of abasic sides thereby leaving a gap in one of the two DNA strands. This type of damage seems to affect purine bases much more often than pyrimidines and both types of lesions are thought to occur approximately 12,000 or 600 times a day in every cell (reviewed in (Tubbs and Nussenzweig, 2017)).

One of the most potent endogenous DNA damage sources is given by oxidative damage which is caused by radicals arising from mitochondrial metabolism. This crucial biological process produces reactive oxygen species (ROS) that can potentially cause multiple forms of oxidative damage to DNA. Therefore, ROS are considered especially harmful due to the various and heterogeneous types of lesions that can be induced including the modification of bases, inter- and intrastrand crosslinking events, crosslinking of proteins to the DNA and DNA strand breaks (reviewed in (Jena, 2012)). One of the best characterized oxidative damage lesion is 7,8-dihydro-8-oxoguanine (8-oxo-G) which has been implied to play a role in cancer and aging (reviewed in (Loft et al., 2008)).

Not only hydrolytic and oxidative damage but also methylation in non-enzymatic reactions can pose a threat to the integrity of DNA molecules. Non-enzymatically catalysed spontaneous

methylation events can modify bases, especially 3`-methyladenine has been suggested as a harmful modification due to its ability to block replication. The intracellular methyl group donor S-adenosylmethionine (SAM) that functions as a co-substrate can act as a weak alkylating agent under physiological conditions (reviewed in (Lindahl, 1993)).
The above mentioned lesions and aberrations can be caused by endogenously occurring damage, however, they can also be inflicted by exogenous damage sources.

1.2.2 Exogenous DNA damage sources

In addition to endogenous damage occurring under physiological conditions as the consequences of biological processes or due to the chemical properties of individual molecules, cells also have to deal with exogenously induced DNA damage. This comprises both naturally occurring DNA lesions caused for example by UV light or irradiation as well as non-natural damage induced by drug treatment, for example bleomycin and CPT, as used in this study.

1.2.2.1 Radiation and radiomimetic agents

Exposure to ultra-violet (UV) radiation also contained in sunlight induces the formation of bulky adducts in the DNA double strands caused mainly by the creation of cyclic photoproducts and dimers between bases. These lesions lead to an altered symmetry of the DNA double helix thereby hampering transcription and replication and their removal is a major task for NER (reviewed in (Costa et al., 2003; Rastogi et al., 2010)).
X-rays constitute a type of electromagnetic radiation which also includes UV radiation and γ- rays. Like the latter, X-rays possess enough energy to ionize molecules and thereby they have been classified as ionizing radiation. It has been commonly accepted that ionizing radiation induced different types of DNA lesions including damage to bases, single and double strand breaks and intra-strand crosslinks between the two DNA strands. The fact that X-rays cause DNA damage has been exploited for a long time and, amongst other observations, led to the identification of crucial repair factors such as Rad52 (Game and Mortimer, 1974).
Interestingly, the types of lesions caused by ionizing radiation seem to be very similar in their chemical properties to ROS-induced DNA damage. However, it has been estimated that the damage inflicted by endogenous ROS action exceeds the damage load usually delivered to human cells using conventional radiotherapy during cancer treatment (reviewed in (Lindahl, 1993; Markkanen et al., 2012)). One explanation why radiation-induced DNA damage still shows higher cytotoxicity could be the complexity of lesions. Upon irradiation, DNA damage occurs in a bundled or grouped fashion accumulating within one or more DNA helical turns. This is referred to as clustered damage and it is thought to be more difficult to repair and have the potential to be converted into additional DSBs thereby causing cell death ((Costa et al., 2003; Game and Mortimer, 1974) and reviewed in (Loft et al., 2008)). In fact, already soon after the discovery of X-rays in the late 19th century and, shortly after, γ-rays as a natural source of radiation, they were used in radiotherapy for cancer treatment (reviewed in (Connell and Hellman, 2009)). Although for some cancer types, radiotherapy is used as the standard treatment since it allows targeted delivery of the damaging agent and thereby helps to preserve integrity of the surrounding healthy tissue, tumour cells can be resistant to radiation

(radioresistance). This has been mainly attributed to intrinsic properties of individual cell types and the surrounding tumour microenvironment and remains an obstacle especially in the treatment of cancers affecting the central nervous system (reviewed in (Kelley et al., 2016)).
As an alternative to ionizing radiation, genotoxic compounds mimicking radiation-induced DNA damage can be used. These agents are referred to as radiomimetic drugs, although, in contrast to ionizing radiation, they seem to induce damage in a highly specific manner by creating mostly DSBs (reviewed in (Rastogi et al., 2010)). Together with its derivatives phleomycin and zeocin, bleomycin belongs to the class of radiomimetic agents used in chemotherapy and its mode of action leading to the cleavage of the DNA backbone has been revealed several years ago (reviewed in (Burger, 1998)).

1.2.2.2 Camptothecin (CPT)

Although the compound camptothecin (CPT) was isolated in the 1950s, the cellular targets, DNA topoisomerases, were discovered and characterized only several years later, first in bacteria in 1971 (Wang, 1971) and afterwards in mouse cells (Champoux and Dulbecco, 1972). Topoisomerases counteract topological stress in the DNA double helix arising from the requirement to massively compact DNA. Supercoiled DNA is an obstacle to transcription as well as replication and needs to be resolved transiently to facilitate the segregation of replicated DNA strands to the daughter cells before cell division can occur (reviewed in (Pommier et al., 2010)). Eukaryotic topoisomerases have been grouped into two classes, with type I enzymes relieving topological tension by transiently incising one strand of the DNA duplex thereby creating a SSB, and type II enzymes acting by cleavage of both DNA strands thereby forming a DSB. Type I topoisomerases can be further subdivided into type IA and type IB enzymes. *Saccharomyces cerevisiae* contains three genes encoding for the different but highly conserved topoisomerase classes: *TOP3* coding for the type IA enzyme (Kim and Wang, 1992), *TOP1* for the type IB enzyme (Thrash et al., 1985), and the type II topoisomerase encoded by the *TOP2* gene (Goto and Wang, 1984). Yeast Top1 and type IB class members in other species can resolve both positive and negative supercoiling (reviewed in (Wang, 1996)). Mechanistically, type IB enzymes like Top1 utilize several transesterification steps involving their catalytically active tyrosine residue to attack the DNA phosphodiester backbone and transiently form a Top1-DNA complex. This complex has been termed the Top1- cleavable complex (Top1cc) and leads to the formation of a SSB. Subsequently, Top1 can lead to a strand rotation thereby relieving the topological stress. As a last step, the SSB created by Top1 action is re-ligated thereby resolving the Top1-DNA intermediate (Top1cc). The two subclasses type IA and IB are discriminated by the kind of enzyme-DNA linkage that is formed during the reaction and the mechanism by which they lead to DNA relaxation. CPT treatment causes the stabilization of the transient Top1cc by intercalating into the cleavage site between Top1 and the DNA strand. Thereby, CPT prevents the final step of DNA re-ligation and consequently causes a persistent SSB (reviewed in (Pommier et al., 2010)). Since CPT does not directly interfere with Top1's catalytic activity to inhibit its function, the term Top1 poison has been established. As a result of the stabilized Top1-DNA intermediate, RNA and DNA polymerases encounter an obstacle during transcription and replication, respectively. Collision of the polymerases with the Top1cc ultimately leads to a DSB and an irreversible crosslink between Top1 and the DNA.

Although not fully understood, the repair of CPT-induced lesions requires both the removal of the enzyme Top1 and the religation of the free DNA ends (reviewed in (Pommier et al., 2010)). So far, at least three pathways to repair CPT-induced DNA damage have been proposed. The best studied mechanism involves the tyrosyl-DNA phosphodiesterase 1 (Tdp1) (Yang et al., 1996). In order to enable repair of the DNA, Top1 protein has to be removed from the stabilized Top1cc by degradation via the proteasome (Desai et al., 2001; Lin et al., 2009). Subsequently, Tdp1 can process the Top1 tyrosyl remnant left behind (Debethune et al., 2002; Pouliot et al., 1999) . Eventually, polynucleotide kinase/ phosphatase (PNKP in humans and Tpp1 in budding yeast) removes the 3`phosphate left on the DNA strand by Tdp1 to allow subsequent repair.
Alternative pathways have implicated endonucleases in the removal of the CPT-mediated stabilized Top1cc or the reversal of the replication machinery concerted with the action of a helicase (reviewed in (Pommier et al., 2006)).
Analogous to topoisomerase I poisoning by CPT, also the type II topoisomerases can be poisoned using etoposide and its derivatives. As for the topoisomerase I-CPT interplay, etoposide stabilizes the reaction intermediate between enzyme and DNA (reviewed in (Cuya et al., 2017)). Due to their mode of action, CPT and its derivatives require DNA replication to cause cytotoxicity and are therefore considered S-phase specific.
An increasing number of CPT derivatives and analogues has been developed and tested for their suitability in cancer treatment. However, the poor chemical stability of CPT under physiological conditions renders drug development difficult. Moreover, the cellular concentration of CPT is decreased due to the activity of pumps such as breast cancer resistant protein (BRCP) or the efflux transporter p-glycoprotein thereby drastically decreasing CPT efficacy as an anti-tumour drug. Nevertheless, CPT compounds are used in chemotherapy targeting a range of cancer types including lung, cervical and breast cancer (reviewed in (Beretta et al., 2006)).
Combinational therapies including CPT derivatives or analogues appear to be especially promising. For example, concomitant inhibition of Tdp1, which is involved in repair of CPT-induced damage, should enhance CPT efficacy as a chemotherapeutic (reviewed in (Cuya et al., 2017)). Although Tdp1-independent CPT damage repair has been reported and this pathway relies on the DNA damage checkpoint, co-administration of CPT and Tdp1 inhibitor can be beneficial based on the fact that most cancers are (at least partially) checkpoint-deficient (reviewed in (Broustas and Lieberman, 2014)).

1.3 DNA damage arising from dysfunctional telomeres

Not only intrachromosomal lesions but also DNA damage arising from chromosome ends can lead to the activation of the DDC. This is because telomeres, the ends of linear chromosomes, structurally resemble one half of a DSB. However, telomeres are protected from being recognized as damage since inappropriate repair events would lead to lethal repair outcomes such as fusions between chromosomes. How telomeres escape from being recognized as DNA damage to ensure genome stability is outlined below.

1.3.1 Overview of yeast telomeres

1.3.1.1 Structure and maintenance of telomeres

Telomeres in the budding yeast *Saccharomyces cerevisiae* consist of simple but heterogeneous repeats with the sequence abbreviated as $C_{1-3}A/TG_{1-3}$ (Shampay et al., 1984) extending over a total length of approximately 300 bp (+/- 75 bp). Wild type yeast cells express telomerase to maintain telomere length, which functions as a reverse transcriptase adding telomeric repeat sequences by using a bound RNA as a template. Budding yeast telomerase consists of three proteinaceous components called Est1, Est2 and Est3, named after their deletion phenotype ever shorter telomeres, and the telomeric repeat templating RNA moiety, *Tlc1* (reviewed in (Wellinger and Zakian, 2012)). Since DNA sequences at the very ends of linear chromosomes would be lost during DNA replication due to the end-replication problem or by the action of nucleases and therefore, telomeres would shorten in every round of cell division eventually leading to the loss of genetic information. The majority of eukaryotic cells, including *Saccharomyces cerevisiae*, employs telomerase to maintain telomere length (reviewed in (Wellinger and Zakian, 2012)). Importantly, human somatic cells do not express telomerase at detectable levels and therefore only divide a limited number of times, which is referred to as the Hayflick limit (Hayflick, 1965). This difference in telomerase expression amongst eukaryotes could reflect distinct requirements in short-lived *versus* long-lived organisms. Chromosome ends are not blunt-ended but possess a 3`single-stranded overhang extending from the G-rich strand, referred to as the G-tail. While the G-tail only comprises roughly 15 nucleotides during most of the cell cycle (Larrivee et al., 2004), its length is increased (to approximately 30 to 100 nucleotides) during late S and early G2 (Wellinger et al., 1993). This increase in G-tail length is not only due to telomerase action but also caused by a cell-cycle dependent degradation of the opposing C-rich strand and is linked to the semiconservative nature of DNA replication (reviewed in (Bonetti et al., 2013)). Telomeric repeats constitute the terminal part of telomeres and lack nucleosomes (Ichikawa et al., 2014; Wright et al., 1992). In contrast, the sequences oriented towards the centromere are termed subtelomeric regions and are considered very dynamic and prone to undergo recombination events (reviewed in (Wellinger and Zakian, 2012)). Subtelomeric DNA is mostly packaged into nucleosomes (Wright et al., 1992) and interestingly, H2A phosphorylated at serine 129 has been found enriched at subtelomeres. This seems curious since H2A phosphorylation is created in a Mec1/Tel1-dependent manner in response to DNA damage indicating that telomeres are at least transiently recognized as damage ((Kim et al., 2007), reviewed in (Longhese, 2008)).

1.3.1.2 Telomeric binding proteins and their function

Although telomeres are considered histone-free, they are bound by specialized telomeric-binding proteins such as Rap1, which binds the dsDNA part of the telomeric repeats. The Rap1 C-terminus is crucial for its function at telomeres and serves as a binding platform for the silencing proteins Sir3 and Sir4 which establish the heterochromatic status of telomeres by recruiting the histone deacetylase Sir2. Additionally, Rap1 recruits the Rap1-interacting factors, Rif1 and Rif2 functioning in telomere length maintenance by negatively regulating telomerase (reviewed in (Wellinger and Zakian, 2012)).

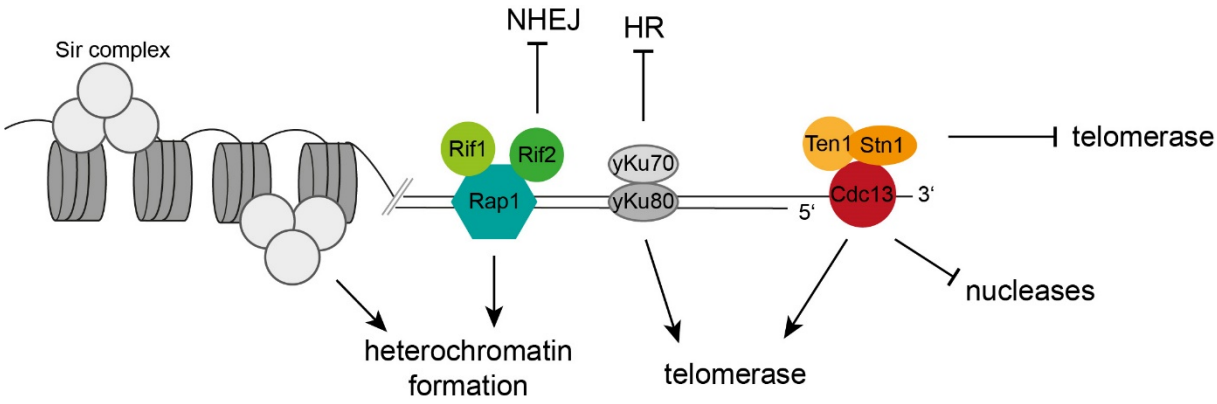


Figure 3. Overview on telomeric binding proteins in *S. cerevisiae* and their functions in telomere biology.

Another protein complex binding to telomeric dsDNA consists of the Yku70 and Yku80 heterodimer referred to as the Ku complex. The Ku proteins play a key role in telomere structure and maintenance (Gravel et al., 1998) and are important regulators of the non-homologous end joining (NHEJ) pathway to repair DNA damage (Boulton and Jackson, 1996; Milne et al., 1996). They have been proposed to localize at the dsDNA-ssDNA border of the telomere (Martin et al., 1999) but also to the subtelomeric region via an interaction between Yku80 and the Sir4 silencing protein (Roy et al., 2004).

The ssDNA portion of the yeast telomere is bound by the essential protein Cdc13 (Lin and Zakian, 1996), identified as a temperature-sensitive mutant affecting cell cycle progression, hence the akronym Cdc for cell division cycle (Hartwell et al., 1973). Cdc13's localization to the telomere and its DNA binding ability is conferred by a C-terminal OB (oligonucleotide/ oligosaccharide binding) domain exhibiting high affinity and specificity for telomeric ssDNA sequences (Mitton-Fry et al., 2002; Mitton-Fry et al., 2004). In addition, Cdc13 harbours more OB folds of which the OB1 domain in the N-terminus is involved in dimerization and has been suggested to bind telomeric ssDNA sequences as well (Mitchell et al., 2010). Cdc13 has a capping function at telomeres preventing them from being recognized as DNA damage by concealing the telomeric ssDNA (Garvik et al., 1995). To accomplish its capping function, Cdc13 cooperates with Stn1 and Ten1 to form the CST (Cdc13-Stn1-Ten1) complex (Grandin et al., 2001b; Grandin et al., 1997). Cdc13 has been reported to prevent homologous recombination at the telomere thereby preserving genome stability (Grandin et al., 2001a). In addition to CST-mediated telomere capping and protection, Cdc13 is also involved in telomere length maintenance. The OB1 fold serves as a binding platform for DNA polymerase α involved in C-strand synthesis (Sun et al., 2011). Within the Cdc13 protein, the OB1 fold is followed by a telomerase recruitment domain (RD) facilitating the interaction with the telomerase subunit, Est1 (Chandra et al., 2001), which leads to the recruitment of the holoenzyme to the telomere to allow elongation. Stn1 has been reported to bind to a similar or adjacent stretch in the Cdc13 protein sequence thereby competing with Est1 and suggesting that Stn1 acts as a negative regulator of telomerase (Grandin et al., 2001b; Grandin et al., 1997). Therefore, a dual role for Cdc13 in telomere length regulation, both positive and negative, has been proposed (see Figure 3 for an overview on telomere binding proteins and their function).

In the temperature-sensitive *cdc13-1* mutant, a proline residue at position 371 is mutated to serine (Nugent et al., 1996). Due to its location within the OB2 domain, this amino acid substitution has been reported to impair Cdc13 dimerization. As a consequence, the interaction between Cdc13 and Stn1 is abolished (Mason et al., 2013) leading to a capping defect which is dramatically exacerbated at elevated temperatures in combination with over-elongated telomeres due to the lack of the negative telomerase regulator, Stn1 (Grandin et al., 1997). Importantly, the CST complex has been shown to be dispensable for telomere capping in non-dividing cells. Rather, the Ku complex in association with Rap1 are responsible for telomere protection in this instance (Vodenicharov et al., 2010). Another protein implicated in telomere length maintenance is Tel1 which also functions in the DDC (Lustig and Petes, 1986). As in the DNA damage response pathway, Tel1 associates with the MRX complex (Ritchie and Petes, 2000) and facilitates the recruitment of telomerase (Goudsouzian et al., 2006).
In addition but not surprisingly given its binding specificity, also the ssDNA binding complex RPA has been proposed to associate with telomeres and positively regulate telomerase action (Luciano et al., 2012).

1.3.2 The DNA damage response at telomeres

Telomeres can be recognized as DNA damage via two different pathways. Firstly, telomere dysfunction can occur immediately, for example by removal of telomere binding proteins. Secondly, in the absence of telomerase as a telomere-lengthening mechanism, chromosome ends shorten continuously which eventually leads to DDC activation resulting in permanent cell cycle arrest termed replicative senescence (AS and Greider, 2003; Lundblad and Szostak, 1989). The senescent state can be overcome by homology-mediated recombination dependent on Rad52 in order to elongate telomeres in the absence of telomerase and re-establish cell division potential (Lundblad and Blackburn, 1993). Telomerase-deficient yeast cells that have escaped senescence employing this pathway were termed survivors and the mechanism of recombination-mediated telomere elongation in telomerase-negative cells was found to be conserved in human cells (Bryan et al., 1997; Bryan et al., 1995). This recombination-mediated and telomerase-independent alternative lengthening of telomeres (ALT) has been studied extensively since the unlimited proliferative capacity characteristic of cancer cells is either based on the ALT pathway (approximately 10% of cancers) or the re-activation of telomerase (reviewed in (Apte and Cooper, 2017)).
Telomere capping mediated by the CST complex prevents DNA damage signalling and consequently protects from undesired repair events such as homologous recombination and non-homologous end joining.
Acute telomere dysfunction elicited for example by using the *cdc13-1* allele causes the uncapping of telomeres. Consequently, this leads to the loading of the 9-1-1 checkpoint clamp onto telomeres aided by the clamp loader to activate Exo1 which leads to the degradation of the C-rich strand (Jia et al., 2004; Zubko et al., 2004). The newly created ssDNA serves as a recruitment platform for DNA damage sensors such as Ddc1 (Melo et al., 2001) and causes DDC activation followed by cell cycle arrest. It has been suggested that Mec1 activates Rad9 to prevent further ssDNA generation via Rad53-dependent and -independent mechanisms (Jia et al., 2004). In addition, a role for additional nuclease(s) upon telomere uncapping has been

proposed which are thought to be regulated by the clamp loader component, Rad24 (Jia et al., 2004). In order to obtain full DDC activation in response to telomere uncapping, the Rad9 checkpoint adaptor is required to couple Mec1 recruitment and activation to the downstream signalling cascade involving Rad53 (Garvik et al., 1995). On the other hand, telomere dysfunction caused by successive telomere shortening in the absence of telomerase requires a second checkpoint adaptor, Mrc1 (Grandin and Charbonneau, 2007). Mrc1 is also required for checkpoint activation in response S-phase specific damage leading to replication stress (Alcasabas et al., 2001). This suggests that telomere dysfunction is also detected via the cellular response to replication stress. In addition, Mrc1 has also been attributed to play a role in telomere end protection in *cdc13-1* mutants, where Mrc1 prevents Exo1 access and checkpoint activation (Tsolou and Lydall, 2007)

Cdc13 prevents Mec1 association with telomeres to prevent Exo1-mediated ssDNA generation and thereby attenuate DDC activation. However, Tel1-MRX recruitment is allowed to occur even at Cdc13-capped telomeres underlining the positive function of Tel1 in telomere length regulation (Hirano and Sugimoto, 2007). This highlights the dual functions of many proteins that play an important role in both response to telomere damage and intrachromosomal damage such as a DSB. Since these two scenarios require differential treatment, a recent publication has proposed the establishment of a threshold by the helicase Pif1 (Strecker et al., 2017). The authors suggest that Pif1, which also acts as a telomerase inhibitor, can bind to break sites that harbour short telomeric sequences representative for an intrachromosomal DSB and prevent telomerase-dependent telomere sequence addition. On the contrary, it is dispensable at break sites flanked by long telomeric sequences resembling a telomere (Strecker et al., 2017). Consequently, the presence of Pif1 at break sites can prevent that these damage sites are treated as telomeres and thereby allow repair events to take place. The same publication showed that the decreased association of Pif1 with breaks sites resembling telomeres was due to the presence of Cdc13 in its function as a telomere binding protein (Strecker et al., 2017). Conversely, recruitment and activation of Mec1 at intrachromosomal break sites prevents Cdc13 binding to these sites via a Mec1-dependent phosphorylation thereby blocking the recognition of intrachromosomal damage as a telomere (Zhang and Durocher, 2010).

Why is it crucial to promote repair at intrachromosomal DSBs but prevent it at telomeres?

Accidental DSBs in intrachromosomal regions need to be repaired in order to facilitate unobstructed DNA replication and transcription. On the other hand, unscheduled repair events at telomeres can have detrimental outcomes for cell survival. There are two main pathways in place to repair DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ). While the occurrence of HR at telomeres is required to some extent for telomere length maintenance in the absence of telomerase, full activation of HR is considered harmful due to increased rates of sister-chromatid exchanges and the appearance of telomeric circles (reviewed in (Claussin and Chang, 2015)). Telomeric sister-chromatid exchanges have been linked to accelerated rates of replicative senescence (Blagoev et al., 2010) and the formation of extrachromosomal circles containing telomeric DNA can lead to loss of genetic material (Wang et al., 2004). NHEJ of telomeres lead to telomere fusion events that can eventually cause circular or dicentric chromosomes. These structurally aberrant chromosomes are unable to segregate properly to daughter cells during mitosis and therefore precede copy number

alterations. Rap1 has been reported to play a major role in preventing NHEJ at telomeres (reviewed in (Marcand, 2014)).

1.4 DNA damage repair pathways

In order to facilitate optimal repair for the many different kinds of lesions that can affect DNA integrity and function, cells have evolved distinct pathways involving specialized repair proteins. Outlined below are the two major pathways to ensure repair of DSBs: NHEJ and HR, as well as a short summary of pathways involved in repair of base damages.
Interestingly, in addition to other factors like cell cycle stage and the type of lesion, also the localization of the damage site with respect to the nuclear space influences repair pathway choice and efficiency (Lee et al., 2016; Lemaitre et al., 2014). Moreover, inefficiently repaired or even irreparable damage sites were shown to localize to the nuclear periphery. This process is mediated by repair factors associated with the lesion and the nuclear envelop protein, Mps3 or nuclear pore components suggesting that the nuclear periphery is a specialized repair environment (reviewed in (Freudenreich and Su, 2016)). Likewise, eroded telomeres eliciting a DDR have been reported to re-localize to nuclear pore complexes (Khadaroo et al., 2009).

1.4.1 Non-homologous end joining (NHEJ)

Non-homologous end joining (NHEJ) describes the direct ligation of two DSB ends regardless of sequence homology and is therefore considered an error-prone repair pathway. Consequently, homology-based repair pathways are favoured if homologous sequences are present. The homologous sequences suitable as repair templates are available either after DNA replication in S and G2 phase or throughout the cell cycle in diploid yeast cells. As a result, NHEJ is only occurs in G1 phase of the cell cycle and in haploid yeast cells. This is in contrast to mammalian cells, where NHEJ is enabled throughout the cell cycle except for mid-S phase (Karanam et al., 2012; Mao et al., 2008). Ploidy-dependent repression of NHEJ is accomplished by the presence of the a1-α2 heterodimer expressed from the heterozygous mating locus in diploids. The a1-α2 heterodimer represses the transcription of *NEJ1*, encoding a crucial NHEJ factor (Frank-Vaillant and Marcand, 2001; Kegel et al., 2001; Valencia et al., 2001).
The core proteins facilitating NHEJ comprise the Ku complex, DNA Ligase IV (Dnl4), Lif1, Nej1 and the MRX complex (reviewed in (Emerson and Bertuch, 2016)). The process underlying NHEJ can be roughly divided into 3 steps consisting of tethering the DSB ends together and protecting them, assembling the protein complex and finally, ligating the ends. The Ku70/80 heterodimer together with the MRX complex have been described to localize rapidly in a first response to the DSB (Wu et al., 2008). The Ku complex prevents nucleolytic processing of the DSB (Clerici et al., 2008; Mimitou and Symington, 2010) thereby funnelling the repair pathway towards NHEJ. MRX has been proposed to function as a linker between the DBS ends (Chen et al., 2001) thereby tethering the ends together in cooperation with Tel1 and Rif2 (Cassani et al., 2016). In the next step, DSB ends are stabilized by strand annealing (if there is homology) and the formation of the NHEJ repair complex. This is facilitated by a scaffolding function of Ku, which recruits the Dnl4-Lif1 complex and Nej1 to the break site (Chen and Tomkinson, 2011; Zhang et al., 2007). Dnl4 is stabilized by Lif1 (Herrmann et al.,

1998) and while it is recruited via the Ku80 C-terminus it can conversely also stabilize the Ku proteins at the DSB (Chen and Tomkinson, 2011; Zhang et al., 2007). The accessory protein Lif1 interacts with Nej1 (Ooi et al., 2001), which is the subject of mating locus-mediated NHEJ repression in yeast diploids. Nej1 has been proposed to reinforce Ku`s function in preventing nucleolytic processing (Zhang et al., 2007) and assist in the recruitment and stimulation of other downstream NHEJ factors including Pol4 (Yang et al., 2015). The Pol4 polymerase functions in gap filling subsequent to the Dnl4-mediated ligation of the two DSB ends (Bebenek et al., 2005; Tseng and Tomkinson, 2002; Wilson and Lieber, 1999). Pol4 has a high propensity to induce errors and exhibits low processivity making it suitable for filling in small gaps and tolerating the limited sequence homology found during NHEJ (Bebenek et al., 2005). The involvement of the MRX complex which functions in resection seems counterintuitive given the fact that nucleolytic processing of DSB ends channels the break site into recombination-based repair pathways (reviewed in (Emerson and Bertuch, 2016)). Clb-mediated Cdc28 activity is required to initiate resection which serves as the initiation step for homology-based repair pathways (Aylon et al., 2004; Ira et al., 2004). This is facilitated by a Cdc28-mediated phosphorylation of the nuclease Dna2 to enable long-range resection at DSBs (Chen et al., 2011b). However, how cell-cycle mediated regulation of short-range resection is achieved is not fully understood. Since activity of the Clb-Cdc28 complex is low during G1 phase resection is inhibited thereby establishing NHEJ as the favoured repair pathway. These findings seem to contradict a role for the MRX complex in resection at DSB in G1 phase, yet the involvement of MRX during NHEJ is crucial. Rather, MRX has been suggested as a bridging factor thereby helping to hold the two broken DNA ends together. This seems consistent with the structure of the MRX complex or, more precisely, its Rad50 subunit (reviewed in (Emerson and Bertuch, 2016)). Moreover, MRX recruitment to intrachromosomal DSBs has been proposed to act as a switch for repair pathway choice dependent on the cell cycle stage (reviewed in (Mathiasen and Lisby, 2014)).

1.4.2 Homology-directed repair (HDR)

As outlined above, ploidy and cell cycle stage regulate repair pathway choice. The latter is achieved by regulation of resection on a post-translational level by Cdc28-mediated phosphorylation of Sae2 and Dna2 in S and G2 phase. In addition, association of Cdc28 with the G1-specific Cln3 regulates resection factors on a transcriptional level (reviewed in (Mathiasen and Lisby, 2014)).
The resection step necessary for HDR can be subdivided into two steps: a short-range nucleolytic processing carried out by MRX and Sae2, followed by an extensive long-range processing mediated by Exo1 and Dna2 (reviewed in (Mathiasen and Lisby, 2014)).
After the initial critical resection step during homologous recombination, the newly created ssDNA will be bound efficiently by RPA. Coating of the ssDNA is necessary for protection from further nucleolytic processing and prevents the formation of secondary structures that could be an obstacle for the downstream events (Alani et al., 1992; Sugiyama et al., 1997). However, RPA has to be removed by the crucial HR mediator protein, Rad52 (Game and Mortimer, 1974; Sugiyama et al., 1997; Sung, 1997a), in order to allow the loading of Rad51, a central factor during HR (Game and Mortimer, 1974; Shinohara and Ogawa, 1998; Sugiyama

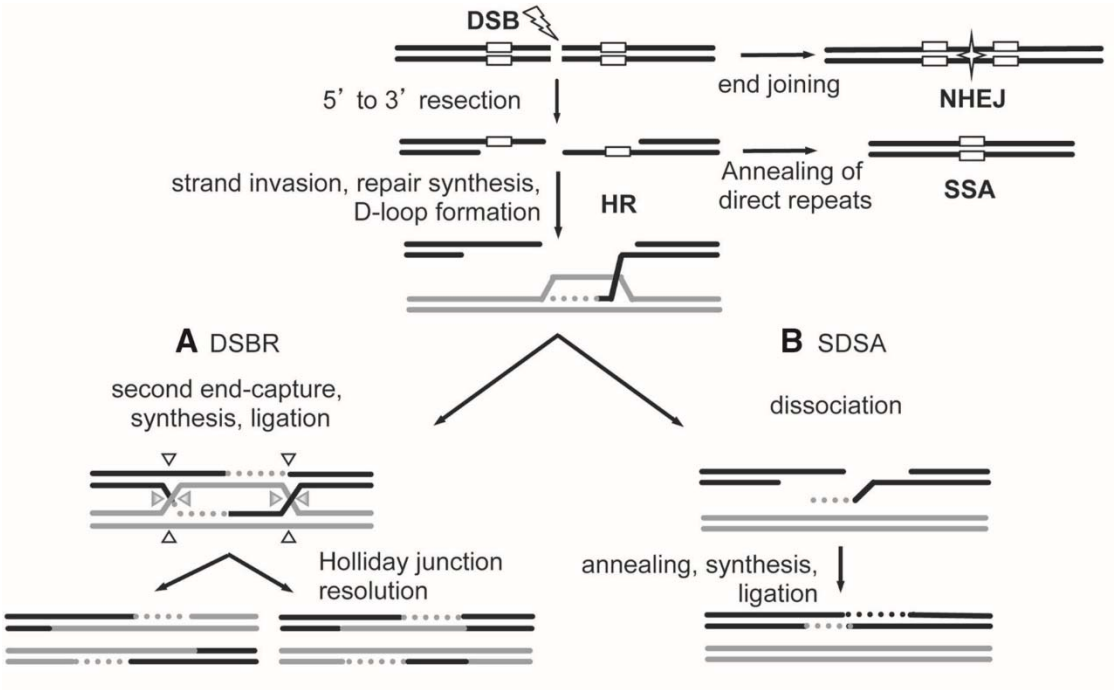


Figure 4. DNA repair by non-homologous end joining (NHEJ) and homologous recombination (HR). The two different scenarios, DSBR and SDSA, following strand invasion during HR are also outlined in panel A and B, respectively. DSBR: Double-strand break repair; SDSA: synthesis-dependent strand annealing. Figure taken from (Do et al., 2014).

and Kowalczykowski, 2002; Sung, 1997a). The Rad51-loading function carried out by Rad52 is assisted by Rad55 and Rad57 (Sung, 1997b), which are paralogs of Rad51 (Lovett, 1994). A direct interaction of Rad52 and Rad51 has been documented, although this has been found to be dispensable for Rad52`s HR mediator function (Krejci et al., 2002). Subsequently, Rad51 binds to the ssDNA to form a stable nucleoprotein filament which is actively protected by the Shu complex from disassembly by the helicase and anti-recombinase Srs2 (Bernstein et al., 2011; Godin et al., 2013). The Rad51-containing nucleoprotein filament then scans the genome searching for an intact homologous sequence that can be used for repair. The movement of the broken ends caused by a DSB searching for a homologous repair template has been tracked in live cells (reviewed in (Mine-Hattab and Rothstein, 2013)) and seems dependent on DDC factors as well as proteins involved in HR (Dion et al., 2012). Once a homologous stretch has been found, Rad51 catalyses the displacement of one strand of the intact homologous duplex to create a displacement loop (D-loop) which allows the invasion of the ssDNA portion of the broken end (reviewed in (Symington et al., 2014)). After invasion, the structure is stabilized by Rad52-mediated base-pairing with one strand of the intact homologous duplex (Mortensen et al., 1996) and by RPA coating the displaced strand of the intact duplex (Eggler et al., 2002; Sugiyama et al., 1997). Following strand invasion, two possible mechanisms have been proposed. On one hand, during the pathway described as classical DSB repair (DSBR), Rad54-mediated removal of Rad51 is necessary to make the invading strand accessible for the replication machinery (Li and Heyer, 2009). The DNA polymerase responsible for DNA synthesis in a D-loop during HR is DNA polymerase δ ((Maloisel et al., 2008), reviewed in (Symington et al., 2014)). Subsequently, HR intermediate structures called Holliday junctions

(HJ) need to be resolved in order to allow subsequent undisturbed chromosome segregation. Removal of interlinked DNA molecules arising during the HR process is achieved mainly by two different mechanisms. First, the Sgs1-Top3-Rmi1 helicase complex together with the helicase Mph1 can function in HJ dissolution and secondly, nucleases including Mms4 and Mus81 can resolve repair intermediates that remain after helicase-mediated processing (reviewed in (Bizard and Hickson, 2014; Talhaoui et al., 2016)).
In a second alternative scenario that can follow strand invasion and which has been termed synthesis-dependent strand annealing (SDSA), no HJ are formed since the D-loop migrates with DNA synthesis and therefore, no HR intermediates have to be resolved afterwards (reviewed in (Symington et al., 2014), see Figure 4 for an overview).
As information encoded in the damaged DNA sequence is copied from an intact homologous repair template, the HR repair pathway is considered largely error-free.

1.4.2.1 The role of Rad52 in budding yeast and mammalian cells

It has been suggested that yeast Rad52 has two distinct roles at different steps during HR: it can act as a mediator protein to enable Rad51-mediated nucleoprotein filament formation and later on, assist in strand annealing after a homologous repair template has been invaded. Therefore, Rad52 is a core factor for seemingly all types of HR processes (reviewed in (Mortensen et al., 2009)). Rad52 has been identified in a genetic screen for X-ray sensitive mutants and is the founding member of the *RAD52* epistasis group including also the HR protein, Rad51, and the central checkpoint kinase, Rad53 (Game and Mortimer, 1974).
In light of the high conservation amongst eukaryotes in HR, it was surprising that mammalian Rad52 was shown to be dispensable for resistance to genotoxins including irradiation (Rijkers et al., 1998). It has been proposed that human Rad52 might serve as a back-up protein since loss of Rad52 in cells lacking other HR factors was found to be synthetically lethal (Feng et al., 2011; Lok et al., 2013). Human Rad52 has been implicated in facilitating strand annealing during HR, while the mediator function of yeast Rad52 has been attributed to human BRCA2 (reviewed in (Liu and Heyer, 2011)). Together with *BRCA1*, *BRCA2* has been identified as a hereditary breast cancer susceptibility gene and mutations in these genes predispose to a variety of cancers including breast but also ovarian, prostate and pancreatic cancers (reviewed in (Prakash et al., 2015)).

1.4.2.2 Mammalian BRCA2 and its involvement in cancer treatment resistance

BRCA1 is a central player in the mammalian HR pathway and engages in multiple protein complexes to fulfil numerous critical steps during the repair process. For example, BRCA1 associates with BRCA2 and others to facilitate strand invasion (reviewed in (Lee et al., 2017)). This encourages the idea that human BRCA2 acts solely as a mediator and is supported by the fact that BRCA2 seems to lack a strand annealing function ((Jensen et al., 2010), reviewed in (Liu and Heyer, 2011)). In summary, cells lacking the *BRCA* gene products are highly impaired in their DNA repair proficiency thereby providing a target pathway to eradicate these cells.
Especially platinum-based chemotherapeutics such as carboplatin have proven as valuable agents against *BRCA*- deficient cancer cells. These agents introduce DNA damage by forming

adducts with DNA bases and causing intrastrand crosslinks (reviewed in (Siddik, 2003)). Additionally, treatment with inhibitors of the PARP enzymes were found to be very effective in these settings. PARP1 (poly-(ADP-ribose)-polymerase 1) localizes to DNA damage sites and adds poly-ADP-ribose (PAR) molecules to various DDR proteins, a posttranslational modification referred to as PARylation. Eventually, PARP1 will PARylate itself as well and therefore mediate its release from the damage site. There are two PARP enzymes present in mammalian cells which are activated by DNA damage and all inhibitors developed to date target both PARP1 and PARP2. Inhibitors of PARP enzymes have been tested for their efficacy as a single-agent therapy ((Bryant et al., 2005), reviewed in (Ohmoto and Yachida, 2017)) but they are also used as sensitizing agents for other cancer treatments like radiation but mostly with chemotherapeutics such as carboplatin (reviewed in (Drean et al., 2016)). Since loss of one or both *BRCA* genes renders cells unable to use HR-mediated repair pathways upon DNA damage, concomitant inhibition of PARP directs towards error-prone repair mechanisms. Thereby, cells are driven into cell cycle arrest or apoptosis ((Farmer et al., 2005), reviewed in (Ohmoto and Yachida, 2017)). However, *BRCA*- deficient and therefore HR- impaired cancers often acquire resistance to chemotherapeutic treatment (reviewed in (Lord et al., 2015)). Mainly, cells are *BRCA*-deficient due to deletions in the coding region(s) leading to the expression of non-functional truncation mutants of BRCA1 and/or BRCA2. Therefore, a major pathway towards resistance is the acquisition of secondary mutations that are able to restore *BRCA* function and thereby re-establish the HR repair pathway (Edwards et al., 2008; Sakai et al., 2008). Such a process causing the re-gain of BRCA protein function is exacerbated in a treatment scheme that favours error-prone repair pathways and is applied over a longer period of time thereby allowing for the selection of resistant cancer cells that are able to avoid the treatment.

1.4.2.3 Rad52- independent repair

As described above, *BRCA* deficiency in human cancers is often reverted by secondary mutations that lead to the expression of a mutant but at least partially functional BRCA protein. Furthermore, human Rad52 is not strictly required for HR. Also yeast cells lacking the central HR gene *RAD52* have been described to utilize alternative Rad52-independent repair pathways instead. Especially when extensive homologous sequences flank the DSB site, for example at the rDNA locus, Rad52 has been shown to be dispensable for strand annealing (Ozenberger and Roeder, 1991). Moreover, telomerase-negative yeast survivors that use a HR-based telomere maintenance pathway can form in the absence of Rad52. In this case, telomerase- and HR-deficient cells were shown to maintain their chromosome ends using palindromic sequences that protected from loss of essential genetic material (Maringele and Lydall, 2004). A second report studying telomere length maintenance in the absence of both telomerase and Rad52 proposed an end-joining mechanisms and termed the novel pathway ILT for inherited long telomere (Grandin and Charbonneau, 2009). In *rad52* mutants, recombination events were still found to occur but their outcomes were changed indicating the occurrence of an alternative HR pathway (Haber and Hearn, 1985). Moreover, upon UV-induced damage, *rad52* mutants showed similar recombination products as wild type yeast cells. The same study showed a requirement for the MRX member Rad50 in both spontaneous and UV-induced recombination

and for the Rad52 paralog, Rad59, for spontaneous recombination in the absence of Rad52 (Coic et al., 2008).
Taken together, these results highlight the existence of Rad52-independent HR events that seem to be influenced by the environment of the damage site in terms of DNA sequence as well as the type of damage which requires recombination-mediated repair.

1.4.3 Other pathways involved in DNA damage repair

1.4.3.1 Break-induced replication (BIR)

Break-induced replication (BIR) constitutes a sub-pathway of homologous recombination. As other HR-mediated repair events, BIR usually requires Rad51-mediated strand invasion to form a D-loop. BIR occurs after replication fork collapse or at dysfunctional telomeres (reviewed in (Malkova and Ira, 2013)). It is thought to take place when only one end of a DSB can successfully invade a duplex with homologous sequence or if there is only one free end such as in the case of a SSB. Subsequently, a replication fork is assembled containing similar protein components as canonical replication forks initiated from origins of replications. For example, the MCM helicase and DNA Polα-primase complex as well as DNA polymerase δ have been reported to be involved in BIR (Lydeard et al., 2007; Lydeard et al., 2010). In addition, the Pif1 helicase was reported to promote DNA Pol δ-mediated replication and deal with topological stress during the BIR process (Wilson et al., 2013). Importantly, two different models regarding the BIR mechanism and progression have been proposed depending on whether the initial D-loop resulting from strand invasion is resolved or maintained during replication (reviewed in (Malkova and Ira, 2013)). Although based on presumably error-free homologous recombination, BIR is a highly mutagenic process (Deem et al., 2011). Furthermore, BIR has been linked to genome rearrangements since the replication fork mediating BIR can dissociate and restart during the process thereby leading to template switching (Smith et al., 2007). Finally, BIR events can lead to translocations and cause loss of heterozygosity (reviewed in (Malkova and Ira, 2013)).

1.4.3.2 Mismatch repair (MMR)

Mismatch repair (MMR) contributes to DNA replication fidelity by dealing with mismatched bases or mispairing due to insertions or deletions up to 12 mispaired bases (reviewed in (Kolodner and Marsischky, 1999)). MMR proteins are also involved in other DNA repair pathways such as BER, DSBR and interstrand crosslink repair. A critical step during MMR is the discrimination between the parental DNA strand containing the correct base and the nascent DNA strand in which the mispairing has occurred. In prokaryotes, DNA methylation that is yet missing on the nascent daughter strand can serve as a discrimination signal. Contrastingly, in eukaryotes several replication characteristics have been proposed as such signals including nicks occurring during lagging strand synthesis, incorporated ribonucleotides or asymmetrically loaded PCNA (reviewed in (Liu et al., 2017a)). In brief, mismatches are recognized by the Msh2-Msh6 heterodimer and the Msh2-Msh3 heterodimer, which seem to have overlapping functions in the recognition process. Subsequently, the Mlh1-Pms1 heterodimer is recruited and introduces a nick in the newly replicated daughter strand

containing the mismatch. The newly created nick then serve as the access point for an exonuclease such as Exo1. The resected strand is subsequently filled in by DNA polymerase δ and the remaining nick is ligated to finish the reaction (reviewed in (Reyes et al., 2015)). In order to allow immediate repair of mismatches, the Msh heterodimeric complexes travel along with the replication fork mediated by an interaction with PCNA (Clark et al., 2000).
Loss of or mutations in MMR genes lead to a drastic increase in mutation frequency and are therefore associated with cancer development. In fact, especially colon cancer has been linked to MMR deficiencies. Furthermore, increased mutational rates leading to genome instability and possibly increased DNA damage loads are associated with pre-mature aging, however, the exact role and impact of MMR has not been fully understood yet (reviewed in (Hsieh and Yamane, 2008)).

1.4.3.3 Nucleotide excision repair (NER)

Nucleotide excision repair (NER) deals with bulky DNA lesions including ultraviolet (UV) light-induced pyrimidine dimers but also other helix-distorting aberrations that are obstacles to transcription and replication. In addition, NER is implicated in the removal of lesions caused by oxidative stress (reviewed in (Boiteux and Jinks-Robertson, 2013)). NER comes in two flavours: one is transcription-coupled (TC-NER) while the other sub-pathway acts globally and genome-wide (GG-NER). Both sub-branches share common players involved in later repair steps but seem to differ in proteins responsible for damage recognition.
GG-NER is initiated when the Rad4-Rad23-Rad33 heterotrimer (equal to XPC, HRAD23B and CEN2 in mammalians) recognizes a lesion (den Dulk et al., 2006; Jansen et al., 1998). The recognition step could be mediated by interactions of Rad4, a critical player in both NER pathways, with chromatin remodelling factors (Gong et al., 2006). In addition, a heterodimeric complex consisting of Rad7 and Rad16 (DDB1 and 2 in mammalians), both unique for GG-NER, is recruited (Verhage et al., 1994). This complex exhibits high affinity for UV-damaged DNA, a Rad16-mediated histone acetylase activity and has been linked to different interacting partners (Guzder et al., 1997; Teng et al., 2008). One interaction involves Afb1 allowing DNA translocation and conformational changes. Moreover, Rad7-Rad16 can interact with an E3 ubiquitin ligase forming a functional complex mediating the ubiquitination of Rad4 and other proteins in response to UV (reviewed in (Boiteux and Jinks-Robertson, 2013)). The essential initiation factor TFIIH, a multi-subunit complex required for RNA polymerase II-mediated transcription, is an important player of NER, both due to its helicase function and because of its interactions with other NER proteins (reviewed in (Egly and Coin, 2011)). Furthermore, also the ssDNA binding protein RPA is a crucial player in the NER pathway due to its ability to bind the undamaged DNA strand (de Laat et al., 1998). The aforementioned steps are often summarized as pre-incision complex formation and are followed by the actual incision of the lesion-containing DNA strand. This has been proposed to occur in a dual incision step flanking the lesion and is exerted by Rad2 (mammalian XPG) and Rad1-Rad10 (mammalian XPF and ERCC1) which are structure-specific endonucleases (Habraken et al., 1993; Tomkinson et al., 1993). Rad1-Rad10 interacts directly with Rad14 (XPA in mammals), a zinc finger domain protein with high affinity to UV-damaged DNA (Guzder et al., 1993, 1996). DNA polymerase

δ or ε are thought to accomplish re-synthesis of the excised DNA and finally, the nick is sealed by Cdc9, yeast DNA ligase I, to finish NER (reviewed in (Boiteux and Jinks-Robertson, 2013)). In contrast to GG-NER, TC-NER is specifically activated upon RNA polymerase II stalling. After the initial damage recognition, TC-NER utilizes the same factors as GG-NER. Then, TC-NER is divided into two sub-branches with one being dependent on Rad26 and the second dependent on the RNA polymerase II subunit, Rpd9 (reviewed in (Boiteux and Jinks-Robertson, 2013)). Interestingly, Rad26 is phosphorylated by Mec1 upon polymerase stalling to increase the efficiency of TC-NER (Taschner et al., 2010). Upon transient stalling of the transcription machinery, Rad26 has been suggested to promote damage bypass. On the other hand, at difficult obstacles or upon permanent stalling of the RNA polymerase complex, Rad26 leads to chromatin remodelling and recruitment of additional TC-NER components. Similarly, Rpb9 has been proposed to have comparable functions in the absence of Rad26 (reviewed in (Boiteux and Jinks-Robertson, 2013)).
In human cells, defects in TC-NER lead to a neurodegenerative disease condition termed Cockayne syndrome and deficiencies in GG-NER proteins are causative for xeroderma pigmentosum which is characterized by extreme sensitivity to sunlight and cancer predisposition (reviewed in (Garfinkel and Bailis, 2002)).

1.4.3.4 Base-excision repair (BER)

In order to deal with lesions caused by endogenous damage such as base deaminations or oxidative base damage, base-excision repair (BER) utilizes the sequential activity of repair complexes. The basic procedure of BER is initiated by cleavage of the N-glycosidic bond and therefore release of the damaged base from the DNA backbone by a DNA N-glycosylases. In yeast, two types of DNA N-glycosylases have been identified: one functions solely as a glycosylase, for example Ung1, while the second type can also act as an endonuclease to introduce a nick. Ogg1 belongs to the latter type (Sandigursky et al., 1997) and is involved in the removal of 8-oxo-G, a well-studied oxidative damage lesion. In the next step, a nick is introduced at the newly created abasic site by an endonuclease/lyase. *APN1* and *APN2* encode the two yeast endonucleases required for nick introduction. Before gap filling can take place, the SSB ends have to be processed by different enzymes including Rad27 and Tpp1 in order to produce a 3`hydroxyl group or 5`phosphate. Finally, DNA polymerase ε fills in the gap that is sealed in a last step by Cdc9, DNA ligase I (reviewed in (Boiteux and Jinks-Robertson, 2013)). Interestingly, PARP1 has often been implicated in mammalian BER due to its ability to bind a SSB and the observation that cells lacking *PARP1* could not complete BER in *in vitro* assays. However, instead of being a member of the canonical BER pathway, PARP1 has been suggested to play a more general role in response to SSB promoting their repair (Strom et al., 2011).

1.5 Termination of the checkpoint: recovery and adaptation

After DNA damage has been encountered, a cell cycle arrest is elicited in order to allow time for repair. To do so, a plethora of repair pathways has evolved to deal with the different kinds of lesions introduced by endogenous or exogenous damages.
If repair succeeds, the DDC has to be shut off for the cell to resume the cell cycle and continue proliferation. This process is referred to as checkpoint recovery.
Conceptually different from the recovery process is checkpoint adaptation. The term describes checkpoint termination followed by cell cycle progression despite the continuous presence of damage. This scenario can occur in a repair-defective or -impaired setting and has been described in many organisms.

1.5.1 Checkpoint recovery

The necessity to terminate the DDC and to abrogate the associated cell cycle arrest has obvious physiological relevance for a cell. Only after the DDC has been inactivated, cell cycle progression is possible and cell growth and division can be resumed. In the following section, I will outline factors involved in terminating the checkpoint after successful repair of the lesion.
In order to study effectors of DNA damage recovery, mostly the HO cut system has been used. In this case, the HO (*homothallic switching*) endonuclease has been employed which naturally introduces a DSB within the mating locus to facilitate mating type switching. Repair can take place using the silenced *HML* or *HMR* loci thereby leading to a switch from one mating type to the other (reviewed in (Haber, 1998)).
In such an experimental set-up, cells lacking the helicase Srs2 were recovery-defective (Vaze et al., 2002). The *srs2* recovery defect as evidenced by the inability to form colonies was restored when DDC function was abolished after successful repair using caffeine, a general inhibitor for PIKK kinases including Mec1 and Tel1. This suggested that Srs2 is indeed required for recovery and it was hypothesized that Srs2 function was required for removal of checkpoint proteins such as the Mec1-Ddc2 dimer or the 9-1-1 checkpoint clamp from the break site or for restoring normal chromatin structure thereby terminating the DDC (Vaze et al., 2002).
Since the DDC is mainly signalling via phosphorylation events, a series of phosphatases has been implied in recovery that could terminate the checkpoint after repair by reverting the activating phosphorylation signal. Consistently, the PP2C phosphatase family member Ptc2 has been implicated in acting at the level of Rad53 phosphorylation (Marsolier et al., 2000). Further investigations confirmed that Ptc2 as well as Ptc3, another PP2C family member, bind to and dephosphorylate Rad53 and thereby facilitate checkpoint recovery (Leroy et al., 2003). How exactly the phosphatases are regulated in order to switch off the DDC at the appropriate time or if an equilibrium of Rad53 phosphorylation and dephosphorlyation is shifted by Ptc2/Ptc3 activity remains elusive.
Another candidate for DDC inactivation is Pph3, which is part of the PP4 phosphatase complex (Keogh et al., 2006). Pph3 was shown to dephosphorylate histone H2A at serine 129 and thereby remove of one the most upstream signals for checkpoint activation. However, Pph3 targeting of phosphorylated H2A seems to occur after its removal from the damage site (Keogh

et al., 2006) suggesting that the mechanism provided by Pph3 is not the initial trigger for checkpoint recovery.
Since the creation of ssDNA is a very early and critical event in DDC signalling, proteins affecting resection could be also involved in DDC termination. Such an example is given by the endonuclease Sae2, which is phosphorylated in a checkpoint-dependent manner to restrict MRX action in a negative feed-back loop (Baroni et al., 2004; Clerici et al., 2006).

1.5.2 Checkpoint adaptation

The term adaptation in its basic meaning describes the adjustment to a specific environment or stimulus and thereby often implies a desensitization.
In the case of DNA damage, repair can be impossible for example due to mutations in or lack of crucial repair factors such as *RAD52* in yeast or *BRCA1* in human cells. Furthermore, also the location of the damage within a heterochromatic region can influence repair (reviewed in (Feng et al., 2016)). In such a scenario, cells can adapt to prolonged checkpoint activation and cell cycle arrest without prospective repair. As for checkpoint recovery, the DDC gets terminated in order to allow cell cycle resumption. Adaptation has been first observed in yeast (Sandell and Zakian, 1993) but was also found to occur in higher eukaryotes such as *Xenopus laevis* and human cancer cells. Adaptation might present a reasonable attempt for repair in a different cell cycle stage. For multicellular organisms, adaptation might also provide a possibility to remove cell progeny that harbours damaged DNA via apoptosis.

1.5.2.1 Major regulators

1.5.2.1.1 Casein Kinase II (CKII)

A genetic screen for regulators of adaptation in yeast identified *CKB1* and *CKB2* (Toczyski et al., 1997), which encode for the two non-essential regulatory subunits of casein kinase 2 (CK2) (Reed et al., 1994). In addition to Ckb1 and Ckb2, CK2 comprises the catalytic subunits Cka1 and Cka2 (reviewed in (Glover et al., 1994)). CK2 plays an important role in the regulation of cell morphology and size in yeast and mammalian cells (reviewed in (Canton and Litchfield, 2006)). Moreover, CK2 plays a critical role in cell cycle progression. First, it is required for G1/S transition and later again for the progression of G2/M (Hanna et al., 1995). Consistently, CK2 phosphorylates Cdc28 at an N-terminal residue (serine 46) (Russo et al., 2000) and it was suggested that CK2-mediated phosphorylation might attenuate Cdc28`s binding to G1 cyclins in an unfavourable environment or until a critical cell size has been reached (Russo et al., 2001). However, it has been demonstrated that ATP and substrate binding by CK2 seems unaffected by the nutritional status (Tripodi et al., 2011). In addition to a direct modulation of Cdc28, CK2 also phosphorylates Sic1, an inhibitor of Cdc28 in G1 (Coccetti et al., 2006). Furthermore, CK2 has also been reported to facilitate entry into S phase at a transcriptional level (Tripodi et al., 2013). Another layer of cell cycle regulation by CK2 is given by its activating phosphorylation of Cdc34 (Coccetti et al., 2008), an ubiquitin-conjugating enzyme required for the G1 to S transition (Willems et al., 1996).
After the initial finding that repair-defective cells lacking *CKB1* or *CKB2* fail to adapt to damage (Toczyski et al., 1997), further analysis of the mutants revealed more insights into their possible role during adaptation. CK2 has been found to phosphorylate Ptc2 at a critical site

(T376) which facilitates Ptc2's interaction with the Rad53 FHA1 domain (Guillemain et al., 2007) and thereby promote checkpoint termination. However, *ckb1* and *ckb2* mutants showed a more severe adaptation defect than the Ptc phosphatase mutants indicating that CK2 has other functions during checkpoint adaptation (Guillemain et al., 2007), a notion supported by increasing numbers of CK2 targets.

1.5.2.1.2 Cdc5

Together with CK2, a second adaptation defective allele was characterized and identified as a variant of Cdc5 (Toczyski et al., 1997). Originally discovered in a screen for cell cycle mutants (Hartwell et al., 1973), Cdc5 is now known as one of the master regulators of mitosis and belongs to the highly conserved Polo kinase family found in all eukaryotes except plants (reviewed in (Archambault and Glover, 2009)).

1.5.2.1.2.1 Cdc5 key functions during an unperturbed cell cycle

Cdc5 protein levels start to accumulate during S and G2 phases and remain high until cells divide. Cdc5 activity, however, was reported to be highest during mitosis suggesting a posttranslational regulation of its kinase activity (Cheng et al., 1998; Hardy and Pautz, 1996; Kitada et al., 1993). Indeed, kinase activity of Cdc5 depends on phosphorylation by Cdc28 at multiple sites including a highly conserved threonine residue (Thr242) residing in the Cdc5 kinase domain (Mortensen et al., 2005). Upon its activation, Cdc5 participates in every critical step throughout mitosis: mitotic entry, chromosome segregation, mitotic exit and cytokinesis. To initiate entry into mitosis, Cdc5 localizes to the bud neck and phosphorylates Swe1 (Asano et al., 2005), a negative regulator of the Clb2-Cdk1 interaction necessary to initiate mitosis (Booher et al., 1993). Importantly, Cdc5 substrate recognition via its Polo-binding domain has been shown to require priming phosphorylations by other kinases, including Cdc28 (Elia et al., 2003). Cdc5 activity is also required in the nucleus to facilitate the onset of mitosis. By phosphorylating the Mcm2-Fkh2-Ndd1 transcription factor complex, transcription of the *CLB2* cluster is facilitated (Darieva et al., 2006). The correct attachment of sister chromatids to the mitotic spindle (spindle assembly checkpoint, SAC) serves as a trigger for APC^{Cdc20} activation and subsequent targeting of Pds1 (securin) for proteasomal degradation (Cohen-Fix et al., 1996). During metaphase, Cdc5 binds to chromatin and targets the cohesion subunit Scc1 (Alexandru et al., 2001), however, this phosphorylation is counteracted by $PP2A^{Cdc55}$ (Yaakov et al., 2012) to prevent premature sister chromatid separation. After Pds1 destruction, Esp1 (separase) can cleave the Cdc5-phosphorylated Scc1 (Ciosk et al., 1998) and simultaneously downregulate $PP2A^{Cdc55}$ to eventually allow separation of sister chromatids. Cdc5 is involved in two regulatory networks, termed FEAR (Cdc Fourteen early anaphase release) and MEN (mitotic exit network) to complete and exit mitosis. In brief, the main purpose of these two signalling cascades is the release of the Cdc14 phosphatase from the nucleolus into the cytoplasm to inactivate Cdc28 and revert Cdc28 phosphorylation events (reviewed in (Weiss, 2012)). At anaphase onset, both Cdc28-Clb2 and Cdc5 phosphorylate the Cdc14 nucleolar anchoring protein, Net1, (Liang et al., 2009; Visintin et al., 2003; Yoshida and Toh-e, 2002) to trigger a first short wave of Cdc14 phosphatase activity during FEAR. Further on in late anaphase, Cdc5 acts at the spindle pole body (SPB) to trigger MEN by phosphorylating the Bfa1/Bub1 GTPase-activating protein (Geymonat et al., 2003; Hu et al., 2001) which leads to

Tem1 and subsequently Cdc15 activation to ultimately exit from mitosis (Jaspersen et al., 1998; Visintin and Amon, 2001). It has been demonstrated recently, that another Cdc28-mediated phosphorylation in the Cdc5 N-terminus is required for its functions during mitotic exit (Rodriguez-Rodriguez et al., 2016). Finally, Cdc5 also participates in cytokinesis by promoting the assembly of the contractile actin ring (Yoshida et al., 2006) and inactivating Cdc42, a requirement for cytokinesis (Atkins et al., 2013).
Control over Cdc5 localization is a key factor in regulating kinase activity and specificity towards its targets. As outlined above, Cdc5 is localized at the SPB after S phase and is shuttled to the nucleus in order to promote mitotic entry. Afterwards, a re-localization form the nucleoplasm to the cytoplasm is required to ensure proper mitotic exit and cytokinesis (reviewed in (Botchkarev and Haber, 2017)).

1.5.2.1.2.2 The role of Cdc5 after DNA damage and during adaptation

When cells encounter DNA damage, for example induced by telomere dysfunction, Cdc5 is phosphorylated by the checkpoint proteins Mec1 and Rad53 mediated by Rad9 (Cheng et al., 1998). At the same time, Cdc5 activity was found to be downregulated after damage (Zhang et al., 2009). Moreover, nuclear localization of Cdc5 is maintained upon damage to prevent the activation of MEN (Valerio-Santiago et al., 2013). Consistently, a recent study found Cdc5 spatially separated from its MEN target, Bfa1, after damage (Botchkarev et al., 2017).
The decision to undergo checkpoint adaptation appears to be tightly regulated. Especially the damage load has been suggested as a critical factor since cells experiencing a single DSB will adapt after 8 to 12 hours of checkpoint arrest, while already two DSB will lead to a permanent cell cycle arrest (Lee et al., 1998; Sandell and Zakian, 1993). Interestingly, also *cdc13-1* cells grown at the non-permissive temperature will eventually undergo adaptation (Toczyski et al., 1997) suggesting that DNA damage elicited by telomere dysfunction could be sensed or evaluated slightly differently. In line with this, it has been hypothesized that the amount of ssDNA that is generated serves as a measure for damage and determines the decision to undergo adaptation. Consistently, many mutants altering the initial processing of DSBs were found to be adaptation-defective, for example *yku70* mutants exhibiting excessive amounts of ssDNA (Lee et al., 1998).
Many insights into the regulation of checkpoint adaptation in yeast could be obtained by either overexpression of Cdc5 while cells were experiencing damage or by using a *CDC5* allele termed *cdc5-ad* that was found to be specifically adaptation-defective in response to DNA damage (Toczyski et al., 1997). The *cdc5-ad* allele harbours a leucine to tryptophan substitution at position 251 which is located within the kinase domain but outside of the well-characterized T-loop required for Cdc5 activity (Toczyski et al., 1997). Consistently, the *cdc5-ad* mutant has been found to be kinase-proficient in assays using a heterologous substrate (Charles et al., 1998; Rawal et al., 2016). However, while high Cdc5 levels lead to the complete loss of Rad53 phosphorylation despite of persistent damage in a manner dependent on Cdc5 kinase activity, overproduction of *cdc5-ad* fails to completely abolish Rad53 phosphorylation (Vidanes et al., 2010). Nevertheless, *cdc5-ad* is not simply a defective allele of *CDC5*, which is an essential gene in budding yeast. Levels of Cdc5 (and presumably activity) are dose dependent for adaptation (Vidanes et al., 2010), however, *cdc5-ad* seems expressed and degraded during the cell cycle comparable to the wild type protein (Rawal et al., 2016). As mentioned before,

overproduction of Cdc5 affects the ability of Rad9 to promote Rad53 autophosphorylation and thereby interferes with checkpoint maintenance (Vidanes et al., 2010) but Cdc5 also plays a role at several steps upstream, such as DSB processing (Donnianni et al., 2010). In agreement with the idea that the decision to undergo adaptation is dependent on the amount of damage present in the cell and since ssDNA serves as a measure for this, *CDC5* overexpression was shown to delay resection after a nuclease-induced DSB (Donnianni et al., 2010). Since a defect in overall kinase activity does not seem to account for the *cdc5-ad* phenotype, it has been hypothesized that these mutants fail to recognize a subset of targets or activate the APC to initiate proteasomal degradation (Cheng et al., 1998; Toczyski et al., 1997).
Taken together, basic insights into the regulation of Cdc5-driven adaptation could be obtained but the specific functions hampered in the *cdc5-ad* allele remain elusive.

Given the high conservation of both the DNA damage response and Cdc5/PLK1, it is not surprising that also in mammalian cells several parallel pathways have been identified that facilitate crosstalk between damage and PLK1. An early observation stated that PLK1 is inactivated by the DNA damage checkpoint (Smits et al., 2000) and subsequently degraded via the proteasome (Bassermann et al., 2008) and this was shown to depend on ATM and ATR (van Vugt et al., 2001). Key PLK1 phosphorylation events including the activating modification at Thr210 (corresponding to Thr242 in budding yeast) were found to be abolished upon DNA damage (Tsvetkov and Stern, 2005). It was shown later that loss of the activating phosphorylation carried out by the Aurora A kinase in association with its cofactor Bora is due to a CHK1-dependent inhibition of Aurora A activity (Krystyniak et al., 2006) as well as ATR-mediated degradation of Bora (Qin et al., 2013). In summary, multiple proteins involved in PLK1 regulation are targeted in response to damage in order to allow cell cycle arrest and repair.

1.5.2.2 Other factors involved in adaptation

As mentioned above, ssDNA generation has been suggested as a mechanism influencing the decision to undergo adaptation. Therefore, loss of the Ku70/80 complex, a negative regulator of resection, leads to extensive ssDNA creation accompanied by an adaptation defect which can be restored by loss of resection factors such as MRX components (Lee et al., 1998). Moreover, mutations affecting subunits of the ssDNA binding protein RPA, such as *rfa1-t11* or phosphomimetic mutations in the Rfa1 N-terminus, can re-establish adaptation in *yku70* mutants (Ghospurkar et al., 2015; Lee et al., 1998). These findings indicate that measurement of ssDNA levels plays an important role in the regulation of adaptation. However, the adaptation defect observed in *cdc5-ad* mutants is not abolished in a *rfa1-t11* background suggesting that Cdc5 promotes adaptation by a mechanism different from assessment of ssDNA levels (Pellicioli et al., 2001). Therefore, also the nuclease Sae2 which is involved in resection displays both a recovery- and an adaptation defect (Clerici et al., 2006). A similar function in the assessment of ssDNA levels has been suggested for Tid1 (also called Rdh54), a helicase previously proposed to participate in HR (Lee et al., 2001b) and its homolog, the chromatin remodelling factor Fun30 (Eapen et al., 2012). Also the Ino80 chromatin remodeller was shown

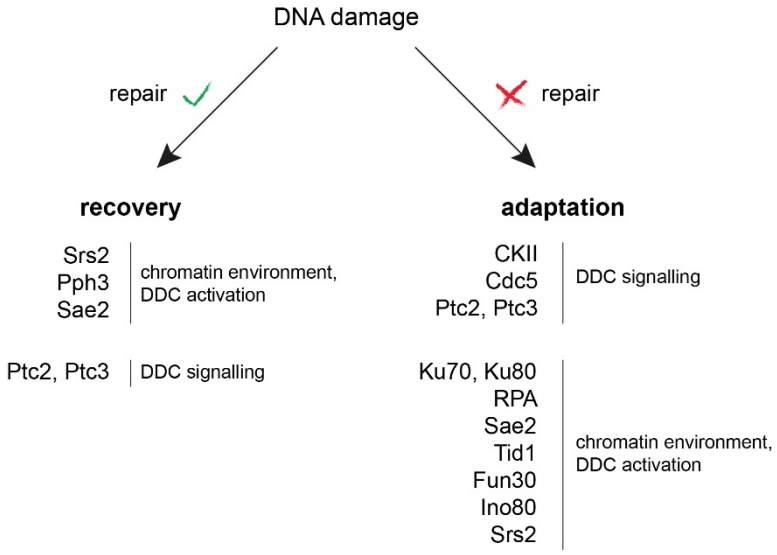


Figure 5. Summary of proteins involved in checkpoint termination either during recovery or adaptation as mentioned in the main text. The step or process that is likely affected during checkpoint termination is indicated.

to be required for adaptation, presumably because the loss of Ino80 leads to lower levels of phosphorylated H2A and increased incorporation of a histone variant in the vicinity of lesions (Papamichos-Chronakis et al., 2006).

In addition to being recovery-defective when damage is repairable, cells lacking the helicase Srs2 are also unable to adapt to an irreparable lesion (Vaze et al., 2002). The same holds true for the phosphatases Ptc2 and Ptc3 (Leroy et al., 2003).

In summary, factors involved in early steps of damage processing and the assessment of ssDNA levels as well as proteins influencing the chromatin environment have been shown to influence checkpoint adaptation (see Figure 5 for a summary of proteins that influence adaptation).

Interestingly, not only nuclear factors play a role during adaptation. Also cytoplasmic processes such as autophagy are regulated during DNA damage. It has become clear that autophagy is induced in response to DNA damage leading to vacuolar degradation of Pds1 resulting in mislocalized Esp1 (Dotiwala et al., 2013; Eapen and Haber, 2013). Importantly, Pds1 has a dual function in regulating Esp1: first, Pds1 is required to shuttle Esp1 into the nucleus where its proteolytic activity is desired and in a second step, Pds1 destruction is necessary to liberate and activate Esp1 (Agarwal and Cohen-Fix, 2002). It was found that DNA damage triggers a specific sub-pathway of autophagy, termed genotoxin-induced targeted autophagy (GTA), and this response is dependent on checkpoint factors such as Mec1 and Rad53 and requires proteins involved in selective autophagy (Eapen et al., 2017). It has been proposed that DNA damage leads to a transient induction of autophagy causing Pds1 degradation and nuclear exclusion of Esp1in order to arrest cells prior to anaphase. However, if autophagy is hyperactivated, cell cycle arrest becomes permanent and cells display an adaptation defect even when the damage-induced phosphorylation of Rad53 is lost. Adaptation can be re-established when autophagy is blocked, especially when affecting factors required for selective autophagy, or when Esp1 is artificially driven into the nucleus (Dotiwala et al., 2013; Eapen and Haber, 2013; Eapen et al., 2017).

1.6 When repair fails: genome instability, chromosome instability and aneuploidy

When cells fail to repair and attempt cell division, chromosome missegregation can be the consequence. Losses or gains of whole chromosomes resulting in an aberrant and unbalanced chromosomes number is referred to as aneuploidy. However, aneuploidy cannot only affect whole chromosomes but it can also comprise translocations, deletions, amplifications or other chromosomal rearrangements, described as segmental aneuploidy (reviewed in (Geigl et al., 2008)). It has been proposed that imbalances in chromosome segregation or genome maintenance factors resulting from aneuploidy as well as DNA damage created by aneuploidy are causative for overall genome instability which is defined as an increased mutation rate and considered a hallmark of cancer (reviewed in (Giam and Rancati, 2015; Hanahan and Weinberg, 2011)). The term genome instability includes different instability phenotypes including chromosomal instability (CIN). CIN describes the cell-to-cell variability in chromosome gains or losses or parts of chromosomes (reviewed in (Geigl et al., 2008)).

1.6.1 Aneuploidy-associated phenotype

Transcriptome analysis of aneuploid yeast cells revealed that the majority (approximately 90%) of genes are expressed according to their copy number (Torres et al., 2007). At the proteome level, protein abundance was mostly positively correlated with gene and mRNA dosage suggesting that there is no general dosage compensation occurring in aneuploid yeast cells (Torres et al., 2007; Torres et al., 2016). In contrast, another study had revealed that up to 40% of the genes found amplified due to aneuploidy showed expression levels that were lower than expected from their gene dosage implying that, to a certain extent, dosage compensation takes place (Hose et al., 2015). However, both studies reported that proteins that are possibly subjected to dosage compensation are components of multi-protein complexes (Hose et al., 2015; Torres et al., 2007; Torres et al., 2016). In order to preserve stoichiometry, supernumerous components of multi-protein complexes have to be degraded and thereby challenge the cellular protein quality control system (Oromendia et al., 2012; Torres et al., 2007). This leads to proteotoxic stress and renders aneuploid cells sensitive to compounds that further impair protein degradation or protein folding (Oromendia et al., 2012; Tang et al., 2011; Torres et al., 2007). In line with these findings, loss of a deubiquitinating enzyme, Ubp6, alleviates the higher proteasomal work load observed in aneuploid yeast cells and thereby improves their fitness (Torres et al., 2010). Moreover, a general transcriptional pattern in response to aneuploidy has been identified in a number of organisms including yeast, plants, mice and humans (Sheltzer et al., 2012). In yeast, the aneuploidy-induced gene expression pattern overlaps greatly with a previously identified transcriptional profile defined as the general environmental stress response (ESR) (Gasch et al., 2000). In addition to impaired overall fitness, aneuploid yeast cells display a prominent cell cycle delay due to an inability to activate the Cln3-Cdc28 complex (Thorburn et al., 2013) and a more subtle delay during S and G2/M phases (Beach et al., 2017). Consistently, also human aneuploid cell lines display a G1 and S phase delay (Stingele et al., 2012). The delay in later phases of the cell cycle has been explained by increased and persistent DNA damage both in yeast and human aneuploid cells (Blank et al., 2015; Passerini et al., 2016) suggesting a link between aneuploidy and checkpoint

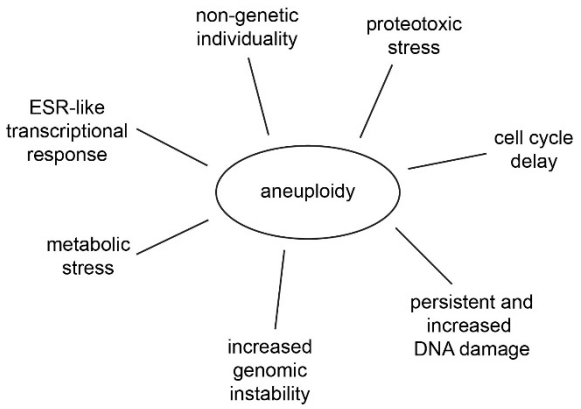


Figure 6. Aneuploidy-associated phenotypes identified in yeast and mammalian cells. ESR: environmental stress response

adaptation. Importantly, adaptation has been shown to cause genome instability (Galgoczy and Toczyski, 2001) and *vice versa*, aneuploid cells show increased genome instability (Sheltzer et al., 2011). In agreement with these observations, also aneuploid human cells suffer from increased genome instability and show elevated levels of autophagy (Ariyoshi et al., 2016; Passerini et al., 2016). The involvement of autophagy as a mechanism to counteract aneuploidy is supported by the finding that aneuploid human cells are very sensitive to autophagy inhibitors (Tang et al., 2011). At the same time, aneuploid cells are hypersensitive to compounds increasing metabolic stress, including the starvation-mimetic drug rapamycin (Tang et al., 2011; Torres et al., 2007). Another striking hallmark of aneuploidy is a high level of non-genetic heterogeneity, both in response to intracellular and extracellular stimuli ((Beach et al., 2017), see Figure 6 for a summary of the aneuploidy-associated phenotype).

1.6.2 The relationship of aneuploidy and genome instability

Although it has become clear that an intimate relationship between aneuploidy and genome instability exists, it has not been well established yet which is the cause or consequence. On one hand, aneuploidy can lead to genome instability phenotypes but on the other hand, pre-existing genome instability can facilitate aneuploidy (reviewed in (Giam and Rancati, 2015)). Strikingly, most solid tumours and about 50% of cancers affecting the hematopoietic system have been shown to be aneuploid (reviewed in (Pariente, 2012)). A recent report proposed aneuploidy as a cause of genomic instability in tetraploid mouse cells (Thomas et al., 2017).
It has been noted though that aneuploidy is not equal to chromosomal instability as seen in individuals with Down syndrome (trisomy 21). Although these patients carry an extra copy of chromosome 21 in every cell rendering the cells aneuploid, there is no cell-to-cell variability in chromosome composition and therefore no chromosomal instability (reviewed in (Geigl et al., 2008)). Although linked to additional phenotypes and malignancies of varying degrees, a few aneuploid karyotypes are viable in humans including the trisomy of chromosome 13, 18, 21 or the sex chromosomes or monosomy for the X chromosome (reviewed in (Hassold and Hunt, 2001)).

1.7 TOR nutrient signalling pathway

Cellular growth is a tightly regulated mechanism in all organisms in order to utilize nutrients present in the environment in the most optimal way. In yeast, a critical cell size has to be reached in order for the cell to commit to cell division. A central and highly conserved regulatory network for growth in eukaryotes is organized by the TOR kinase. TOR (target of rapamycin) belongs to the PI3K-related kinase family including also ATM/ATR and Mec1/Tel1.
In yeast, TORC1 is considered as the main sensor for the availability of nitrogen including amino acids, while a second nutrient sensory pathway involving Protein Kinase A (PKA) is the major glucose sensor (reviewed in (Zaman et al., 2008)).

1.7.1 TOR in yeast

Unlike all other eukaryotes, the budding yeast genome encodes for 2 TOR kinases, *TOR1* and *TOR2*. Loss of *TOR1* can be tolerated, while loss or mutation of *TOR2* is lethal suggesting partially overlapping but also unique functions of the two kinases. Indeed, Tor1 or Tor 2 can form the TOR complex 1 (TORC1) which also comprises the accessory proteins Kog1, Lst8 and Tco89. On the other hand, Tor2 builds up the TOR complex 2 (TORC2) together with Lst8, Bit61 and the Avo proteins 1, 2 and 3. Both TORC1 and TORC2 are huge protein complexes of about 2 MDa and most likely exist as homodimers (reviewed in (Wullschleger et al., 2006)). TORC1 regulates temporal aspects of cell growth, while TORC2 is also involved in the spatial regulation of growth and plays a role in actin skeleton polarization (Loewith et al., 2002; Schmidt et al., 1996). Importantly, only TORC1 is sensitive to rapamycin (Loewith et al., 2002), a fungal metabolite with antibiotic and immunosuppressive activity (reviewed in (Schreiber, 1991)). In fact, the search for the cellular targets conferring rapamycin sensitivity has led to the identification of the Tor proteins (reviewed in (Loewith and Hall, 2011)). To inhibit TORC1, rapamycin binds to an intracellular cofactor, FKBP12 (FK506-binding protein of 12 kDa) (Heitman et al., 1991a). FKBP12 is a highly conserved peptidyl-prolyl *cis/trans* isomerase encoded by the *FPR1* gene in yeast (Heitman et al., 1991b). The rapamycin-FKBP12 complex binds to the FRB domain including a highly conserved serine residue (S1972 in Tor1 and S1975 in Tor2) which is adjacent to the Tor kinase domain (Stan et al., 1994). Although the FRB domain is also part of Tor2 and therefore present in the TORC2 complex, rapamycin is specifically inhibiting TORC1 which has been explained by a masking of the respective binding site in Tor2 by the additional proteins present in TORC2 (Loewith et al., 2002). TORC2 has been mainly found at the plasma membrane (Wedaman et al., 2003), while TORC1 localizes constitutively to the vacuolar membrane where it is catalytically active (Binda et al., 2009; Urban et al., 2007). Active TORC1 promotes anabolic processes including translation initiation and ribosome biogenesis but counteracts catabolic processes such as autophagy and the expression of genes required for metabolizing less favourable carbon sources (reviewed in (Loewith and Hall, 2011), see Figure 7 for an overview on TOR complexes).
Since rapamycin acts specifically on TORC1, I will further focus on this branch of the Tor signalling network.

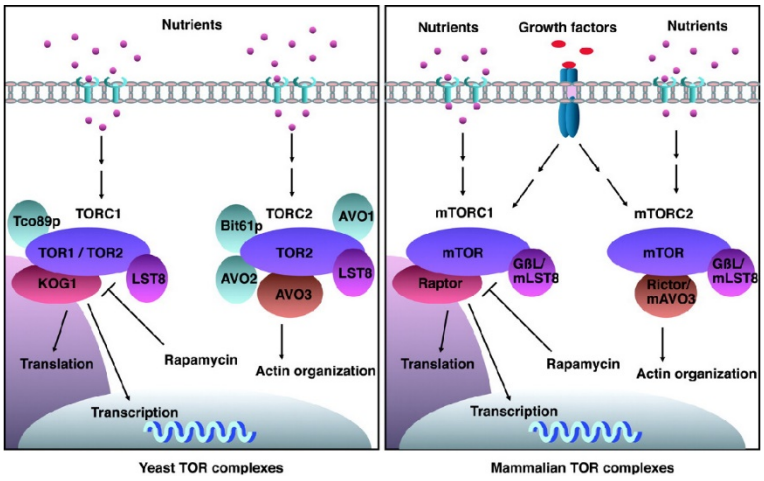


Figure 7. Protein composition and cellular functions of the two TOR signalling complexes in *S. cerevisiae* and mammalian cells. Of note, only the TORC1 complex is sensitive to rapamycin. Figure taken from (Inoki et al., 2005).

1.7.1.1 Upstream factors regulating TORC1 activity

TORC1 can sense nutrient availability in response to different environmental cues, including carbon, nitrogen, phosphate and amino acids. TORC1 seems to integrate both the abundance but also the quality of available nutrients, however, the exact molecular mechanisms have not been fully elucidated (reviewed in (Loewith and Hall, 2011)). In addition to nutrients, TORC1 activity is also regulated by extracellular stimuli such as high temperature or redox stress (Urban et al., 2007). In adverse conditions or in the presence of stresses including DNA damage, TORC1 activity is downregulated. However, in favourable, nutrient-rich conditions, active TORC1 signalling promotes cell mass accumulation thereby facilitating cell division. Importantly, treatment with rapamycin induces the same cellular response as TORC1 inhibition upon starvation (Barbet et al., 1996). Nutrient-deprived cells including yeast cells enter stationary phase or quiescence (termed G0) characterized by a metabolically active but non-dividing state. A screen to identify mutants unable to recover from rapamycin-induced growth arrest revealed the EGO (escape from rapamycin-induced growth arrest) complex as an upstream regulator of TORC1 (Dubouloz et al., 2005). Like TORC1 itself, the EGO complex consisting of Ego1, Ego3, Gtr1 and Gtr2 is localized to the cytoplasmic face of the vacuolar membrane (Binda et al., 2009). Gtr1 and 2 are Ras-like GTPase (Kim et al., 2008) and the nucleotide-binding status of Gtr1 seems determinant of TORC1 activity in response to amino acids, especially leucine (Binda et al., 2009) and glutamine (Crespo et al., 2002). Gtr1 GTPase activity seems regulated by the guanine-nucleotide exchange factor (GEF) Vam6 residing at the vacuolar membrane (Binda et al., 2009). However, a corresponding GTPase activating enzyme (GAP) remains to be identified. It is also unclear how exactly Gtr1 activity is triggered in response to nutrients, however, the vacuolar localization suggests that intravacuolar amino acid levels could be the link between TORC1 and the EGO complex. The TORC1 component Tco89 seems to be the interaction interface coupling the two protein complexes (Binda et al., 2009). In addition to responding to extracellular nutrient levels, TORC1 activity is also increased in response to treatment with the translation inhibitor cycloheximide suggesting that TORC1 can

also sense free amino acids in the cytoplasm (Beugnet et al., 2003; Binda et al., 2009; Urban et al., 2007).

1.7.1.2 Downstream effectors and consequences of TORC1 activity

1.7.1.2.1 Sch9

The kinase Sch9 is one of the best characterized downstream effectors of active TORC1. Sch9 belongs to the AGC kinase family which also comprises the mammalian Sch9 ortholog and TORC1 target, S6K1 kinase. Phosphorylation of Sch9 in its C-terminus by TORC1 is required for Sch9 activity in order to regulate translation initiation, ribosome biogenesis and prevent entry into stationary phase (Urban et al., 2007). In addition to TORC1, activation of Sch9 also depends on multiple phosphorylations by Pkh1 and 2, the yeast homologs of mammalian PDK1 (3-phosphoinositide-dependent kinase 1) (Roelants et al., 2004).

1.7.1.2.2 PP2A

In parallel to the Sch9 branch, TORC1 signalling involves type 2A phosphatases including PP2A as well as 2A-related phosphatases such as Sit4 (reviewed in (Loewith and Hall, 2011)). The phosphatase complex PP2A comprises one of three redundant catalytic subunits Pph21, Pph22 or Pph3 associated with a scaffolding protein Tpd3 and a regulatory subunit encoded either by *CDC55* or *RTS1*. Likewise, the related 2A-like phosphatase contains catalytically active Sit4 bound by a regulatory subunit which is either Sap155, Sap190 or Sap185 (reviewed in (Zaman et al., 2008)).

PP2A regulation by TORC1 involves Tap42 which binds to and inactivates the phosphatase complex under nutrient-rich conditions facilitated by TORC1 phosphorylation (Di Como and Arndt, 1996; Jiang and Broach, 1999). Upon nutrient starvation or rapamycin treatment leading to TORC1 inactivation, Tap42 becomes dephosphorylated causing the liberation and activation of PP2A (Di Como and Jiang, 2006; Jiang and Broach, 1999; Yan et al., 2006). Similar to TORC1, also the Tap42-PP2A complex resides at the vacuolar membrane (Yan et al., 2006). Taken together, these findings suggested Tap42 as a negative regulator of PP2A activity, however, also a phosphatase-activating role for Tap42 has been proposed (Duvel et al., 2003) highlighting the complexity of TORC1 downstream signalling. How exactly Tap42 is regulated by TORC1 is currently not fully understood, both direct and indirect mechanisms have been proposed. On one hand, Tap42 has been shown to be directly phosphorylated by TORC1 (Jiang and Broach, 1999). However, a genetic screen identified Tip41 as a Tap42-interacting protein and found that Tip41 can both positively and negatively regulate TORC1 signalling (Jacinto et al., 2001). Together with the Sch9 kinase, PP2A has been suggested as the major downstream effector to coordinate TORC1 signalling.

1.7.1.2.3 Ribosome biogenesis and protein synthesis

TORC1 activity is a positive regulator of ribosome biogenesis and 5`cap dependent translation initiation. Protein synthesis and translation initiation are influenced by TORC1 via two different downstream effectors, the Tap42-PP2A complex and Sch9.

For efficient ternary complex formation during translation, GDP-bound translation initiation factor eIF2 has to be exchanged with GTP-bound eIF2. However, nutrient starvation or rapamycin treatment lead to the phosphorylation of a conserved serine residue (serine 51 in yeast) in the α subunit of eIF2 which is encoded by *SUI2*. Phosphorylation of eIF2α hampers the interaction with its GEF and thereby decreases the efficiency of translation initiation and consequently general protein synthesis (reviewed in (Aitken and Lorsch, 2012)). *GCN2* encodes the sole yeast eIF2α kinase and its phosphorylation at serine 577 for decreases Gcn2 kinase activity (reviewed in (Loewith and Hall, 2011)). In response to TORC1 inhibition, Gcn2 is dephosphorylated at serine 577 in a Tap42-PP2A-dependent manner (Cherkasova and Hinnebusch, 2003). Consequently, Gcn2 becomes activated and phosphorylates Sui2 (eIF2α) at serine 51 which ultimately leads to decreased 5`cap dependent translation under nutrient-poor conditions or upon rapamycin treatment. Which kinase is responsible for Gcn2 phosphorylation and therefore inactivation to promote protein synthesis under nutrient-rich conditions remains unclear, however, a role for Sch9 as a TORC1 effector has been excluded (reviewed in (Loewith and Hall, 2011)). In addition to its role in regulating translation initiation via eIF2α, TORC1 has also been implicated in phosphorylating other translation factors such as the eIF4G scaffolding protein thereby regulating its stability (Berset et al., 1998) or the eIF4E-associated protein, Eap1 (Cosentino et al., 2000).

TORC1 is the major regulator of ribosome biogenesis and this regulation is mostly brought about at the transcriptional level involving the Sch9 branch of TOR signalling (Huber et al., 2009). Ribosome biogenesis includes the transcription and maturation of rRNA, tRNA, ribosomal proteins and other ribosome biogenesis (RiBi) factors involving all RNA polymerases and is considered the rate-limiting step for cell growth. Moreover, a large fraction of the cell's energy is devoted to the production of ribosomes and therefore, tight regulatory mechanisms of this costly process exist (reviewed in (Warner, 1999)). TORC1 inhibition impairs ribosome biogenesis in multiple pathways. Short-term effects of TORC1 inactivity affects the translation of ribosomal proteins by decreasing protein synthesis rates in general (see above), but in the long range, inactive TORC1 also impacts on RNA polymerase I-mediated transcription of rDNA by degrading the essential transcription factor Rrn3 (Claypool et al., 2004; Reiter et al., 2011). Furthermore, the TORC1 effector kinase Sch9 phosphorylates the RNA polymerase III repressor protein Maf1 leading to the stimulation of RNA polymerase III-dependent transcription of rDNA under nutrient-rich conditions (Lee et al., 2009; Upadhya et al., 2002; Vannini et al., 2010). In addition to influencing rDNA transcription, TORC1 also impinges on the transcription of ribosomal proteins and RiBi factors. For example, TORC1 activity leads to the phosphorylation of the transcription factors Stb3, Dot6 and Tod6 via the Sch9 signalling branch (Liko et al., 2010; Lippman and Broach, 2009). Loss of phosphorylation due to TORC1 inhibition allows the recruitment of the three transcription factors to their respective target promoter elements and leads to the recruitment of the RPD3L histone acetylase complex to repress transcription of RiBi factors. Likewise, TORC1 also regulates the transcription of ribosomal proteins by influencing the association of the transcription factor Fhl1 with either Ifh1 to allow transcription or Crf1 to repress it (reviewed in (Loewith and Hall, 2011)). An additional layer of control over ribosome biogenesis is given by post-transcriptional regulations via TORC1. Catalytical steps of ribosome assembly are controlled by TORC1 as

well as some aspects of mRNA stability and pre-mRNA splicing (reviewed in (Loewith and Hall, 2011)).

1.7.1.2.4 Autophagy

Autophagy is a major catabolic pathway devoted to the degradation of cytoplasmic and nuclear components as well as organelles within the vacuole in yeast or lysosomes in higher eukaryotes. Non-selective autophagy is referred to as macroautophagy, however, also selective microautophagy involving receptors and targeting organelles or nuclear compartments exist as well as non-canonical but selective autophagy pathways such as the cytosol-to-vacuole (Cvt) pathway (reviewed in (Reggiori and Klionsky, 2013)). Autophagy constitutes the second option of protein degradation in addition to the proteasome. The latter mainly deals with short-lived or misfolded proteins by marking them for degradation via the attachment of ubiquitin. While the proteasome is constitutively active, autophagy is mostly an inducible process and responds to starvation but also other stimuli (reviewed in (Lilienbaum, 2013)).

In nutrient-rich conditions triggering TORC1 activity, Atg13 is hyperphosphorylated by TORC1 which prevents its binding to Atg1 kinase, whose catalytic activity is required for autophagy and depends on Atg13 (Kamada et al., 2000; Matsuura et al., 1997). When TORC1 is inhibited by starvation or rapamycin treatment, phosphorylation of Atg13 is lost (Scott et al., 2000) by active dephosphorylation by the PP2A phosphatase complex (Yeasmin et al., 2016). However, a more recent study implied that the binding of Atg1 and Atg13 is constitutive (Kraft et al., 2012). Atg17, Atg29 and Atg31 form a stable ternary complex that is required for the maximal activation of the Atg1 kinase (Kabeya et al., 2009; Kamada et al., 2000; Kawamata et al., 2008). These steps are required for autophagy induction and formation of the autophagosome, the vesicular structure that engulfs the substrate for degradation. Atg9 is a transmembrane protein which is strictly required for autophagosome formation (Noda et al., 2000). Atg9 is translocated to the endoplasmic reticulum and passed on to the Golgi apparatus after synthesis to be sorted into vesicles (Geng et al., 2010) and it likely acts as a scaffold and provider of initial membrane surface required to initiate autophagosome formation (Mari et al., 2010; Yamamoto et al., 2012). Atg12, a small, ubiquitin-like protein, is conjugated to Atg5 and subsequently interacts with Atg16. This complex has been proposed to act as an interaction platform and E3-like enzyme that facilitates the lipidation of Atg8 to form the Atg8-PE conjugate in an ubiquitin conjugation-like enzymatic reaction (Hanada et al., 2007; Noda et al., 2013). The lipidated Atg8 protein marks both the growing and complete autophagosome membrane (reviewed in (Reggiori and Klionsky, 2013)). Conjugation of LC3 (or LC3-I), the mammalian homolog of Atg8, to the membrane lipid PE, which is then referred to as LC3-II, is a commonly used read-out for autophagy induction (reviewed in (Tanida et al., 2008)). Studies of Atg8 or its mammalian counterpart LC3 have provided first insights into the mechanism of selective autophagy.

One pathway of selective cargo recognition and degradation by autophagy is the cytosol-to-vacuole (Cvt) pathway. In brief, the protein machinery used for non-selective macroautophagy appears to be largely the same as in selective autophagy. The scaffold protein Atg11 as well as the cargo receptors Atg19 and Atg34 are Cvt-specific components (reviewed in (Reggiori and Klionsky, 2013)). The Cvt pathway is required for the delivery of several hydrolases to the vacuole thereby employing the core autophagy proteins. However, the Cvt pathway also

requires additional proteins such as Vps53 and Vps51 (for vacuolar protein sorting) which are involved in vesicle formation and tethering during Cvt. The four proteins Vps51, 52, 53 and 54 assemble a complex that is referred to either as VFT after its eponym Vps Fifty Three or GARP for Golgi-associated retrograde protein transport (reviewed in (Lynch-Day and Klionsky, 2010)).
In our experiments, we employed deletions of *ATG5* to abolish non-selective macroautophagy or *VPS51* to negatively interfere with the Cvt pathway.

As outlined previously, autophagy is emerging as an important player in the DNA damage response causing the nuclear exclusion and degradation of cell cycle regulators like Esp1 and Pds1. In addition, an autophagy pathway engulfing small parts of the nucleus has been identified first in yeast (Roberts et al., 2003) and was then found to be conserved in higher eukaryotes. This selective autophagy pathway has been termed piecemeal autophagy of the nucleus (PMN) and requires an interaction between the vacuolar membrane protein Vac8 and the nuclear envelop component Nvj1 in addition to core macroautophagy proteins (reviewed in (Luo et al., 2016)). Although PMN could serve as an important interface between the nuclear and cytoplasmic response after several stimuli including DNA damage, its regulation and consequences have not been fully understood yet.
In addition, autophagy induction has been shown to occur in response to histone deacetylases (HDAC) inhibitors (Shao et al., 2004). This has been attributed to an Atg19-dependent degradation of the resection and recombination protein Sae2, implicating the Cvt pathway in the DNA damage response and repair pathway (Robert et al., 2011).

1.7.1.2.5 Cell cycle progression

TORC1 signalling via Sch9 appears to be a major determinant of cell size which is mediated by the stimulation of ribosome biogenesis ((Jorgensen et al., 2002), reviewed in (Loewith and Hall, 2011)). In yeast, cell size is seen as a threshold for cell division and therefore, TORC1 has been proposed as a coordinator of cell size and division. Indeed, treatment with high doses of rapamycin causing TORC1 inhibition leads to a cell cycle arrest in G1 due to insufficient translation of Cln3 (Barbet et al., 1996). Surprisingly, rapamycin-induced G1 arrest was found to be accompanied by cell size increase which has been explained by the swelling of the vacuole due to the induction of autophagy (Granot and Snyder, 1991; Heitman et al., 1991a). TORC1 also promotes transition through S phase by regulating deoxyribonucleotide homeostasis, especially in response to DNA damage (Shen et al., 2007). Finally, TORC1 regulates transition through G2/M by influencing the localization of Cdc5 via Tap42-PP2A (Nakashima et al., 2008). Mislocalized Cdc5 fails to degrade Swe1, an inhibitor of Clb2-Cdc28, and consequently delays entry into mitosis (reviewed in (Botchkarev and Haber, 2017)). In the presence of DNA damage, TORC1 inhibition by rapamycin prevents checkpoint adaptation (Klermund et al., 2014). Although the induction of autophagy is sufficient for preventing adaptation, macroautophagy is not required in this process (Dotiwala et al., 2013; Klermund et al., 2014).

1.7.2 TOR in mammalian cells (mTOR)

1.7.2.1 Functions and regulation of mTOR

In contrast to yeast, mammalian cells contain only one gene encoding the *TOR* kinase but still harbour two different TOR complexes depending on the accessory proteins. The mammalian equivalent of the rapamycin-sensitive yeast TORC1 contains mTOR itself as well as raptor (yeast Kog1), mLST8 (yeast Lst8), PRAS40 and DEPTOR and is called mTORC1. The mammalian counterpart of yeast TORC2, termed mTORC2, is composed of mTOR, rictor (yeast Avo3), mLST8 and the putative homolog of yeast Avo1, hSIN1. In multicellular organisms, mTOR is a coordinator of development and cell growth (reviewed in (Loewith and Hall, 2011)).
Like yeast TORC1, mTORC1 responds to nutrients including glucose and amino acids and stress stimuli such as hypoxia. Another layer of mTORC1 upstream regulators in multicellular organisms is given by the presence of growth-promoting factors. In brief, insulin and insulin-like growth factors serve as activating signals for mTORC1 which in turn recruits PI3K (phosphoinositide 3-kinase). The PI3K pathway is triggered by many different classes of receptors responding to extracellular cues and has been proposed to be the most commonly activated signal transduction pathway in cancers (reviewed in (Liu et al., 2009)). PI3K phosphorylates the membrane lipid PIP2 to create PIP3 and this step is counteracted by the lipid phosphatase PTEN. PIP3 can recruit the kinase PDK1 and its substrate, the kinase PKB (also known as Akt) thereby leading to PKB activation (Alessi et al., 1997). Active PKB/Akt signals to mTORC1 via the negative TOR regulators, TSC1 and TSC2. The TSC heterodimer functions as a GAP for the small GTPase Rheb which activates mTORC1 when Rheb is bound to GTP. Therefore, promotion of Rheb GTPase activity by the TSC heterodimer keeps mTORC1 inactive (reviewed in (Wullschleger et al., 2006)).
Comparable to yeast, mTORC1 positively regulates 5`cap dependent translation via S6K1, the mammalian homolog of yeast Sch9 (Urban et al., 2007), and 4E-BP1, a translation repressor that is inactivated by mTORC1 signalling (Gingras et al., 1998). In addition, mTORC1 promotes ribosome biogenesis via multiple pathways and represses autophagy. Like yeast TORC2, mTORC2 is involved in actin cytoskeleton organization (reviewed in (Wullschleger et al., 2006)).

1.7.2.2 mTOR in disease and cancer

Rapamycin has been first described to possess immunosuppressive functions and is therefore used after organ transplantations as well as in the treatment of autoimmune diseases. In addition, rapamycin or pharmacologically optimized derivatives thereof have been proposed in the treatment of cardiovascular diseases as well as metabolic disorders including diabetes (reviewed in (Wullschleger et al., 2006)).
The majority of research devoted to mTOR signalling is fuelled by the observation that up to 70% of cancers exhibit hyperactive mTORC1 (reviewed in (Xie et al., 2016)). In line with this, a recent report suggested that the failure of cancer cells to down-regulate mTOR activity in the presence of DNA damage promotes cancer cell survival. In this study, aberrantly active mTOR suppressed spontaneous DNA damage and replication stress by controlling the levels of CHK1

(Zhou et al., 2017). Consequently, inhibition of mTOR activity in cancer cells could serve as a potent anti-tumour strategy. Indeed, initial studies showed that rapamycin and its derivatives such as everolimus or sirolimus (first generation rapamycin orthologues) decrease tumour growth in several animal models, however, they failed to show significant effects when used as a single anti-tumour drug (reviewed in (Xie et al., 2016)). Rather, TORC1 inhibitors have been suggested as sensitizing agents to be used in combination with damage-inducing agents. The rationale for this was given by the finding that rapamycin was shown to suppress repair of DSBs in human cancer cells, both via NHEJ and HR, and in addition increase the frequency of chromosome breakage even in the absence of exogenous damage (Chen et al., 2011a). Recent reports have shown that the combination of mTOR and PARP1 inhibition is very effective in targeting cancer cells, especially *BRCA*-deficient breast cancers that are prone to acquire resistance resulting in relapse of the tumour. The combinational treatment with mTOR and PARP1 inhibitors renders cells unable to repair DNA damage due to the simultaneous inhibition of DSB and SSB repair (Osoegawa et al., 2017). Rapamycin treatment hampers HR by impairing the recruitment of RAD51 and BRCA1 (Chen et al., 2011a) but also by decreasing the levels of SUV39H1, a histone methyltransferase implicated in HR (Mo et al., 2016).

1.7.2.3 The role of TOR signalling in longevity and ageing

Strikingly, reduced TORC1 activity increases lifespan in all model organism tested so far. This can be achieved even in nutrient-rich conditions by mutations or pharmacologic inhibition of TORC1 signalling. In addition, also dietary restriction decreases TORC1 activity leading to lifespan extension (reviewed in (Evans et al., 2011)). However, the combination of dietary restriction and mutations in the TORC1 pathway did not lead to a synergistic effect on lifespan extension. Dietary restriction is known to slow down aging and delay aging-related diseases and it has been proposed that also mTORC1 inhibition could have these positive effects (reviewed in (Johnson et al., 2013)).
In yeast, extension of chronological lifespan by rapamycin-mediated TORC1 inhibition requires a functional DDC. Taken together with the finding that rapamycin prevents checkpoint adaptation these observations suggest that decreasing TORC1 activity promotes lifespan extension by increasing genome stability (Klermund et al., 2014).

1.8 Rationale and scope of the thesis

An early link between DNA damage (in this case elicited by dysfunctional telomeres) and nutrient signalling was reported in 2008 (Qi et al., 2008). Further investigation in our lab has revealed that decreased TORC1 signalling, a highly conserved nutrient sensor, prevents adaptation to the DNA damage checkpoint (Klermund et al., 2014). As adaptation describes the termination of the DNA damage checkpoint despite unrepaired DNA damage, adaptation is considered as an attempt to preserve cell viability although it is associated with genome instability. Additional studies by many research groups have strengthened the intriguing connection between nutrient signalling and the damage response. For example, Cdc5, a main regulator of checkpoint adaptation, is mislocalized when TORC1 activity is compromised (Nakashima et al., 2008). Moreover, quantitative mass spectrometry has identified mTOR as a regulator of the DDR (Bandhakavi et al., 2010) and, *vice versa*, many studies, especially in mammalian cells, have revealed a downregulation of TOR signalling in response to DNA damage. Intriguingly, mTOR inhibition also involves functional p53 (Cam et al., 2014), the most commonly mutated tumour suppressor in human cancers (Kandoth et al., 2013). A second pathway to inhibit mTOR after DNA damage relies on the PKB/Akt network which itself is heavily involved in cancerogenesis (reviewed in (Carnero, 2010)). Deregulated energy metabolism is an emerging hallmark of cancers as well as genome instability. The latter is accompanied by chromosomal instability and aneuploidy which can themselves fuel further genomic instability in a vicious cycle. Furthermore, cancers often harbour mutations and alterations in DNA repair pathways and therefore readily undergo adaptation as the only route to escape permanent cell cycle arrest. This suggests adaptation as a major threat to genome stability and loops back to TOR signalling as a regulator of adaptation.

Building on these observations, we could show previously that TOR inhibition prevents checkpoint adaptation in yeast (Klermund et al., 2014). However, this study used telomere dysfunction to elicit DNA damage. We therefore wanted to explore the role of adaptation in the case of irreparable damage and chose yeast strains lacking the central HR protein Rad52 as a model. Yeast Rad52 is the equivalent of human BRCA2, providing us with a powerful model system to study the interplay between adaptation in response to genotoxins and genome instability. An earlier study had already revealed that adaptation causes genome instability in yeast (Galgoczy and Toczyski, 2001) but how exactly adaptation is regulated with respect to external cues including the nutritional status remains poorly understood.
We could show that adaptation in response to telomere-elicited DNA damage can be modulated via the Tap42-PP2A axis downstream of TORC1 signalling. Although the exact molecular mechanism of Cdc5 regulation in response to damage is unclear, we could show that rapamycin treatment appears to regulate Cdc5 protein levels. Using repair-deficient (*rad52*) yeast cells, we were able to show that the prevention of adaptation both genetically and pharmacologically using rapamycin sensitizes these cells to genotoxic treatment. Importantly, repair-proficient cells did not experience any disadvantages when adaptation was inhibited. Furthermore, if adaption is allowed to occur, repair-deficient *rad52* mutants acquire multidrug resistance which could be directly translated to chemoresistance observed in *BRCA*-deficient human cancers. In addition, our analysis of *rad52* cells that had adapted and acquired genotoxin resistance revealed that these cells had become aneuploid. Consistently, they display a variety of

aneuploidy-associated phenotypes including chronic DNA damage. Therefore, we could provide a link between adaptation, drug resistance and aneuploidy, however, the order of events establishing cause and consequence in this set-up is not yet fully understood. Nevertheless, the observation that repair-defective cells acquire an aneuploid karyotype provides useful insights in how to target these cells after their emergence. Notably, inhibition of TORC1 signalling could play two important functions: rapamycin treatment can prevent adaptation but it can also sensitize adapted and aneuploid repair-defective cells.

Taken together, the results presented here provide basic insights into the involvement of nutrient signalling in adaptation. Moreover, we could strengthen the link between acquisition of genotoxin resistance and aneuploidy, which we suggest as results of adaptation.

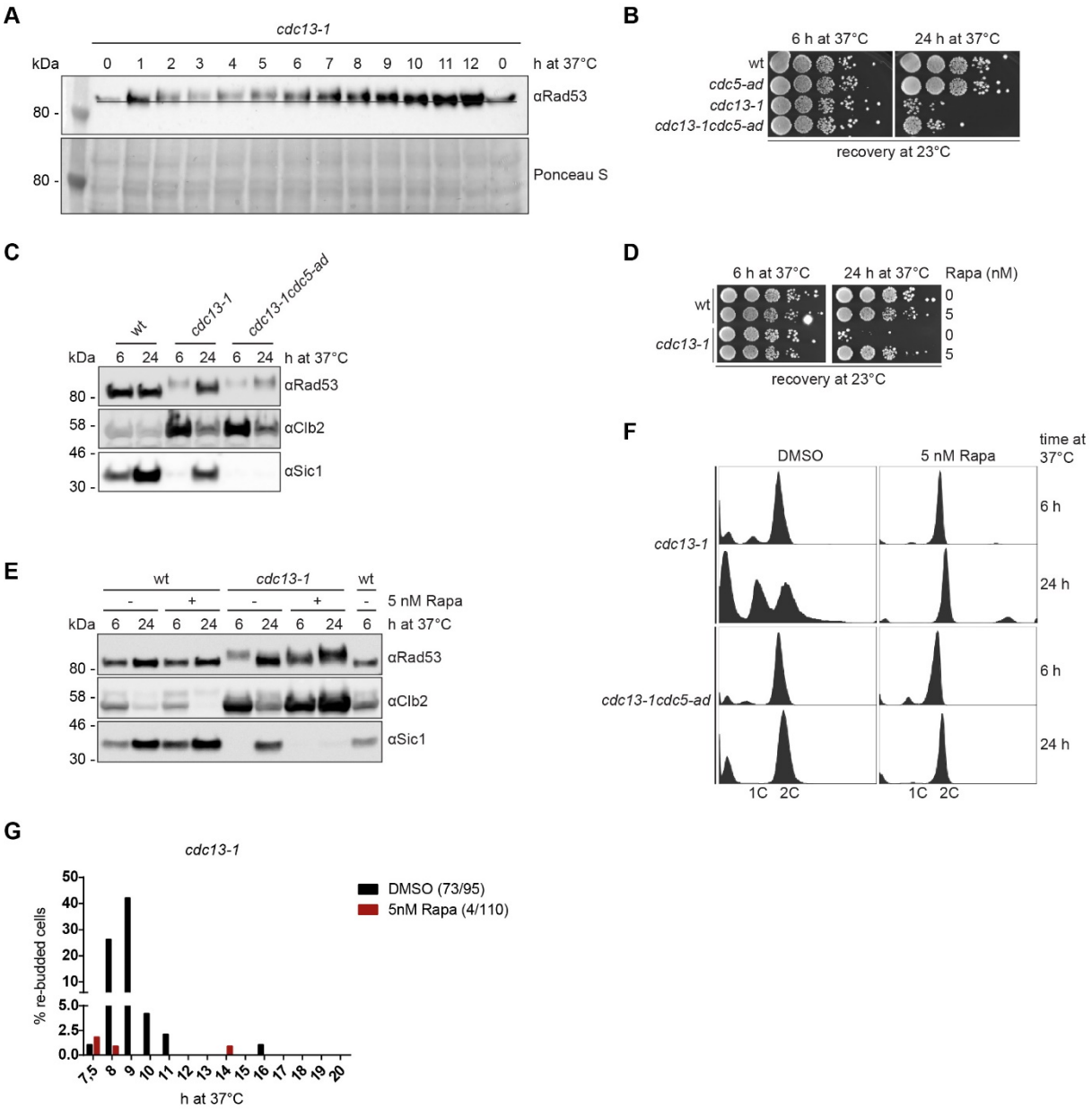
2 Results

2.1 Preventing adaptation in repair-proficient cells improves cell viability

In order to investigate the regulatory pathways influencing checkpoint adaptation, we used the budding yeast *Saccharomyces cerevisiae* as a model organism. Occurrence of DNA damage leads to a highly orchestrated and conserved order of events termed the DNA damage response in order to arrest the cell cycle and allow appropriate damage repair.

Yeast cells harbouring the temperature-sensitive *cdc13-1* allele of the telomere binding protein Cdc13 activate the DNA damage response when shifted to the restrictive temperature accompanied by telomeric ssDNA accumulation and G2/M cell cycle arrest (Garvik et al., 1995; Hartwell et al., 1973; Lin and Zakian, 1996). Interestingly, after a prolonged period of telomere dysfunction, *cdc13-1* mutants downregulate the DNA damage checkpoint as evident from the dephosphorylation of the checkpoint kinase Rad53 (Figure 8 A, (Klermund et al., 2014; Ratsima et al., 2016)). Shifting *cdc13-1* cells to the restrictive temperature for a short period of time (6 hours) did not result in loss of cell viability when cells were returned to the permissive temperature to restore Cdc13 protein function and hence allow cellular recovery (Figure 8 B). However, prolonged incubation at the restrictive temperature (24 hours) eventually caused a loss of viability in *cdc13-1* cells (Figure 8 B, (Toczyski et al., 1997)). These findings suggest that downregulation of checkpoint activity in the presence of persistent DNA damage may lead to lethality. The yeast polo-like kinase Cdc5 has been shown to override the DDC at the level of Rad53 phosphorylation (Vidanes et al., 2010) and by acting on the upstream signalling cascade (Donnianni et al., 2010). The observation that the DDC can be “overridden” despite persistent DNA damage is referred to as (checkpoint) adaptation and Cdc5 has been identified as a main driver of adaptation (Toczyski et al., 1997). To test the idea that *cdc13-1* cells undergo checkpoint adaptation and therefore lose viability, we made use of the checkpoint adaptation-defective *cdc5-ad* allele harbouring a single point mutation (L251W). While cell cycle progression in unperturbed conditions is unaffected, *cdc5-ad* mutants are unable to undergo checkpoint adaptation (Toczyski et al., 1997). We found that in *cdc13-1cdc5-ad* double mutants, cell viability was preserved even after long-term incubation at the restrictive temperature (Figure 8 B). This is consistent with previous findings (Toczyski et al., 1997) and implicates checkpoint adaptation as the major cause for the loss of cell viability in *cdc13-1* cells exposed to prolonged DNA damage.

The onset of adaptation has been linked to the loss of Rad53 phosphorylation which correlates with the inactivation of Rad53 kinase activity (Pellicioli et al., 2001). Analysing the phosphorylation status of Rad53 in adaptation-proficient *cdc13-1* cells by Western Blot revealed that although the checkpoint had been fully activated at the 6 h time point, it was downregulated after prolonged DNA damage (Figure 8 C). This was consistent with the expression of cell cycle markers: the G2/M cyclin Clb2 was present at the early 6 h time point supporting the accumulation of cells in the G2/M phase of the cell cycle due to checkpoint activation. Accordingly, expression of Sic1, a G1-specific CDK inhibitor, was absent at the 6 h time point. However, after prolonged damage at the 24 hour time point, the majority of the Rad53 pool was found dephosphorylated which coincided with lower levels of the G2/M marker Clb2. At the same time, Sic1 accumulated suggesting that at least a subpopulation of *cdc13-1* cells had dephosphorylated and therefore inactivated Rad53 to override the DNA



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damage checkpoint and progressed through the cell cycle. In the presence of the adaptation-defective *cdc5-ad* allele, Rad53 remained phosphorylated and therefore maintained its checkpoint kinase activity even after a long period of DNA damaging conditions (Figure 8 C). Taken together, these results are consistent with previously published data using an irreparable DSB induced by expression of the HO endonuclease (Pellicioli et al., 2001).

A previous report had already linked nutrient signalling via the highly conserved TORC1 signalling pathway and cell viability after prolonged DNA damage in *cdc13-1* cells (Qi et al., 2008). We therefore set out to confirm the finding that TORC1 inhibition rescues cell viability in *cdc13-1* cells by incubating them at the restrictive temperature in the presence of the TORC1 inhibitor rapamycin. As shown in Figure 8 D, *cdc13-1* viability was not compromised after short-term telomere dysfunction (6 h) when cells were returned to the permissive temperature to allow colony formation. However, as seen previously, only very few *cdc13-1* cells were viable and able to proliferate after a 24 h incubation at the restrictive temperature. Importantly,

Figure 8. Checkpoint adaptation occurs after prolonged telomere dysfunction and is prevented by the *cdc5-ad* allele and rapamycin treatment. (A) *cdc13-1* cells were arrested in G1 by treatment with 1 nM αFactor at 23°C for 2.5 h. Cells were then released at the restrictive temperature of 37°C and protein samples were harvested at the indicated time points. Total protein lysates were subjected to Western Blot analysis to detect Rad53. **(B)** Strains of the indicated genotypes were grown overnight in YPD at the permissive temperature of 23°C. Cultures were then diluted and shifted to the restrictive temperature of 37°C. At the indicated intervals, cells were spotted in serial dilutions on YPD agar plates and incubated at the permissive temperature (23°C) to allow colony formation and the assessment of recovery capacity. **(C)** Yeast strains were treated as described in (B), but at the indicated time points, protein samples were harvested and processed for Western Blot. For Rad53 detection, samples were run on 7.5 % polyacrylamide gels while for Clb2 and Sic1 detection, proteins were separated on gradient 4- 15 % polyacrylamide gels. Subsequently, membranes were probed with antibodies specific for Rad53, Clb2 and Sic1 and proteins were visualized using chemiluminescence. **(D)** Wild type and *cdc13- 1* cells were treated as described in (B), but the incubation at the restrictive temperature (37°C) was performed in the presence of either 5 nM rapamycin or DMSO as a vehicle control. **(E)** Cells described in (D) were subjected to Western Blot analysis as described for (C). **(F)** DNA content of the yeast strains described in (D) was analysed by flow cytometry after staining with Sytox® Green. **(G)** *cdc13-1* cells were grown overnight in SC medium lacking tryptophan (SC-Trp) at the permissive temperature (23°C) and subsequently diluted in SC-Trp and shifted to the restrictive temperature of 37°C for 6 h to arrest them in G_2/M. Afterwards, the culture was split and treated with either 5 nM rapamycin or DMSO and cells were imaged at the restrictive temperature. Re-budding events were counted manually using Fiji Software. Numbers in brackets denote the number of re-budded cells with respect to the total number of cells analysed. Panels **(B)** and **(F)** have been published in (Klermund et al., 2014) and my master thesis (Bender, K., 2013). The observation in panel **(C)** has been published in (Klermund et al., 2014). Experiments shown in panels **(D)** and **(E)** have been performed by Julia Klermund and have been published in (Klermund et al., 2014).

the presence of rapamycin drastically improved cell viability in *cdc13-1* cells (Figure 8 D). We then analysed the phosphorylation status of Rad53 by Western blot in *cdc13-1* cells at the restrictive temperature in the presence and absence of rapamycin. In the absence of rapamycin, *cdc13-1* cells activate the DNA damage checkpoint after 6 h at the restrictive temperature, however, after prolonged DNA damage, the checkpoint was turned off as evidenced by the loss of Rad53 phosphorylation (Figure 8 E). In contrast, the presence of rapamycin prevented checkpoint inactivation even after long-term DNA damage (24 h). As shown in Figure 8 C, the maintenance of Rad53 phosphorylation correlated well with the expression of Clb2 indicating that the cells remained arrested in the G2/M phase of the cell cycle and with an active checkpoint. This result was further confirmed by DNA content analysis using flow cytometry. As seen in Figure 8 F, *cdc13-1* cells arrested with a 2C DNA content after 6 h incubation at the restrictive temperature both in the absence and presence of rapamycin. However, without rapamycin, cells progressed further through the cell cycle after long-term DNA damage as seen by the appearance of a 1C DNA peak and a large fraction with even lower DNA content (sub-G1 DNA content) indicative of cell death. In the presence of rapamycin, cells remained arrested with a 2C DNA content even at the 24 h time point. Of note, the slight distortion of the 2C DNA content peak towards the right might be explained by the enlarged cell size due to a prolonged cell cycle arrest (Johnston et al., 1977). This suggests that TORC1 inhibition using rapamycin can improve viability in *cdc13-1* mutants by preventing checkpoint adaptation thereby maintaining checkpoint activity and checkpoint-mediated cell cycle arrest. In agreement with this hypothesis and consistent with Figure 8 B and C, *cdc13-1* cells harbouring the adaptation-deficient *cdc5-ad* allele also remained arrested with a 2C DNA content (24 h, Figure 8 F).

Interestingly, the presence of rapamycin could further reduce the small fraction of sub-G1 DNA content (compare *cdc13-1cdc5-ad* DMSO with *cdc13-1cdc5-ad* 5 nM Rapa at 24 h), suggesting that rapamycin could function in one or more additional pathway(s) to prevent checkpoint adaptation.

We then tested whether the timing of checkpoint adaptation is an orchestrated event within a cell population or if it is an individual cellular decision. To address this question, we used live-cell imaging to follow the fate of individual *cdc13-1* cells in a population in the presence or absence of rapamycin over time at the restrictive temperature. Figure 8 G summarizes the distribution of re-budding events observed in *cdc13-1* cells during the incubation at the restrictive temperature. In the absence of rapamycin, approximately 77% (73 out of 95) cells underwent a re-budding event during the time course although kept at the restrictive temperature, suggesting that they adapted to the persistent DNA damage and progressed further through the cell cycle (Figure 8 G). The majority of cells adapted in a time window of 8 to 10 hours after the shift to the restrictive temperature. This suggests that the exact timing of adaptation is an individual cellular decision. However, the fact that the majority of cells adapted within a 3 hour time period could be an indication for the existence of a more general counting mechanism that regulates the onset of checkpoint adaptation. Importantly, in the presence of rapamycin, only approximately 4% (4 out of 110) cells re-budded while the majority remained arrested with a dumbbell morphology characteristic for a G2/M cell cycle arrest. This supports the hypothesis that rapamycin prevents checkpoint adaptation by preserving Rad53 phosphorylation (Figure 8 E) and thereby maintaining the damage checkpoint-induced cell cycle arrest (Figure 8 F and G). Interestingly, the majority of cells that underwent checkpoint adaptation as judged by re-budding event(s) eventually underwent cell lysis (data not shown) which is consistent with previous analyses of temperature-sensitive *cdc* mutants (Hartwell et al., 1973).

2.2 Preventing adaptation requires a functional DNA damage checkpoint

Cdc5 can drive adaptation by overriding the Rad53-mediated DNA damage checkpoint (Donnianni et al., 2010; Vidanes et al., 2010). In agreement with the idea that Cdc5 requires a functional DNA damage checkpoint, *cdc5-ad* mutants are unable to prevent adaptation in a *rad9* background (lacking the Rad9 checkpoint adaptor protein) after a HO-induced DSB (Toczyski et al., 1997). Consistently, when we diminished Rad53 function using the *rad53-11* allele, viability in *cdc13-1* mutants conferred by the *cdc5-ad* allele was decreased (Figure 9 A). The *rad53-11* allele, which is also known as *mec2-1*, harbours a glycine to glutamic acid point mutation at amino acid position 653 (Dohrmann and Sclafani, 2006) and is checkpoint-defective (Weinert et al., 1994), probably due to decreased kinase activity both *in vivo* (Sun et al., 1996) and *in vitro* (Sidorova and Breeden, 2003). Interestingly, in this experiment, also rapamycin was unable to rescue the viability of *cdc13-1cdc5-adrad53-11* triple mutants after

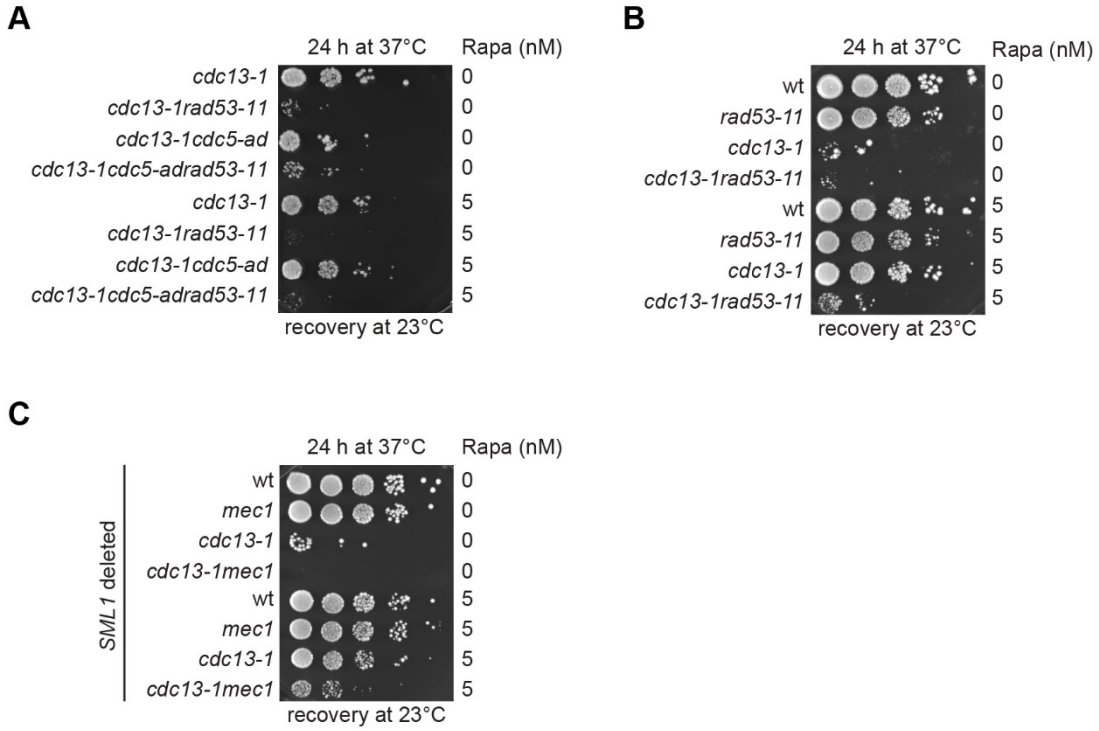


Figure 9. The *cdc5-ad* allele and rapamycin both require a functional DNA damage checkpoint to prevent checkpoint adaptation in *cdc13-1* mutants. (A) Yeast strains with the indicated genotypes were grown overnight at the permissive temperature of 23°C. Subsequently, cells were diluted in the presence of 5 nM rapamycin or DMSO as a vehicle control and shifted to the restrictive temperature (37°C). At the indicated time points, cells were spotted in serial dilutions on YPD agar plates and colony formation was assessed after recovery at the permissive temperature. **(B) - (C)** Yeast cells of the indicated genotypes were treated as described in (A). Panel **(B)** has been published in (Klermund et al., 2014).

prolonged incubation at the restrictive temperature (Figure 9 A). We then repeated the experiment in *CDC5* wild-type cells and found that rapamycin failed to promote cell viability in checkpoint-defective *cdc13-1rad53-11* mutants (Figure 9 B). We also included mutants lacking *MEC1* (and *SML1* as the Suppressor of *mec1* Lethality) in our analysis, which is the most upstream and initial checkpoint kinase. As seen in Figure 9 C, rapamycin failed to preserve cell viability in *cdc13-1* mutants when the DDC cannot be activated. Taken together, these results suggest that preventing adaptation after prolonged telomere dysfunction in *cdc13-1* cells either using the adaptation-defective *cdc5-ad* allele or by rapamycin treatment requires a functional DDC.

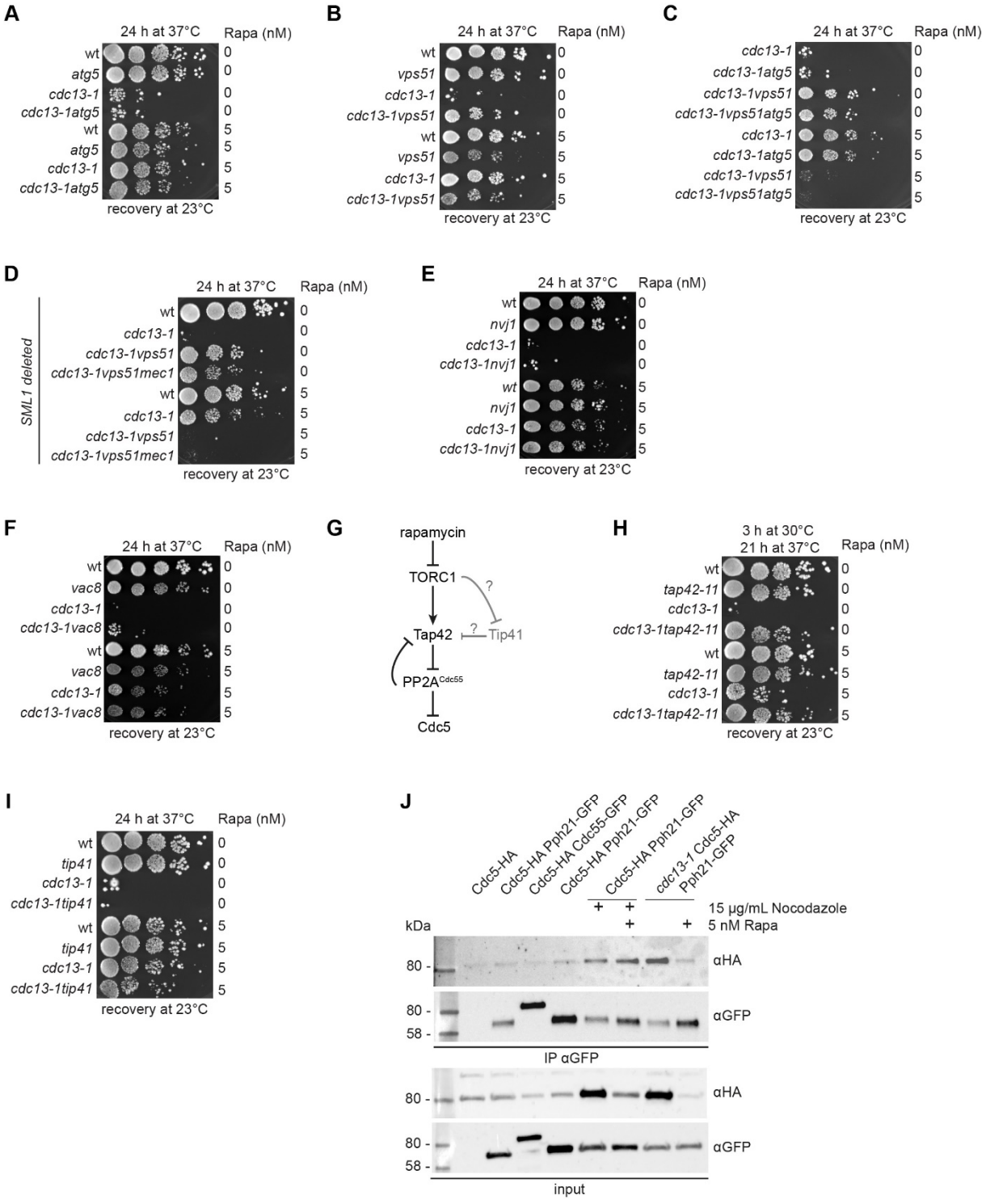
2.3 The role of TORC1 effectors in preventing adaptation in *cdc13-1* mutants

Inhibition of TORC1 signalling by rapamycin mimics nutrient starvation even in standard rich growth conditions. Therefore, diminished TORC1 activity elicits multiple cellular responses including the induction of catabolic processes like autophagy. We set out to determine the role of autophagy in mediating the rapamycin-induced prevention of checkpoint adaptation in *cdc13-1* cells. As shown in Figure 10 A, loss of *ATG5*, which is involved in autophagosome formation, did not abolish the rescue of cell viability upon rapamycin treatment. Also other branches of autophagy and protein sorting have been implied to play a role in checkpoint

adaptation. It was reported that yeast cells lacking *VPS51*, a component of the Golgi-associated retrograde protein (GARP) complex, are adaptation defective after a HO-cut induced DSB although Rad53 phosphorylation was abolished (Dotiwala et al., 2013). The GARP complex resides at the cytosolic face of the Golgi apparatus and functions in retrograde protein trafficking (Bonifacino and Hierro, 2011) and in the cytoplasm-to-vacuole targeting (Cvt) pathway (Reggiori et al., 2003). The adaptation defect in *vps51* mutants has been shown to be caused by the mislocalization of Esp1 and Pds1 to the cytoplasm, thereby preventing the onset of mitosis (Dotiwala et al., 2013). In addition to loss of *VPS51*, rapamycin-mediated induction of autophagy was shown to be sufficient to achieve a permanent cell cycle arrest after damage caused by an irreparable endonuclease-induced DSB (Dotiwala et al., 2013). We therefore tested whether loss of *VPS51* could also rescue cell viability in *cdc13-1* mutants. We found that *cdc13-1vps51* double mutants were able to form colonies after 24 h incubation at the restrictive temperature to a similar extent as *cdc13-1* mutants incubated in the presence of rapamycin (Figure 10 B). A previous study had found that the adaptation defect in *vps51* mutants required bulk autophagy mediated by Atg5 (Dotiwala et al., 2013). In order to test if this was true in the rescue of *cdc13-1* mutants as well, we created a *cdc13-1vps51atg5* triple mutant. We found that the *vps51*-mediated rescue seemed independent of bulk autophagy, since loss of *ATG5* did not decrease *cdc13-1vps51* viability (Figure 10 C). The differences observed regarding the role of Atg5 could be explained by the use of an irreparable single DSB system employed by Dotiwala and colleagues (Dotiwala et al., 2013). Of note, *vps51* mutants were very sensitive to rapamycin treatment and therefore hampered the analysis of cell viability of the respective double or triple mutants in the presence of rapamycin (compare Figure 10 B-D). Since the adaptation defect in *vps51* mutants occurred independently of Rad53 (Dotiwala et al., 2013), we tested the ability of *VPS51* loss to exert its beneficial function in the viability rescue of *cdc13-1* in a *MEC1*-deficient background. We hypothesized that loss of the most upstream checkpoint kinase Mec1 should not affect the *vps51*-mediated rescue. Indeed, mutant *cdc13-1* cells lacking *VPS51*, *MEC1* (and *SML1* as the Suppressor of *mec1* Lethality) were as proficient in colony formation as *cdc13-1vps51* double mutants (Figure 10 D), indicating that the rescue effect conferred by loss of *VPS51* is largely checkpoint independent.

We then aimed to address if nucleus-specific autophagy (piecemeal autophagy of the nucleus, PMN) could play a role in the removal of adaptation-promoting factors from the nucleus. In fact, Cdc5 requires relocalization from the nucleoplasm to the cytoplasm to promote exit from mitosis (reviewed in (Botchkarev and Haber, 2017)). Therefore, we hypothesized that abolishment of the PMN proteins Nvj1 or Vac8 should hamper the rapamycin-mediated rescue of *cdc13-1* mutants. As shown in Figure 10 E and F, defects in PMN did not influence viability after prolonged telomere dysfunction. These results suggested that rapamycin does not prevent adaptation by triggering the selective removal of adaptation-promoting factors from the nucleus via the PMN pathway. However, further experiments are required to exclude a role of PMN in preventing checkpoint adaptation upon telomere dysfunction.

The heterotrimeric phosphatase PP2A constitutes another TORC1 effector and in addition, PP2A plays important roles during cell cycle control. Associated with its regulatory subunit Cdc55, PP2A is involved in retaining Cdc14 in the nucleolus thereby delaying mitotic exit (Queralt et al., 2006). Furthermore, Cdc55-associated PP2A regulates the correct cellular localization of Cdc5 (Nakashima et al., 2008). Under nutrient rich conditions, active TORC1



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binds to the Tap42-PP2A heterodimer and thereby negatively regulates PP2A activity via phosphorylation of Tap42 (Di Como and Arndt, 1996; Jiang and Broach, 1999). Upon TORC1 inhibition, for example by rapamycin treatment, the TORC1-Tap42-PP2A complex dissociates and PP2A causes the dephosphorylation of Tap42 leading to the full activation of PP2A (Di Como and Jiang, 2006; Jiang and Broach, 1999; Yan et al., 2006). A genetic screen identified Tip41 as a Tap42-interacting protein and therefore, also Tip41 was implicated in TORC1-mediated control of PP2A (Jacinto et al., 2001). Although a TORC1-mediated phosphorylation

Figure 10. The influence of TORC1 downstream targets on preventing checkpoint adaptation and preserving viability in *cdc13-1* mutants after DNA damage. (A) – (F) Yeast strains with the indicated genotypes were grown overnight at the permissive temperature (23°C). Cells were then diluted in the presence of either 5 nM rapamycin or DMSO as the vehicle control and shifted to the restrictive temperature (37°C). At the indicated time points, cells were spotted in serial dilutions onto YPD agar plates and colony formation was assessed after recovery at the permissive temperature. **(G)** Schematic model for the regulation of $PP2A^{Cdc55}$ activity. Active TORC1 phosphorylates Tap42 thereby promoting its association with the PP2A complex. Upon TORC1 inhibition, Tap42 phosphorylation decreases and releases $PP2A^{Cdc55}$. Once liberated, $PP2A^{Cdc55}$ activity causes further dephosphorylation of Tap42 and the PP2A phosphatase complex can act on Cdc5. The role of Tip41 in regulating $PP2A^{Cdc55}$ is not fully characterized and putative connections are indicated in dark grey. (**H**) Yeast strains of the indicated genotypes were grown overnight at the permissive temperature (23°C) and diluted in the presence of 5 nM rapamycin or DMSO (as control). Cultures were pre-arrested in G2/M due to checkpoint activation at 30°C for 3 h and then shifted to 37°C to inactivate mutant Tap42 (*tap42-11*). Serial dilutions were spotted onto YPD agar plates and colony formation was documented after recovery at the permissive temperature. (**I**) Yeast strains with the indicated genotypes were treated as described for panels (A) - (F). (**J**) Co-immunoprecipitation of endogenously tagged proteins was performed either from exponentially growing cells or in the presence of 15 µg/mL nocodazole to mimic *cdc13-1*-mediated checkpoint arrest in G_2/M following the temperature shift to 37°C. Cell extracts were incubated with beads coupled to anti-GFP antibody fragments, subjected to SDS-PAGE on gradient 4-15% polyacrylamide gels and transferred to membranes that were subsequently probed with anti-HA or anti-GFP antibodies. Proteins were detected by chemiluminescence. Panel **(A)** and **(H)** have been published in (Klermund et al., 2014).

of Tip41 resulting in a tighter association of Tip41 and Tap42 has been shown, this appears to have a greater impact on the PP2A-like phosphatase, Sit4 (Jacinto et al., 2001). Given the important role of PP2A in mitotic exit control and Cdc5 localization and knowing it as a TORC1 downstream target, we sought to test the idea if PP2A might contribute to the rapamycin-mediated rescue of *cdc13-1* mutants. We hypothesized that rapamycin-mediated TORC1 inhibition would cause the release and activation of PP2A eventually leading to a delayed mitotic exit due to nucleolar Cdc14 retention (see model Figure 10 G). To test this idea, we sought to interfere with PP2A function. However, simultaneous deletion of the two redundant catalytic PP2A subunits *PPH21* and *PPH22* rendered the mutants temperature sensitive and conferred a previously documented growth defect (Sakumoto et al., 2002), thereby hampering the analysis of their effect on *cdc13-1* viability (data not shown). Instead, we chose to use a temperature-sensitive allele of Tap42 (*tap42-11*), which was reported to cause a proliferation defect due to G1 cell cycle arrest at the restrictive temperature (Cherkasova and Hinnebusch, 2003; Di Como and Arndt, 1996). The temperature-sensitive phenotype has been attributed to the inability of *tap42-11* to interact with phosphatases (Wang et al., 2003). We hypothesized that shifting *cdc13-1tap42-11* double mutants to elevated temperatures should lead to PP2A activation and consequently, rescue of the *cdc13-1*-conferred viability loss upon DNA damage. To be able to analyse the effects of *tap42-11* allele upon checkpoint-induced cell cycle arrest and to avoid a G1 cell cycle arrest due to interfering with Tap42 protein, *cdc13-1tap42-11* mutants were first shifted to 30°C, which is sufficient to inactivate Cdc13, and subsequently shifted to 37°C, the restrictive temperature for *tap42-11*. As shown in Figure 10 H, inactivation of Tap42 in *cdc13-1* cells rescued cell viability after prolonged DNA damage even in the absence of rapamycin suggesting that the Tap42-PP2A axis might indeed have an impact on cell viability in the presence of DNA damage. However, we were unable to reliably determine the cell cycle distribution or Rad53 phosphorylation status in *cdc13-1tap42-11* double mutants

and therefore cannot rule out a DDC-independent effect of the *tap42-11* mutation in preserving viability in *cdc13-1* cells.
In addition to using the *tap42-11* allele, we analysed the effect of *TIP41* deletion as a Tap42-regulatory factor. However, *cdc13-1tip41* double mutants were neither viable after prolonged DNA damage nor did *TIP41* loss affect the rapamycin-mediated rescue of *cdc13-1* cells (Figure 10 I). This is likely to be due to Tip41 influencing mainly a different phosphatase, Sit4 or unknown regulatory factors influencing the Tip41-Tap42 interaction. Since interfering with Tap42 to manipulate PP2A activity rescued cell viability in *cdc13-1* mutants and given the fact that PP2A is a phosphatase we wondered whether activated PP2A due to DNA damage might interact with Cdc5. Such an interaction could lead to Cdc5 dephosphorylation at critical activating phosphorylation sites and thereby cause or maintain Cdc5 inhibition. Consequently, the DNA damage checkpoint and cell cycle arrest would be reinforced (see model Figure 10 G). This scenario has been reported for higher eukaryotes where DNA damage increased the interaction between PP2A with its regulatory subunit B55α, the mammalian orthologue of Cdc55, and Polo-like kinase 1 (Wang et al., 2015). In order to test this idea in our *cdc13-1* background, we created epitope-tagged proteins of Cdc5, the PP2A regulatory subunit Cdc55, and one of the two redundant catalytic subunits, Pph21, expressed from their endogenous promoters (Figure 10 J). Using these strains, we performed co-immunoprecipitations followed by Western Blot to reveal a possible interaction between Cdc5 and PP2A complex members. After arresting cells in G2/M with nocodazole to mimic a damage-induced cell cycle arrest, pull-down of Pph21-GFP co-purified Cdc5-HA, an effect that was slightly increased if cells were treated with rapamycin in addition (Figure 10 J). When the *cdc13-1* allele was present and cells were shifted to the restrictive temperature to elicit a DNA damage response, Pph21-GFP and Cdc5-HA were also found to interact, however, this interaction seemed decreased in the presence of rapamycin (Figure 10 J). Importantly, both in the input and co-immunoprecipitation fraction, rapamycin treatment seemed to decrease Cdc5 protein levels. This unexpected finding, together with the observed interaction of Cdc5 with PP2A, implicated that rapamycin might prevent checkpoint adaptation in *cdc13-1* cells by regulating Cdc5 protein levels or by keeping Cdc5 via PP2A phosphatase inactivated to ensure cell cycle arrest and maintain cell viability.

2.4 Regulation of Cdc5 protein levels by rapamycin to prevent adaptation

One possibility to explain the prevention of adaptation by rapamycin could be that rapamycin regulates Cdc5 levels at the protein level and therefore prevents mitotic progression. We based our hypothesis on the involvement of TORC1 in the regulation of cap-dependent translation and its role in controlling autophagy because both processes are potent regulatory pathways for protein abundance. In order to test our idea, we analysed Cdc5 protein levels by Western Blotting in the absence and presence of rapamycin. To be able to compare Cdc5 protein levels in *cdc13-1* mutants in the presence and absence of rapamycin, we used G1-synchronized cultures and released them into the cell cycle at the restrictive temperature (37°C), thereby ensuring that all cells in the population arrested uniformly in G2/M due to DDC activation. After two hours, when *cdc13-1* cells had arrested at the restrictive temperature, we split the culture and treated with rapamycin or the solvent DMSO (Figure 11 A). As seen in Figure 11 B, Cdc5-HA levels were very low in G1-arrested cells and increased as cells arrested in G2/M.

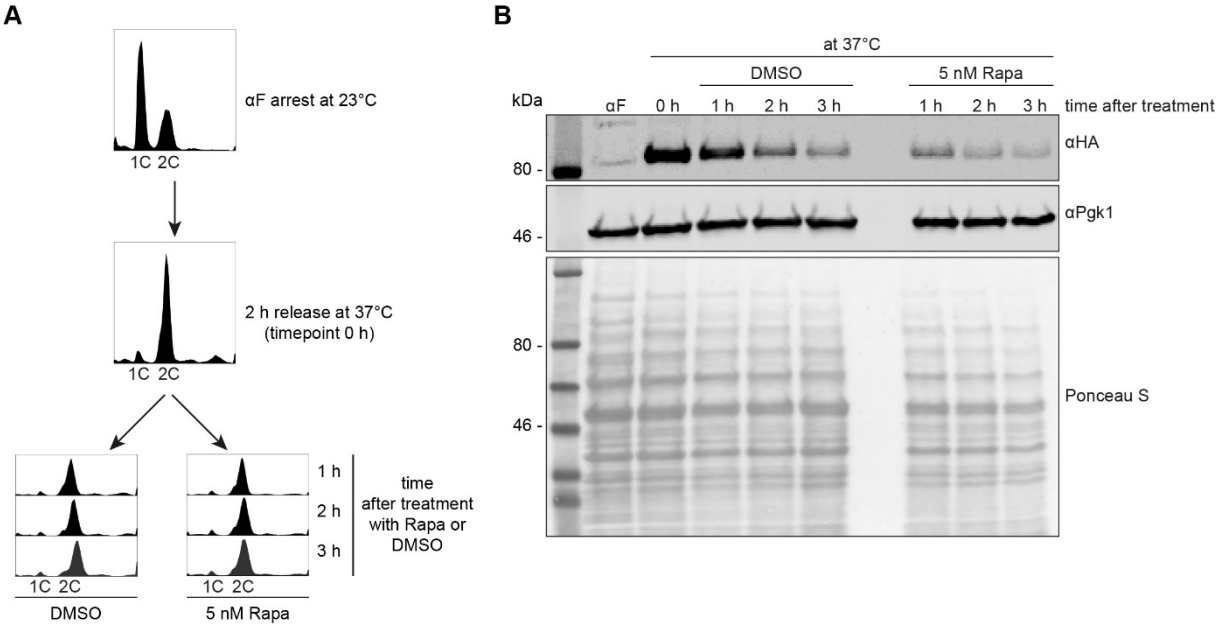

Figure 11. Regulation of Cdc5 protein levels by rapamycin as a possible mechanism to prevent checkpoint adaptation. (A) DNA content analysis of *cdc13-1* cells expressing endogenously tagged Cdc5-HA protein after αFactor (αF) arrest (1 nM) and release at the restrictive temperature. After a 2 h release, the culture was split and treated either with 5 nM rapamycin or DMSO as the vehicle control. Samples for protein extracts and flow cytometry were taken at the indicated time points after splitting the culture. **(B)** Total protein from samples collected in (A) were separated on gradient 4-15% polyacrylamide gels. After transfer, anti-HA antibody was used to detect Cdc5-HA protein and anti-Pgk1 antibody was used as a loading control and proteins were detected using fluorescence-coupled secondary antibodies.

This is consistent with Cdc5 protein levels peaking in mitosis before anaphase and subsequently declining during anaphase and telophase (Cheng et al., 1998; Hardy and Pautz, 1996). In the presence of DNA damage, Cdc5 protein levels were slowly reduced in control treated cells over time (Figure 11 B, DMSO). However, in the presence of rapamycin, there was a strong decrease in Cdc5 protein levels observed already one hour after treatment, an effect that was exacerbated during the time course (Figure 11 B, 5 nM Rapa). Of note, also total protein levels seemed to decrease upon rapamycin treatment as evident by the weaker signal for total protein observed after Ponceau S staining. This is likely to be due to the general translation inhibitory role of rapamycin. In summary, these observations suggest a possible regulation of Cdc5 protein levels mediated by rapamycin in order to maintain checkpoint activation and arrest induced by *cdc13-1* growth at the restrictive temperature.

2.5 Preventing adaptation sensitizes repair-defective cells to genotoxins

The chemotherapeutic agent camptothecin (CPT) creates DNA damage by trapping the topoisomerase 1 (Top1) enzyme on the DNA backbone (Hsiang et al. (1989)). Subsequently, the initial covalent cleavable complex can lead to DSBs after collisions between the transcription machinery and the Top1-DNA complex and due to the stalling of replication forks (Hsiang et al., 1989; Pommier et al., 2010). Rad52-mediated homologous recombination (HR) plays an important role in the repair of CPT-inflicted DNA damage (Pouliot et al., 2001). It is

known that HR-deficient *rad52* mutants that are unable to undergo checkpoint adaptation due to the presence of the adaptation-defective *cdc5-ad* allele are hypersensitive to X-rays (Galgoczy and Toczyski, 2001). We first wanted to confirm this finding by treating homozygous diploids that are either adaptation-proficient (*CDC5*) or adaptation-deficient (*cdc5-ad*) by irradiating with 45 Gy of X-rays. If not indicated otherwise, all yeast strains used in these experiments were homozygous diploids. To assess the effect of checkpoint adaptation on X-ray sensitivity, we spotted the indicated mutants in serial dilutions onto YPD agar plates and irradiated with 45 Gy of X-rays or left the cells untreated as a control. As shown in Figure 12 A, repair-defective *rad52* diploids are sensitive to X-ray treatment, while repair-proficient wild type and *cdc5-ad* diploids are unaffected in their growth after genotoxic treatment. Furthermore, *rad52* diploids harbouring the *cdc5-ad* allele, rendering them adaptation-deficient, are hypersensitive to X-rays as evidenced by the decreased colony formation compared to *rad52* single mutants. YPD agar plates were supplemented with the vital dye Phloxine B, which accumulates in metabolically inactive or dead cells thereby causing a red colony colour (Minois et al., 2005). Colonies formed by repair-defective *rad52* mutants as well as adaptation-defective *rad52cdc5-ad* mutants appeared in a slight pink colour especially after genotoxic treatment (Figure 12 A) underlining that mutants were severely affected in their growth and viability after experiencing irradiation. We then continued to assess the X-ray hypersensitivity of *rad52cdc5-ad* cells in a quantitative manner by plating cells and scoring for their ability to form colonies after X-ray irradiation (Figure 12 B). For the quantitative plating assay, we plated a defined number of cells on either control plates or plates that were subsequently subjected to genotoxic treatment. Afterwards, all colonies visible by eye were counted and survival relative to control cells was calculated. Consistent with Figure 12 A, preventing checkpoint adaptation in a *rad52* diploid background significantly decreased the number of colonies growing after irradiation from 16.8% survival in *rad52* to 2.6% survival in *rad52cdc5-ad* diploids (Figure 12 C). At the same time, repair-proficient cells remained unaffected in their survival after X-ray treatment independently of their ability to undergo checkpoint adaptation (Figure 12 C, compare wild type to *cdc5-ad* cells). We then continued to assess the effect of adaptation deficiency on cell survival in *rad52* diploids in response to CPT treatment. In accordance with Figure 12 A, spotting cells in serial dilutions onto drug-containing plates revealed a strong sensitivity of *rad52* diploids to CPT that was further increased by preventing adaptation using the *cdc5-ad* allele (Figure 12 D). Consistently, plating cells onto plates containing CPT and comparing to control plates showed a significant decrease in cell survival of *rad52cdc5-ad* cells compared to adaptation-proficient *rad52* cells (29.8% in *rad52cdc5-ad vs* 80.6% survival in *rad52* mutants) (Figure 12 E). In agreement with our previous findings, adaptation deficiency in a repair-proficient background did not affect cell survival after CPT treatment (Figure 12 A and D). Of note, due to counting errors during cell number determination and technical inaccuracies while plating, cell viability (expressed as % survival normalized to control conditions) exceeds 100 % in some experiments. Importantly, *rad52* diploids and, more rarely, *rad52cdc5-ad* diploids only formed colonies on CPT-containing plates after 8 days, which was not due to CPT inactivation since CPT plates incubated for 7 days before using them were still effective in hampering the growth of *rad52* diploids (data not shown). Taken together, HR-defective *rad52* diploids are able to form colonies after X-ray irradiation or in the presence of CPT due to undergoing checkpoint

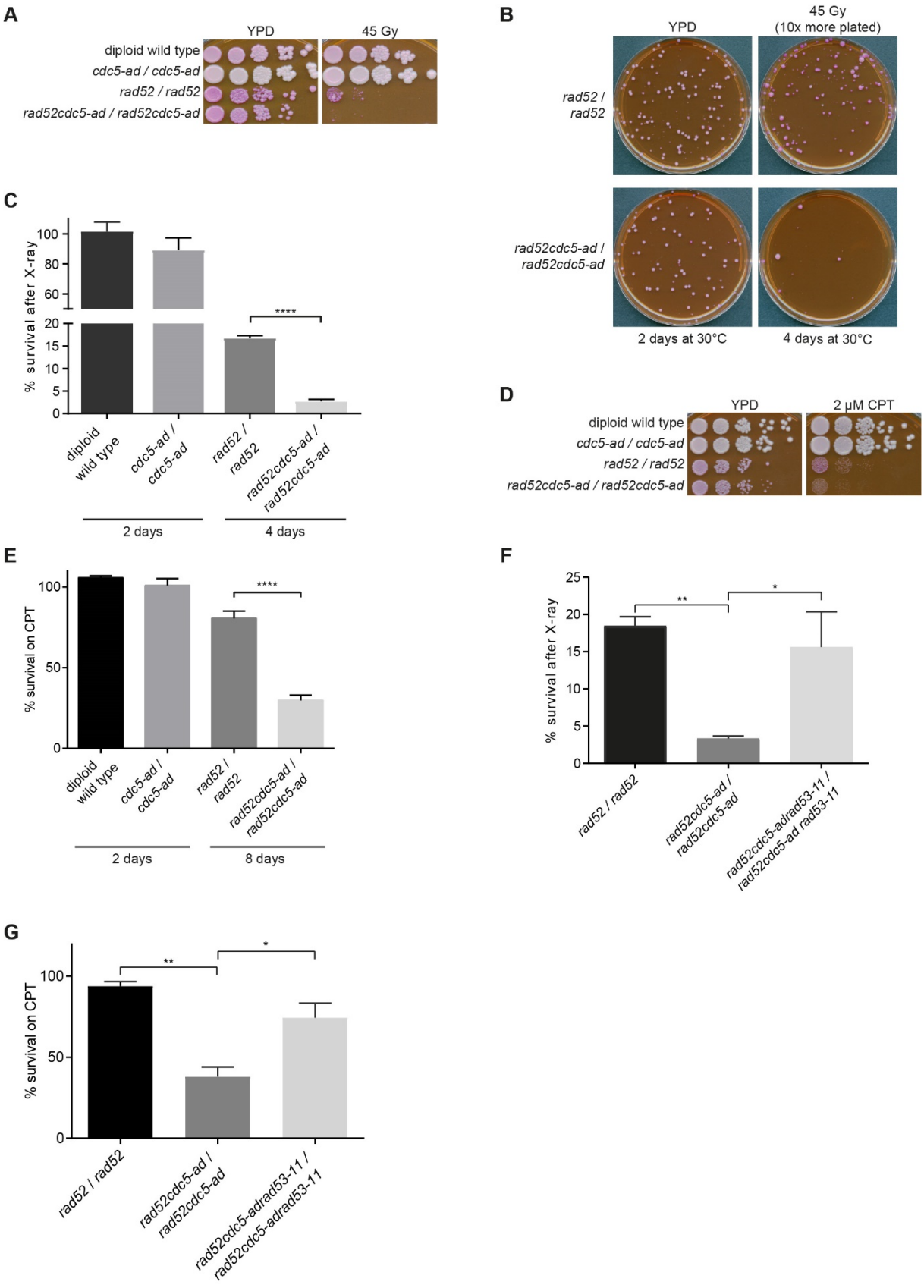

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Figure 12. **Preventing checkpoint adaptation using the *cdc5-ad* allele sensitizes repair-defective *rad52* mutants to genotoxic agents in a checkpoint-dependent manner. (A)** Diploid yeast strains of the indicated genotypes were spotted in serial dilutions onto YPD agar plates and either left untreated or irradiated with a dose of 45 Gy of X-ray. Colony formation was assessed after 3 to 4 days incubation at 30°C. Plates contained 8 µg/mL Phloxine B to visualize cellular metabolic activity. **(B)** Yeast cells of the indicated genotypes were plated onto YPD agar plates (supplemented with 8 µg/mL Phloxine B) and either left untreated or irradiated with 45 Gy of X-rays. Plates were then incubated for the indicated periods at 30°C and colony number was analysed by manual counting. Of note, the cell number plated on X-ray treated plates was ten-times higher than the cell number plated on control plates. **(C)** Quantification of plating assays as shown in (B). **(D)** Spotting assay was performed as described in (A) but YPD agar plates contained 2 µM CPT (in DMSO) or DMSO as control. All agar plates contained 8 µg/mL Phloxine B. **(E)** Quantification of colony numbers after plating assays with CPT as a genotoxic agent as described in (B). **(F) – (G)** Quantification of plating assays as described in (B) and (C) using yeast strains of the indicated genotypes either in response to X-ray exposure (F) or in the presence of CPT (G). Note the different axis scale in panel (F). Data are represented as mean values and the error bars indicate the SEM of 3 independent experiments. Statistical analysis was performed using one-way ANOVA with ** as $p \leq 0.01$, *** as $p \leq 0.001$ and **** as $p \leq 0.0001$. Experiments shown in panels **(A) – (E)** have been performed by Julia Klermund and Stefanie Grimm.

adaptation. Therefore, preventing checkpoint adaptation can decrease cell survival of repair-defective mutants following genotoxic treatment.

As described earlier, Cdc5 promotes checkpoint adaptation by inactivating or bypassing the checkpoint pathway (Pellicioli et al., 2001; Toczyski et al., 1997; Vidanes et al., 2010). In order to strengthen the link between checkpoint adaptation and the hypersensitivity of *rad52* to genotoxins, we investigated the role of a functional Rad53-dependent DNA damage checkpoint. Therefore, we introduced the checkpoint-defective *rad53-11* allele and plated the cells onto CPT-containing plates or YPD plates that were subjected to X-ray irradiation and colonies were counted to assess cell survival. In cells harbouring the *rad53-11* allele, the adaptation-defective *cdc5-ad* allele failed to decrease the colony number both in the presence of CPT and following X-ray treatment (Figure 12 F and G).

In summary, we found that preventing checkpoint adaptation in repair-deficient cells leads to hypersensitivity in response to both CPT and X-ray treatment in a checkpoint-dependent manner. These findings are consistent with a previous report (Galgoczy and Toczyski, 2001) demonstrating the role of adaptation in sensitizing *rad52* diploids to X-rays and underline the role of adaptation occurring in response to various types of damage including X-rays (Galgoczy and Toczyski, 2001), a single HO-mediated DSB (Sandell and Zakian, 1993) and in a *cdc13-1* model system (Klermund et al., 2014; Toczyski et al., 1997).

2.6 Repair-defective cells exhibit drug resistance after genotoxic treatment

It is possible that *rad52* diploids which were able to grow after genotoxic treatment did not get damaged significantly and therefore were able to form colonies. Alternatively, these cells could have acquired resistance towards the genotoxic insult. To discriminate between these two possibilities, we tested if the acquisition of resistance facilitated cell growth after further genotoxic treatment. Therefore, we plated *rad52* mutants on CPT-containing plates (as described for Figure 12 B) and after colonies had formed, challenged these cells again with CPT and other genotoxic insults. As seen in Figure 13 A, *rad52* diploids experiencing CPT

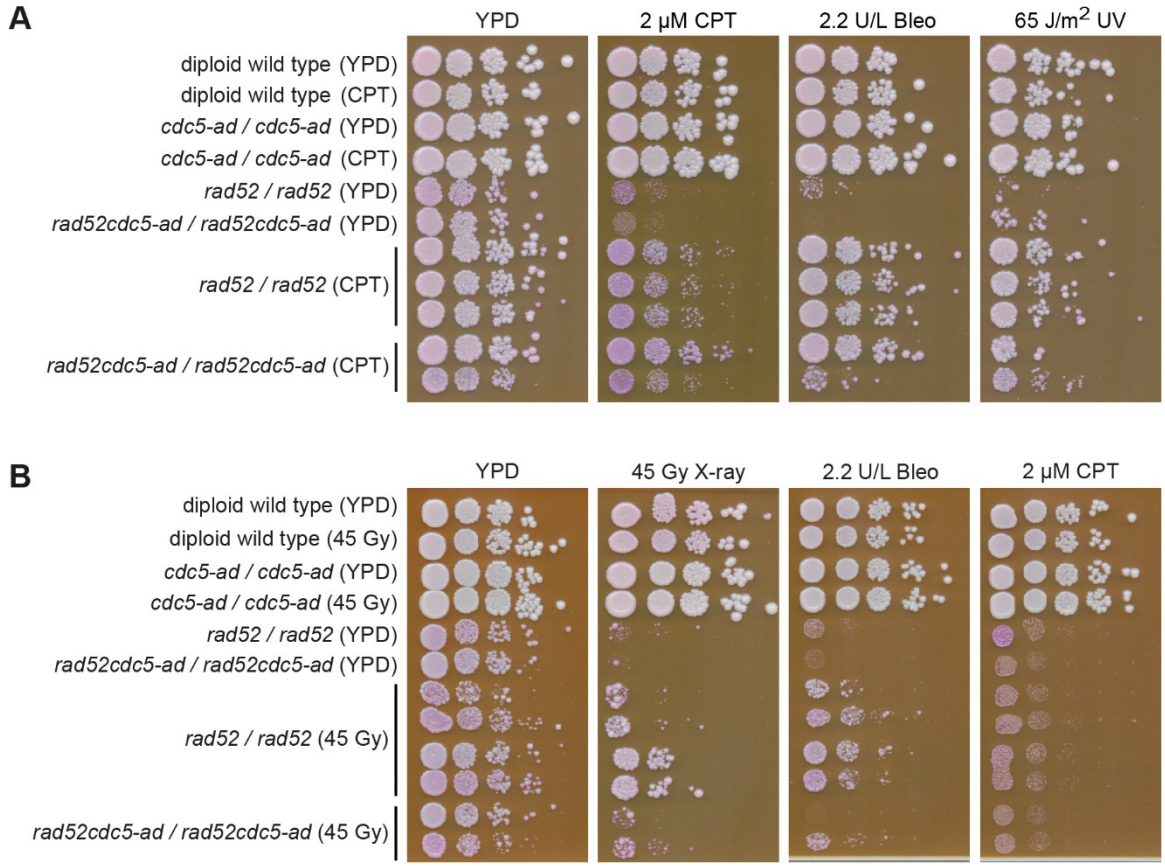

Figure 13. Depending on the genotoxic treatment, *rad52* mutants exhibit different drug-resistance properties. (A) Yeast strains with the indicated genotypes were either derived from control YPD agar plates (denoted as (YPD)) or from YPD agar plates supplemented with 2 µM CPT (denoted as (CPT)) and spotted in serial dilutions onto YPD agar plates. A second round of genotoxic treatment was conducted as indicated: either YPD agar plates contained DNA damaging drugs (2 µM CPT or 2.2 U/L bleomycin) or were subjected to UV treatment (65 J/m^2). Images were taken after 3 to 4 days incubation at 30°C. **(B)** Analogous to the experiment described in (A), yeast strains derived from either control YPD agar plates (denoted as (YPD)) or derived after X-ray treatment (denoted as (45 Gy)) were spotted in serial dilutions in the presence of the indicated drugs (2.2 U/L bleomycin or 2 µM CPT) or were treated with 45 Gy X-ray. All YPD agar plates were supplemented with 8 µg/mL Phloxine B. Experiment in Panel **(A)** has been performed by Julia Klermund.

treatment for the second time (denoted by *rad52* (CPT)) had a drastic growth advantage compared to the parental *rad52* mutants derived from a control YPD plate (denoted by *rad52* (YPD)) suggesting that they had acquired resistance to CPT. Of note, this observation was also true for *rad52cdc5-ad* diploids derived after CPT treatment. However, the frequency with which *rad52cdc5-ad* diploids were able to grow in the presence of CPT was greatly reduced (compare Figure 12 E). Importantly, CPT-derived *rad52* diploids did not only display resistance to CPT but also when spotted on plates containing the radiomimetic drug bleomycin or after UV irradiation (Figure 13 A). This indicated that repair-defective *rad52* mutants had acquired genotoxin resistance after CPT treatment.

We then continued to analyse if the same observation was true for *rad52* diploids forming colonies after X-ray irradiation. In brief, we plated *rad52* mutants and subsequently subjected them to 45 Gy of X-ray irradiation. Subsequently, we challenged the cells that were able to grow after this initial irradiation again with genotoxins. In contrast to our observations using

CPT, only some of the *rad52* mutants derived from X-ray irradiation (denoted by *rad52* (45 Gy) showed resistance to a second X-ray irradiation or bleomycin treatment (Figure 13 B). Moreover, when challenged with CPT as the second genotoxic agent, none of the *rad52* mutants derived from X-ray irradiation showed increased resistance to CPT compared to the *rad52* parental strain grown on a control plate (Figure 13 B). These differences could be explained by the type of damage used to create resistant *rad52* cells: in the case of CPT, damage is persistent while X-rays are a single dose-damaging agent.

2.7 Resistant *rad52* mutants show an altered karyotype after genotoxic treatment

It is known that homozygous diploid *rad52* cells lose chromosomes at high frequencies that can be further increased by X-ray irradiation (Mortimer et al., 1981). Therefore, we analysed the ploidy state of resistant *rad52* cells generated by CPT treatment. After colonies had appeared on drug-containing plates, individual colonies were picked, patched onto YPD plates and then inoculated to subject them to DNA content analysis by flow cytometry. Strikingly, all *rad52* mutants resistant to CPT showed a nearly haploid karyotype despite the parental strain being diploid (Figure 14 A). This indicates that in response to CPT treatment, *rad52* diploids experienced extensive chromosome loss. We then went on to test *rad52* resistant cells derived from X-ray irradiation. Importantly, *rad52* mutants grown after X-ray treatment displayed a variety of ploidy states ranging from diploid (as the parental *rad52* cells) to intermediate and nearly haploid (Figure 14 B). The rare resistant *rad52cdc5-ad* cells we obtained after CPT treatment or X-ray irradiation showed the same karyotypes regarding their ploidy as adaptation-proficient *rad52* cells (Figure 14 C and D). To rule out that the observed extensive chromosome loss leading to a (near-) haploid karyotype after CPT treatment in *rad52* cells was due to meiosis, we employed a *spo11* mutant in repair-defective *rad52* cells. Spo11 is a meiosis-specific endonuclease required for meiotic DSB induction (Keeney, 2001) rendering meiosis in *rad52spo11* diploids highly ineffective. After CPT treatment, *rad52spo11* double mutants formed colonies as efficiently as *rad52* diploids (Figure 14 E) and showed the same karyotypic profiles (Figure 14 F) confirming that the extensive chromosome loss in initially diploid repair-defective cells was not due to meiosis but was rather a consequence of checkpoint adaptation.

We then continued to analyse the chromosome loss events in resistant *rad52* mutants in more detail by determining their karyotype using a whole genome sequencing approach (Figure 15 A). We selected yeast cells that had been either treated with CPT or irradiated with 45 Gy of X-ray and cells grown under control conditions, extracted genomic DNA and performed whole-genome sequencing (WGS). As seen in Figure 15 A, diploid wild type cells maintained their karyotype even after being challenged with genotoxic treatment, a result confirmed by DNA content analysis using flow cytometry (Figure 15 B). Consistently with our observations in Figure 14 A, *rad52* mutants exposed to CPT underwent excessive chromosome loss since they had lost one copy of each homologous chromosome except for chromosome III (chromosome retention displayed in red). The near-haploid karyotype of CPT-resistant *rad52* mutants revealed in our sequencing analysis was also evident with the flow cytometry assay (Figure 15 B). However, single chromosome losses or retentions are unlikely to be detected by

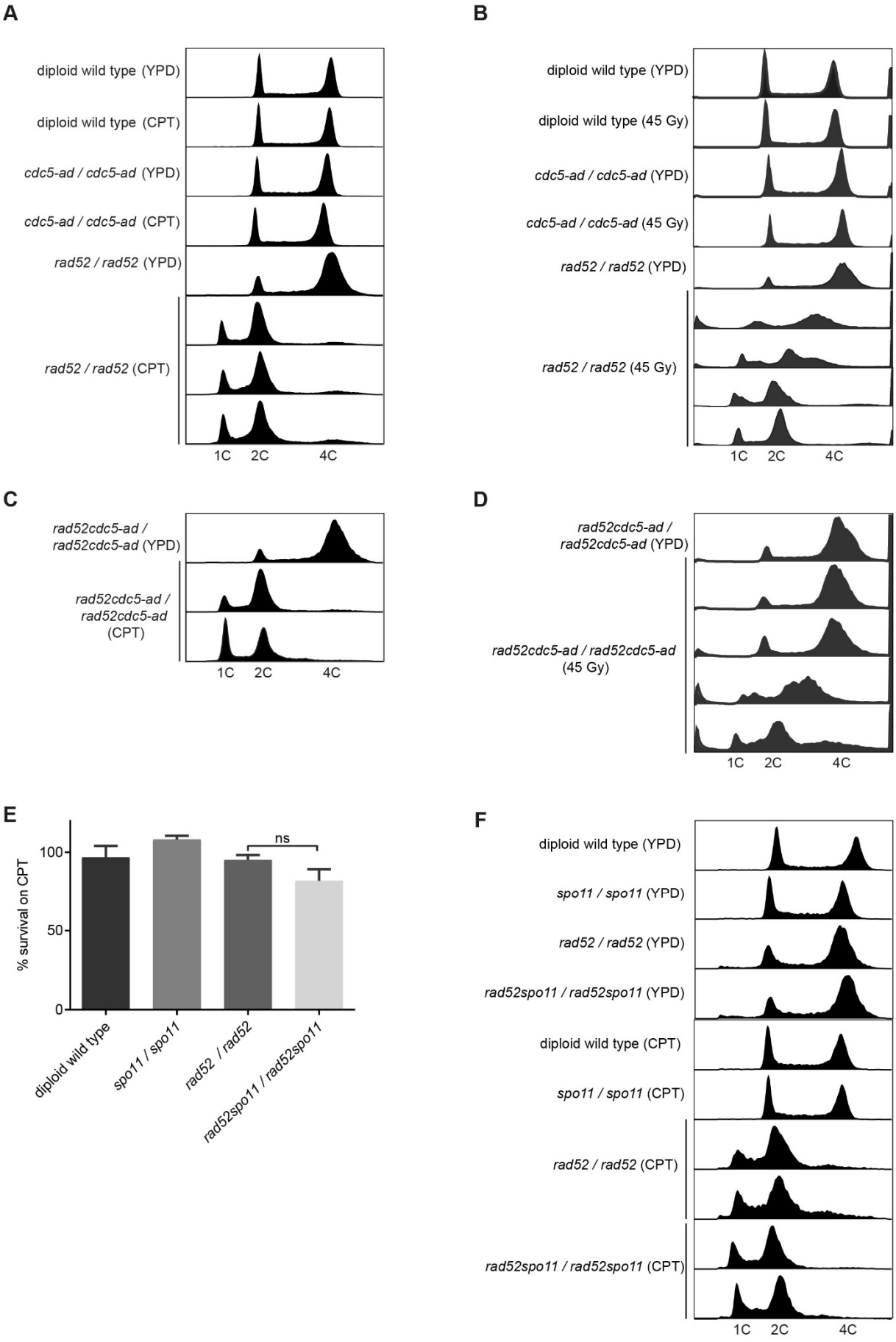

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Figure 14. Upon genotoxic stress, *rad52* mutants experience extensive chromosome loss which is not due to meiosis. (A)- (D) DNA content analysis was performed with yeast strains of the indicated genotypes derived from either control YPD plates (denoted as (YPD)) or after genotoxic treatment (denoted as (CPT) or (45 Gy)). **(E)** Quantification of plating assays. Data are represented as mean values and the error bars indicate the SEM of 3 independent experiments. Statistical analysis was performed using one-way ANOVA. Ns is p = 0.3932. **(F)** DNA content analysis of yeast strains used in (E). Experiments in Panel **(A)-(D)** have been performed by Julia Klermund and Stefanie Grimm.

flow cytometry due to sensitivity limitations. After exposure to 45 Gy of X-ray we detected a range of ploidies in *rad52* mutants, in agreement with our previous analysis (Figure 14 B). While one resistant *rad52* mutant had lost a copy of all homologous chromosomes except for chromosome III and IX, a second *rad52* strain kept chromosomes III, VIII, IX, XI and XIV in two copies but had lost one copy of every other chromosome. A third *rad52* mutant had maintained all chromosomes in two copies except for chromosome I (chromosome loss displayed in green). The different degrees of chromosome loss events resulting in aneuploidy were reflected very well in the flow cytometry analysis (Figure 15 B). Due to the normalization procedure of sequencing reads, the base line for the ploidy is not the same for every individual sample. If cells had retained or lost chromosome can be inferred from the ploidy observed in our flow cytometry assay. Furthermore, we ruled out the event of chromosome gains leading to a 2N+1 karyotype based on our cytometry-based DNA content analysis showing only the loss of genetic material evident as a shift in staining intensity towards the left and based on a previous report documenting only chromosome loss events in *rad52* mutants either spontaneously or exacerbated by X-ray induced DNA damage (Mortimer et al., 1981).
Interestingly, retention of chromosome III was found in *rad52* mutants after both CPT treatment and X-ray irradiation while the retention of chromosome IX was observed after X-ray exposure. Possible effects and causes for this selective retention of individual chromosomes after genotoxic insult are discussed below. In summary, our flow cytometry analysis together with the in-depth DNA sequencing approach led us to the conclusion that *rad52* mutants challenged with genotoxic agents acquired resistance and exhibited an aneuploid karyotype through whole-chromosome losses.

2.8 Resistant and aneuploid *rad52* mutants continue to lose genetic material

We wanted to address whether the chromosome loss events observed in *rad52* mutants after genotoxic treatment would continue when cells were passaged in unchallenging conditions.
In order to address this question, we treated *rad52* diploids with CPT to obtain resistant mutants. Next, we passaged the colonies formed in the presence of CPT on YPD plates in the absence of further DNA damaging agents in patches. With this approach, we did not streak out the cells as single colonies but rather analysed a bulk population. The near-haploid karyotype observed in *rad52* mutants after CPT exposure was observed in 67% (4 out of 6) already after the first passage (Figure 16 A and data not shown). Two of the mutants analysed displayed a more diploid or intermediate karyotype after the first passage that eventually progressed to a near-haploid DNA content over passaging time (Figure 16 A and data not shown). The DNA content shown in the first passage in Figure 16 A (top row of every histogram stack) was analysed by picking the *rad52* colony directly from the plate after genotoxic treatment. The histogram

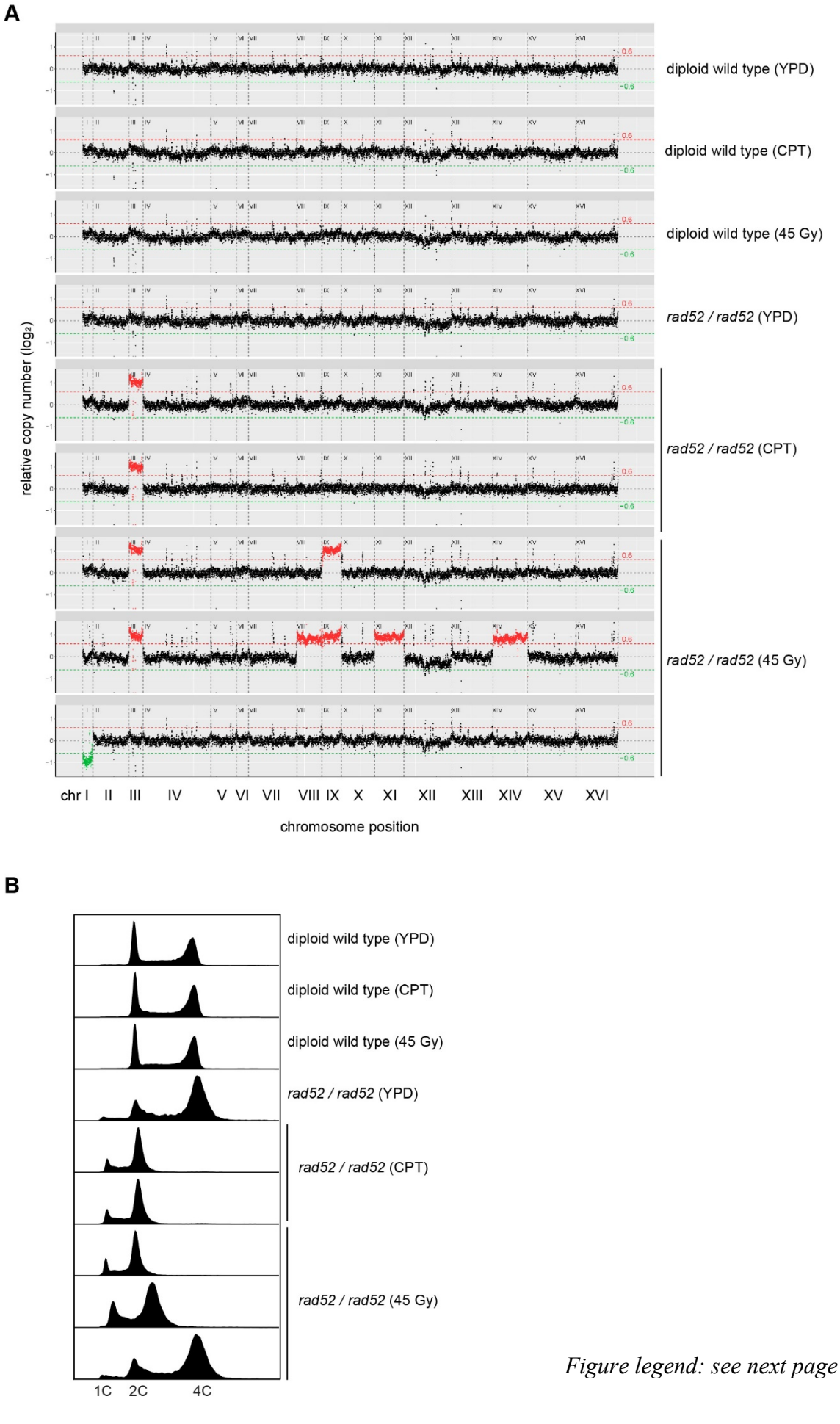

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Figure 15. Analysis of the aneuploidy observed in *rad52* mutants after genotoxic treatment using Whole Genome Sequencing (WGS). (A) Representative segmentation plots depicting chromosome copy number variation in *rad52* mutants derived from either control plates (denoted as (YPD)) or after genotoxic treatment (denoted as (CPT) or (45 Gy)). The segmentation plot depicts the $\log_2$ ratio of binned reads (reads per million (RPM), y-axis) for the respective chromosome position (x-axis) normalized to the sample median number of reads per bin (RPM). Copy numbers above (0.6) or below (-0.6) threshold are coloured in red for chromosome retention and in green for chromosome loss. For more details about normalization, see Materials and Methods section 4.2.10. **(B)** DNA content analysis of the strains displayed in (A). This figure displays a subset of strains used for Figure 20. Bioinfomatical analysis in Panel **(A)** has been done by Anke Busch.

reveals a highly unstable DNA profile with almost indistinguishable peaks reflecting severely damaged genetic material lacking the organization into non-replicated (2C) and replicated (4C) DNA content. However, already after the first round of growth on rich medium (second row of every histogram stack), *rad52* mutants had overcome this unstable karyotype.

We then went on to test *rad52* resistant cells derived from X-ray irradiation. As shown before (Figure 14 A and B and Figure 15), *rad52* mutants resistant to X-ray treatment displayed a variety of ploidy states. We found that *rad52* mutants exhibiting an intermediate ploidy continued to lose chromosomes if further passaged. Of the six X-ray-resistant *rad52* mutants analysed in this experiment, two mutants revealed an intermediate ploidy while the other four mutants still exhibited a diploid DNA content (Figure 16 and data not shown). Interestingly, after three passages on YPD plates the two independent *rad52* mutants with an initially intermediate-type of ploidy eventually displayed a near-haploid karyotype (Figure 16 B and data not shown). This suggests that HR-defective *rad52* mutants continued to lose chromosomes presumably to reach a more stable karyotype, i.e. either a haploid or diploid DNA content, to avoid the negative impact of an unbalanced karyotype.

Our results from analysing CPT resistant *rad52* cells suggested that a (near-) haploid karyotype might be beneficial for the growth in the presence of CPT damage. Likewise, *rad52* mutants with an intermediate karyotype between haploid and diploid after X-ray exposure also continued to lose genetic material to reach a more haploid-like DNA content (Figure 16 B). To test the idea if a haploid karyotype could confer a growth advantage in the presence of CPT or after X-ray irradiation, we compared the growth of diploid and haploid repair-defective *rad52* mutants including near-haploid *rad52* mutants after genotoxic treatment. Interestingly, both initially haploid *rad52* mutants and near-haploid mutants resulting from either CPT treatment (Figure 16 C and D) or X-ray exposure (Figure 16 E and F) showed a growth advantage when challenged again with the same genotoxic agent. This suggests that a haploid DNA content allows repair-defective *rad52* mutants to grow under DNA damaging conditions, possibly by enabling alternative DNA repair mechanisms like NHEJ which is suppressed in diploid yeast cells (reviewed in (Astrom et al., 1999)).

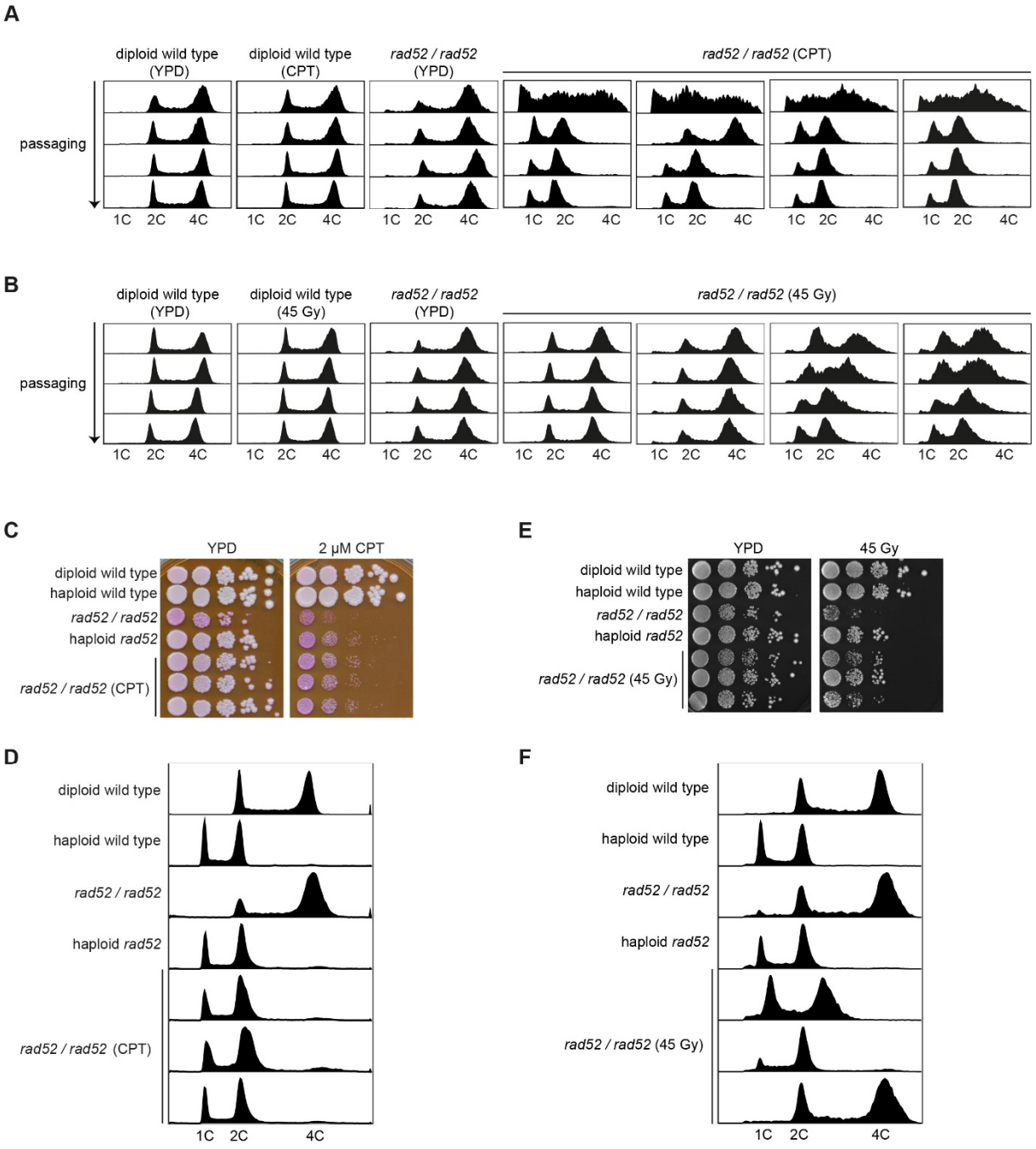

Figure 16. Chromosome loss occurs over time in *rad52* mutants after genotoxic treatment.
(A) Yeast strains with the indicated genotypes were either plated onto YPD agar plates as controls or plated onto YPD agar plates containing 2 µM CPT. After colonies had formed, individual clones were picked and passaged on YPD agar plates. After every round of passage, clones were subjected to DNA content analysis by flow cytometry. **(B)** Experiment was performed as described in (A) but with 45 Gy of X-ray as the DNA damaging agent. **(C)** Diploid and haploid strains of the indicated genotypes were spotted in serial dilutions on either control YPD agar plates or on YPD agar plates containing 2 µM CPT. **(D)** DNA content analysis of the strains used in (C). **(E)** Experiment was performed analogously to (C) but using 45 Gy of X-ray as genotoxic treatment. **(F)** DNA content analysis corresponding to the yeast strains used in (E). Experiments shown in Panel **(C)** and **(D)** were performed by Julia Klermund.

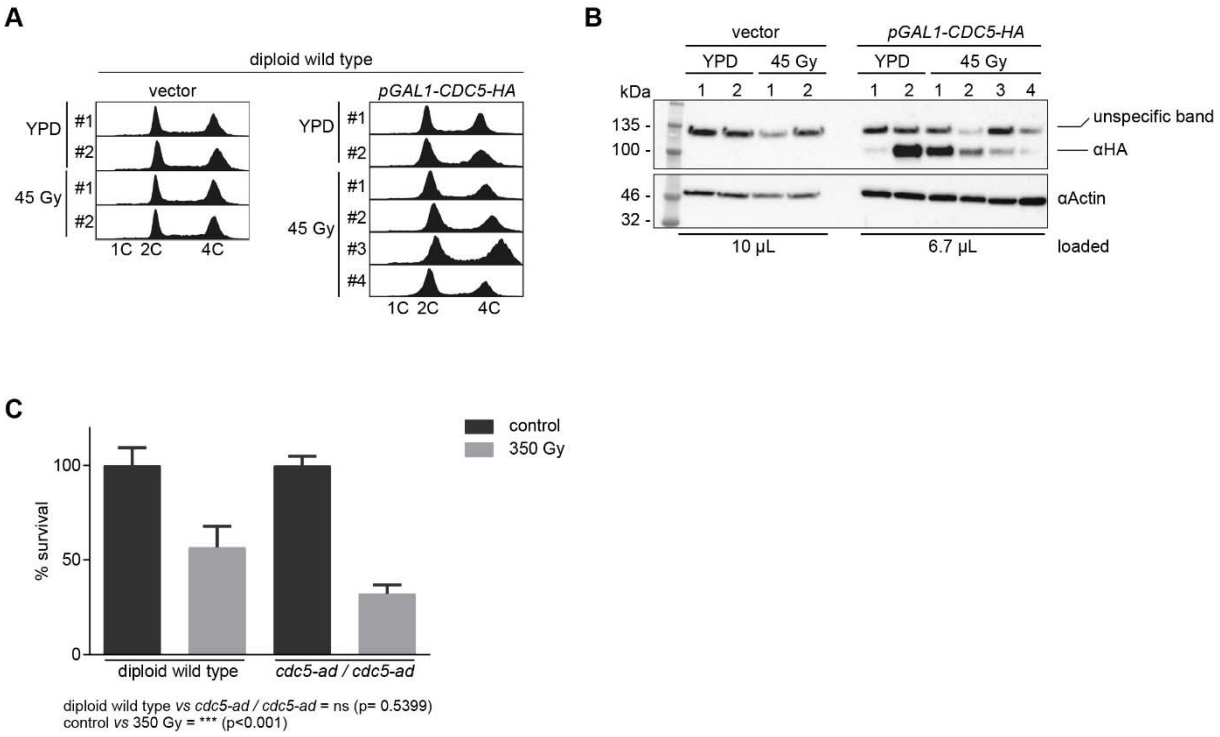

Figure 17. Role of Cdc5 protein levels and adaptation in repair-proficient cells. (A) DNA content analysis of diploid wild type cells transformed either with an empty vector control or a galactose-inducible overexpression plasmid for Cdc5-HA (*pGAL1-CDC5-HA*). **(B)** Western Blot to visualize Cdc5-HA expression levels. Total protein lysates of the strains used in (A) were used and membranes were probed with anti-HA antibody to detect Cdc5-HA fusion protein or anti-actin antibody as a loading control. Of note, only 2/3 of the lysate volume of samples prepared from cells overexpressing Cdc5-HA were loaded compared to control samples. **(C)** Quantification of cell survival determined by plating assays using diploid wild type and adaptation-deficient diploid *cdc5-ad* cells after exposure to 350 Gy of X-rays. Data are represented as mean values and the error bars indicate the SEM of 3 independent experiments. Statistical analysis was performed using a two-tailed unpaired t-test summarizing groups either depending on the genotype or on the treatment. P-value comparing diploid wild type *vs cdc5-ad / cdc5-ad* is ns (p=0.5399), p-value comparing control *vs* 350 Gy treatment is *** ($p \leq 0.001$).

2.9 High levels of Cdc5 do not cause aneuploidy in repair-proficient cells

Cdc5 has been shown to act in a dose-dependent manner to drive checkpoint adaptation (Vidanes et al., 2010) suggesting that the levels of Cdc5 could play an important role in the acquisition of genotoxin resistance and aneuploidy. We hypothesized that if elevated levels of Cdc5 protein drove cells into aneuploidy, we could detect chromosome loss events even in a repair-proficient background. To test this idea, we overexpressed a Cdc5-HA fusion protein under a galactose-inducible promoter from a high copy plasmid (p-Gal1-Cdc5-HA, Figure 17 A). We chose X-ray as a genotoxic treatment since repair-defective *rad52* mutants displayed a variable response in terms of their DNA content to this treatment (Figure 14 and 15). Upon overexpression of Cdc5 we could not detect chromosome loss events indicating an aneuploid karyotype using flow cytometry-based DNA content analysis. However, due to limitations in flow cytometry sensitivity, we cannot exclude single chromosome loss events in this experimental set-up. However, our results indicated that high levels of the adaptation-

promoting Cdc5 were not sufficient to drive wild-type cells into aneuploidy affecting multiple chromosomes after a single dose of X-ray treatment. Importantly, when we checked the expression levels of the Cdc5-HA fusion protein after galactose-mediated induction, we found drastically different protein levels (Figure 17 B) suggesting that Cdc5 might be tightly regulated at the protein level. However, also a highly overexpressing clone (clone #1 after 45 Gy) did not display an aneuploid karyotype. Of note, pro-longed overproduction of Cdc5 was very toxic (data not shown) which is consistent with previous results reporting aberrant cytokinesis and multinucleated cells after Cdc5 overexpression (Bartholomew et al., 2001; Song et al., 2000).
As we could show, preventing checkpoint adaptation sensitizes repair-deficient cells to genotoxin treatment (Figure 12). We were then curious to see if inhibition of adaptation would also sensitize repair-proficient cells to DNA damage. In order to test this idea, we treated adaptation-proficient diploid wild-type cells and adaptation-deficient *cdc5-ad* diploids with a high dose of X-ray (350 Gy) that was sufficient to impair wild-type viability (decrease approximately 50 %). As shown in Figure 17 C, preventing adaptation in such a setting did not increase the sensitivity of adaptation-deficient *cdc5-ad* mutants to X-ray induced DNA damage compared to adaptation-proficient cells. In agreement with our results shown in Figure 12 C, this observation indicates that repair-proficient cells do not experience a growth disadvantage upon preventing adaptation. In contrast, repair-deficient cells (in a *rad52* background) were sensitized to genotoxins by the inhibition of adaptation thereby presenting a way to specifically target them.

2.10 NHEJ is not responsible for drug resistance in *rad52* mutants

The Rad52 protein possesses an annealing activity used for strand invasion during homologous recombination and is required for Rad51 loading onto RPA-coated ssDNA (reviewed in (Krejci et al., 2012)) in order to undergo homology-directed repair events. Therefore, we questioned how *rad52* mutants could form colonies after treatment with either CPT or X-rays. An alternative repair pathway for DSBs is provided by non-homologous end joining (NHEJ). However, NHEJ is normally inhibited in diploid cells due to the repression of the NHEJ genes *NEJ1* and *LIF2* by the a1-α2 repressor encoded by the heterozygous *MATa/MATα* locus also present in our *rad52* mutants (Frank-Vaillant and Marcand, 2001; Kegel et al., 2001; Valencia et al., 2001). To test if NHEJ was occurring and thereby allowing colony formation in *rad52* mutants in DNA damaging conditions, we used *rad52* diploids lacking the crucial NHEJ factor *DNL4* (DNA Ligase IV, (Wilson et al., 1997)). As shown in Figure 18 A and B, mutants defective for both Rad52-mediated HR and NHEJ (*rad52dnl4* double mutants) were able to survive treatment with either CPT or irradiation with 45 Gy of X-rays and formed colonies. Of note, we found a slight but significant decrease in cell survival in *rad52dnl4* double mutants compared to *rad52* single mutants following irradiation (Figure 18 B). This indicates that re-activation of Dnl4-dependent NHEJ in *rad52* mutants most likely did not account for cell survival and the subsequent acquisition of genotoxic resistance accompanied by an aneuploid karyotype.

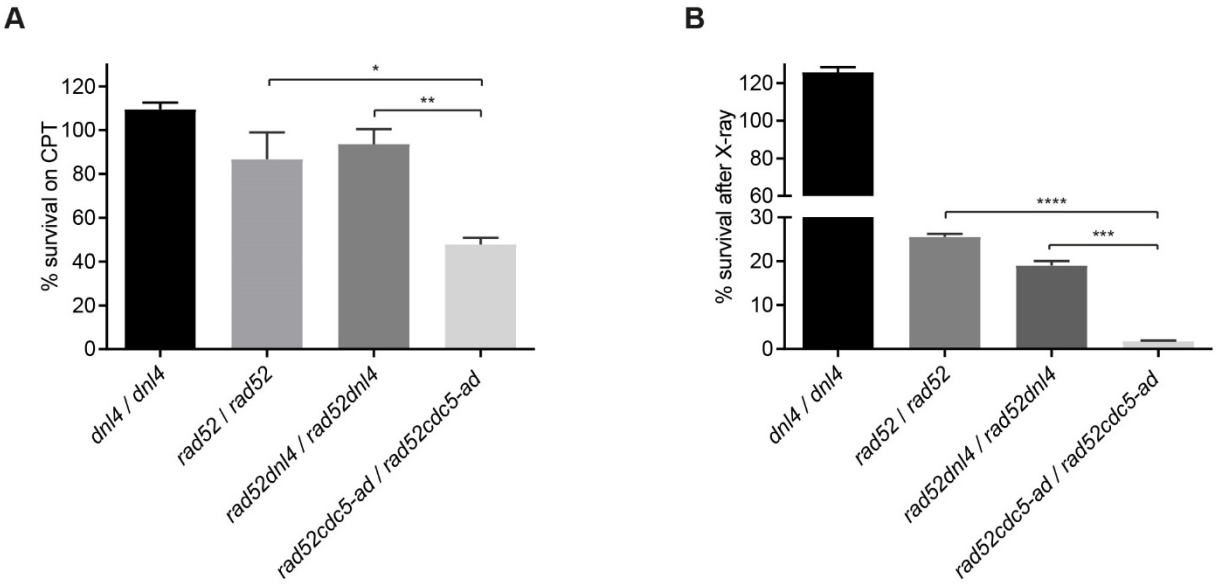

Figure 18. The role of NHEJ in the resistance of HR-defective *rad52* mutants to genotoxic treatment. (A) - (B) Quantification of plating assays using yeast strains with the indicated genotypes in response to either CPT treatment (A) or after X-ray exposure (45 Gy) (B). Data are represented as mean values and the error bars indicate the SEM of 3 independent experiments. Statistical analysis was performed using one-way ANOVA with * as $p \leq 0.05$, ** as $p \leq 0.01$, *** as $p \leq 0.001$ and **** as $p \leq 0.0001$.

2.11 Aneuploid and resistant *rad52* mutants harbour chronic DNA damage

We hypothesized that *rad52* mutants acquire resistance to genotoxins and become aneuploid as a consequence of Cdc5-mediated checkpoint adaptation. Therefore, adapted and resistant *rad52* mutants should still suffer from DNA damage due to their repair defect.

To test this idea, we subjected *rad52* mutants derived from either CPT or X-ray treatment to Western Blot and DNA content analysis after they had grown on rich medium without further genotoxic insults (Figure 19). All resistant *rad52* mutants tested showed slight Rad53 phosphorylation, indicating that even under unchallenged conditions the DNA damage checkpoint was activated. We confirmed this observation by analysing the protein levels of Rnr3. Rnr3 is upregulated in response to DNA damage (Huang et al., 1998) and all resistant *rad52* mutants tested showed increased Rnr3 protein levels after growth on rich medium under non-damaging conditions (Figure 19 A and B). Importantly, treatment with the replication inhibitory drug hydroxyurea revealed that *rad52* mutants were still checkpoint proficient in terms of Rad53 phosphorylation, Rnr3 induction (Figure 19 A and B), and cell cycle arrest in S phase (Figure 19 C and D). Taken together, after genotoxic insult, HR-defective *rad52* mutants proliferate although they display signs of chronic DNA damage and are checkpoint-proficient. The finding that *rad52* mutants divide and form colonies in the presence of cellular DNA damage even though bearing a functional DNA damage checkpoint suggests that checkpoint adaptation plays an important role in the acquisition of resistance to genotoxic treatment and the development of aneuploidy.

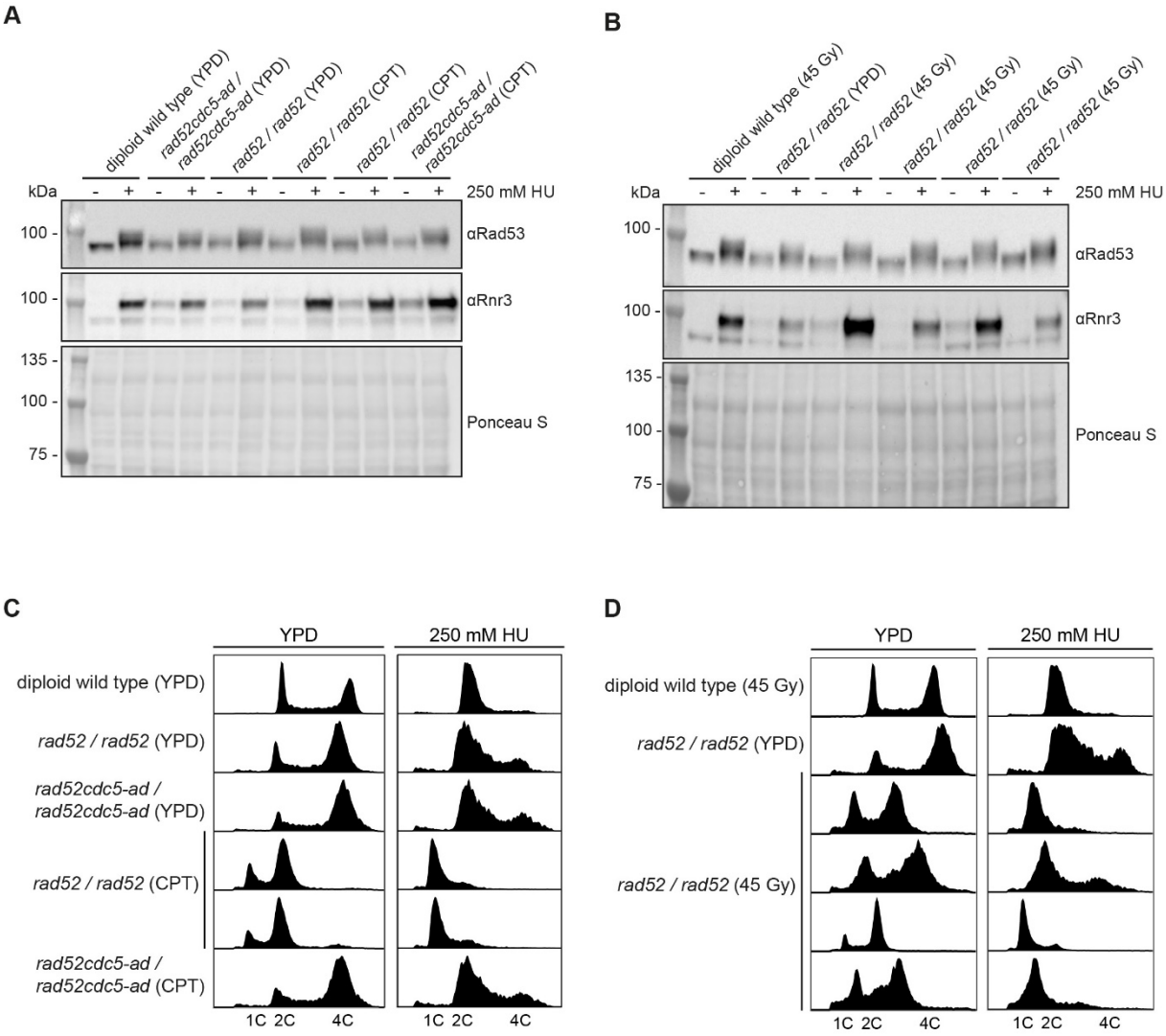

Figure 19. Resistant and aneuploid *rad52* mutants harbour DNA damage and are checkpoint-proficient. (A) Total protein lysates from the indicated strains were prepared after growth in the presence of 250 mM hydroxyurea (HU) or under control conditions. Yeast strains were derived from either control YPD agar plates (denoted as (YPD)) or from plates containing 2 µM CPT (denoted as (CPT)). Total cell lysates were separated on 7.5% polyacrylamide gels and membranes were probed first with an antibody specific for Rad53. Subsequently, the membrane was re-probed using an anti-Rnr3 antibody and the signal was detected by chemiluminescence. **(B)** Experiment was performed as described in (A) but yeast strains were derived from either control YPD agar plates or after exposure to 45 Gy of X-ray (denoted as (45 Gy)). **(C)** DNA content analysis of the strains used in (A). **(D)** DNA content analysis of the strains used in (B).

2.12 Transcriptome analysis of *rad52* mutants after genotoxic treatment

Aneuploidy has been studied extensively in multiple organisms including budding yeast and higher eukaryotes such as plants, mice and humans and it has been found that all aneuploid cells exhibit common characteristics collectively termed the aneuploidy-associated phenotype (reviewed in (Tang and Amon, 2013)). These shared characteristics include altered gene expression profile of genes involved in metabolic processes and protein folding along with increased levels of proteotoxic stress. In line with this, aneuploid cells are sensitive to elevated temperatures and compounds interfering with protein folding as well as agents inflicting energy

stress. In agreement with an altered and imbalanced karyotype, aneuploid cells also display genomic instability and increased levels of DNA damage accompanied by a high variability of aneuploid cells in response to various stimuli (reviewed in (Siegel and Amon, 2012)). In our system, creation of aneuploid cells appears to be random by increasing the frequency of chromosome loss in HR-defective *rad52* diploid cells, as it has been reported previously (Mortimer et al., 1981). This is in contrast to the very controlled creation of aneuploid cells in most studies, especially those using yeast as a model organism. In these studies that were performed mostly in the Amon lab, disomic yeast strains have been employed to investigate the effects of aberrant chromosome numbers on gene expression and cellular physiology. In these instances, the aneuploid cells carried only one or few extra chromosomes, rendering them disomic for an individual chromosome or a small set of chromosomes (for example (Oromendia et al., 2012; Torres et al., 2007)). However, the degree of aneuploidy observed in these disomic yeast strains differs in some cases drastically from the degree of aneuploidy studied in our experimental set-up (Figure 14 and 15). Furthermore, in our study aneuploid cells were created using DNA damage as a driving force and probably simultaneously as a selection marker while others have created aneuploids using less invasive methods. Therefore, we set out to investigate the gene expression profiles in *rad52* mutants after genotoxic treatment. To do so, total RNA was extracted from yeast strains that were subjected to DNA sequencing to assess their chromosome copy numbers (compare Figure 15). After depletion of ribosomal RNA, mRNA expression levels were analysed thereby taking into account the copy number of the respective chromosome harbouring the coding gene (compare Figure 15). Gene expression patterns of *rad52* mutants that had become resistant to either CPT treatment or X-ray treatment are summarized in Figure 13. We first analysed *rad52* mutants that had become resistant to CPT treatment and underwent extensive chromosome loss rendering them in a near-haploid state (compare Figure 14 and 15). Comparing CPT-resistant *rad52* mutants to the parental (and still diploid) *rad52* strain revealed 119 genes that were significantly expressed (Figure 20 A, column 1). We sorted this set of genes based on their fold-change expression ($\log_2$ of their fold change, see legend Figure 20 A) and analysed the expression levels of the same set of 119 genes when comparing X-ray-resistant *rad52* mutants and their parental diploid *rad52* mutants (Figure 20 A, column 2). Although there were only 4 genes whose expression changed significantly after gaining X-ray resistance and only 2 of them were contained in the set of genes identified in CPT resistant *rad52* mutants, we observed a similar trend in the up- and downregulation of genes in response to CPT and X-ray treatment (compare color-coded differences in gene expression in column 1 *versus* 2, Figure 20 A). This indicates that genotoxic treatment of *rad52* cells leads to a fairly uniform transcriptional response. We then also compared the list of genes found altered in CPT-resistant *rad52* mutants (119 genes shown in column 1, Figure 20 A) to the genes that are differentially expressed when comparing the parental and diploid *rad52* strain (derived from control plates, denoted by (YPD)) to an untreated diploid wild type strain (denoted by (YPD)) (column 3, Figure 20 A). Interestingly, many of the genes significantly up- or downregulated in CPT-resistant cells (column 1, Figure 20 A) were also significantly changed in their expression upon loss of *RAD52* even in unchallenged conditions (column 3, Figure 20 A) suggesting that already loss of the *RAD52* gene product, which renders the cells deficient for homologous recombination, causes a dramatic change in gene expression. In order

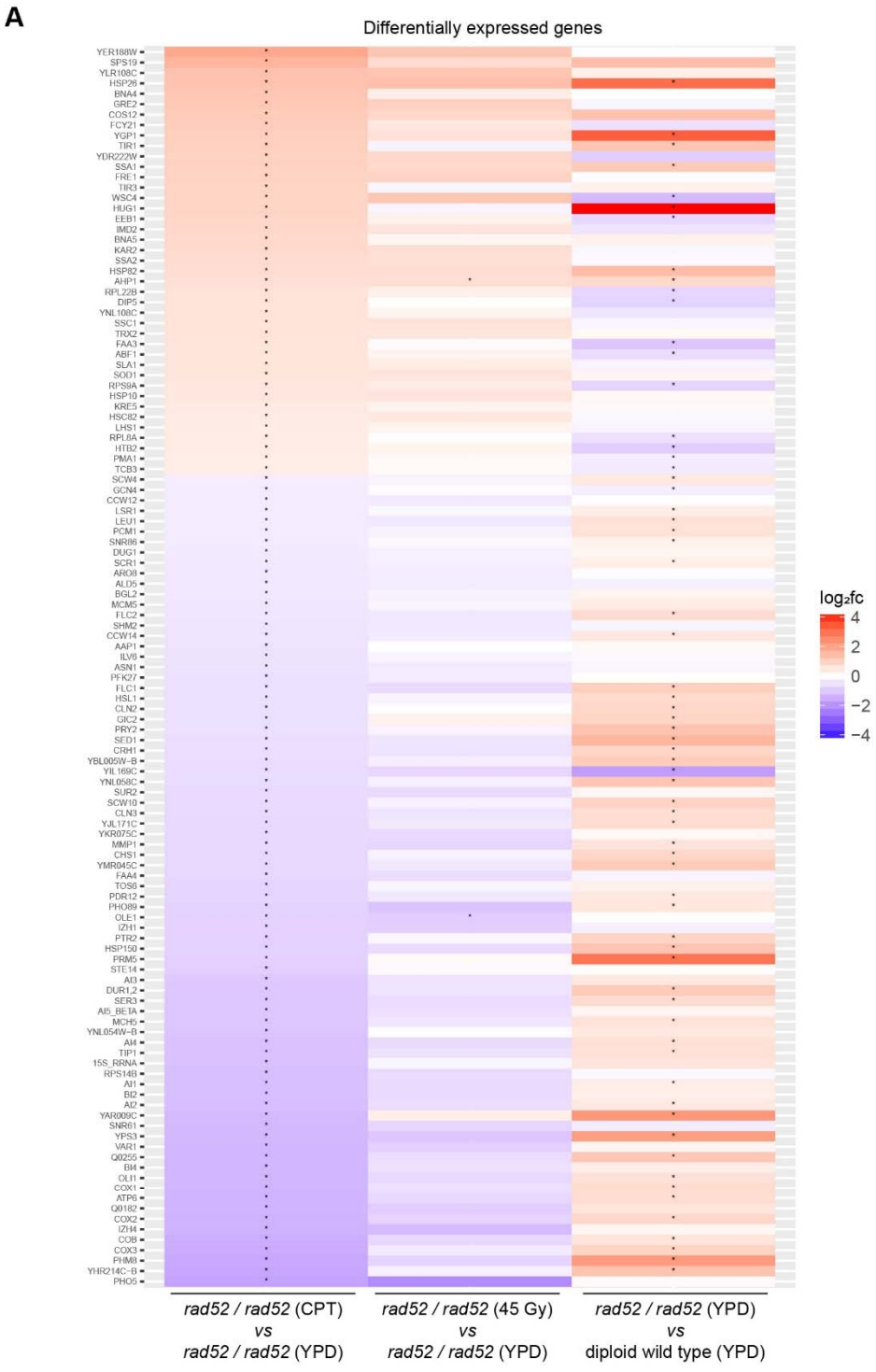

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Figure 20. Transcriptional response of *rad52* mutants after genotoxic treatment shows an aneuploidy-associated gene expression pattern. (A) RNA sequencing results are summarized as a heatmap showing all genes (119 in total) with a significantly differential expression (based on FDR, marked with an asterisk *) comparing *rad52* mutants derived after CPT treatment and *rad52* mutants grown on control plates (YPD) (first column). The expression patterns of the differentially expressed genes displayed in the first column are also plotted for the comparison of *rad52* mutants derived after X-ray treatment (denoted as (45 Gy)) *versus rad52* mutants grown on control (YPD) plates (second column) and for the comparison of *rad52* mutants grown on control (YPD) plates *versus* wild type cells grown on control (YPD) plates (third column). The magnitude of expression differences (log_2 fold change) is color-coded as displayed in the key. Differential gene expression analysis was corrected for chromosome copy numbers. **(B)** Simplified GO term analysis showing the biological processes found enriched in the list of up-regulated genes that were identified when comparing the gene expression pattern in *rad52* mutants derived after CPT treatment *versus rad52* mutants grown on control (YPD) plates. The adjusted p-value is color-coded as displayed in the key. Bioinformatical analysis has been performed by Anke Busch.

to investigate in more detail which genes are differentially expressed when cells acquire resistance to CPT and to get insights into which cellular pathways might facilitate such drug resistance, we used the set of genes found upregulated in CPT-resistant *rad52* mutants in Figure 20 A (column 1) and performed a gene ontology (GO) term enrichment analysis (Figure 20 B). We summarized the top 10 enriched GO terms for biological processes into more broad categories, as displayed in Figure 20 B. All GO terms found enriched were associated with protein folding, refolding or localization to the endoplasmic reticulum, a cellular compartment strongly involved in protein quality control (McCaffrey and Braakman, 2016) (Figure 20 B).
Taken together, this analysis reveals the upregulation of genes involved in protein quality control in aneuploid *rad52* mutants after the acquisition of CPT resistance suggesting that also aneuploid repair-defective mutants created with our experimental set-up exhibit common aneuploidy-associated phenotypes.

2.13 Resistance in *rad52* mutants is not due to slower growth

As outlined above, aneuploid cells display a characteristic aneuploidy-associated phenotype which includes slower cell cycle progression (Beach et al., 2017; Thorburn et al., 2013). Moreover, HR-defective *rad52* mutants exhibit a delayed growth rate compared to wild type cells. This cell cycle delay could be beneficial in the presence of CPT which is toxic in dividing cells due to its mode of action and eventually allow the growth of *rad52* mutants. In order to rule this out, we assessed the emergence of colonies in the presence of CPT at different temperatures. Diploid wild type, *rad52* and *rad52cdc5-ad* cells were spotted on CPT-containing agar plates and colony formation was analysed after growth at low (room temperature, 23°C and 25°C), medium (27°C) and normal (30°C) incubation temperature. As seen in Figure 21, incubation at lower temperatures to slow down the vegetative growth rate did not influence survival in the presence of CPT. In summary with the observation that CPT is still active after prolonged incubation at 30°C (data not shown) this suggests that CPT resistance observed in *rad52* mutants is not due to CPT inactivation and the slow growth phenotype associated with aneuploidy or the repair defect of *rad52* cells.

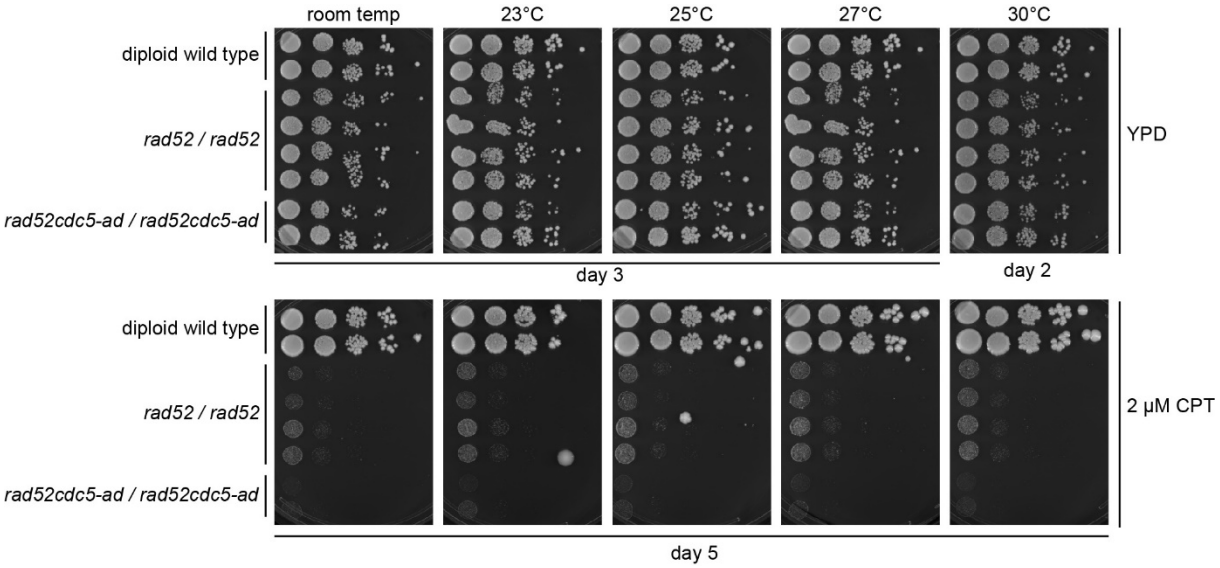
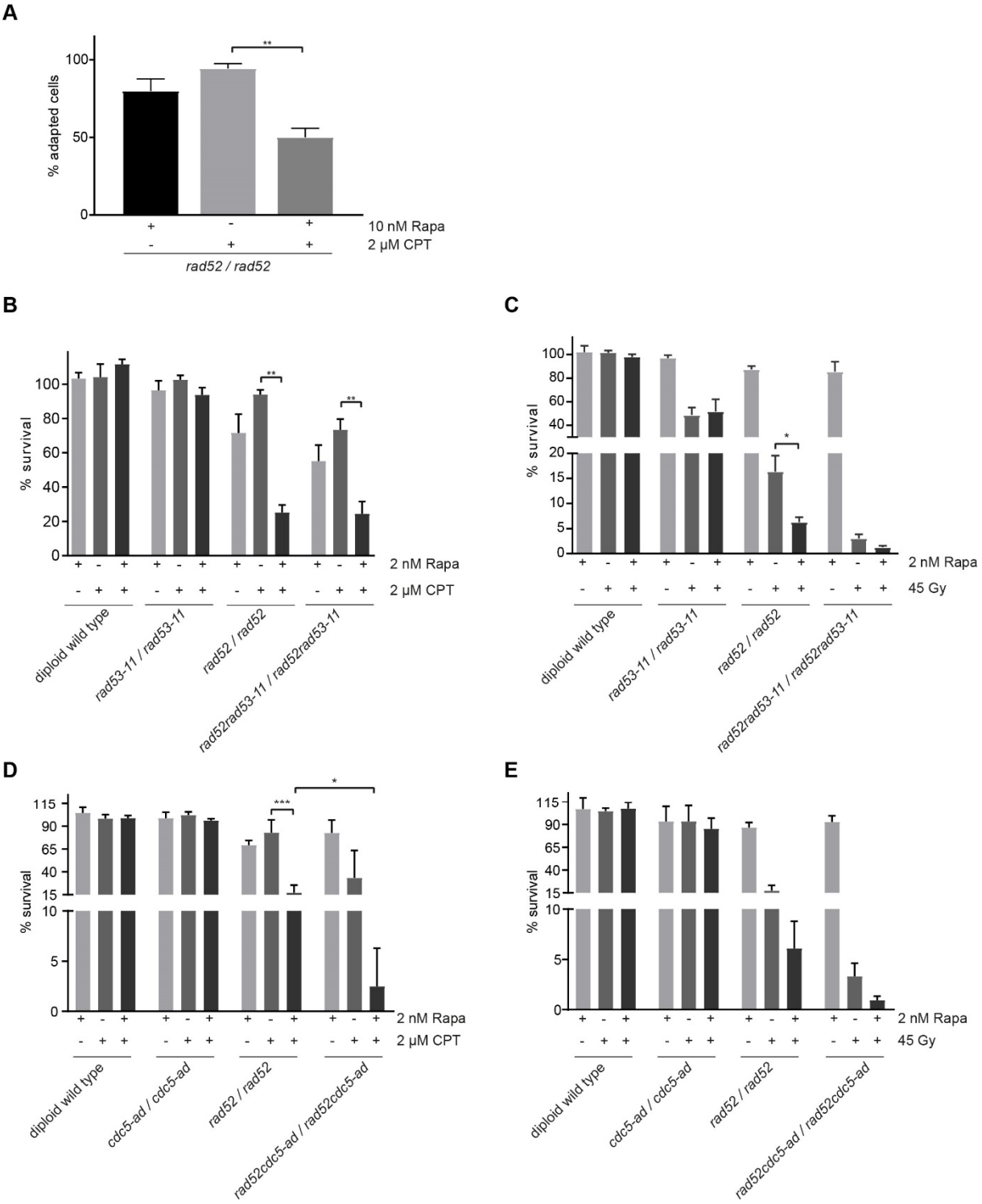


Figure 21. Slower growth does not affect the emergence of CPT resistance in *rad52* mutants. Yeast strains of the indicated genotypes were spotted in serial dilutions on control YPD agar plates or plates containing 2 µM CPT. Afterwards, plates were incubated at the indicated temperatures and pictures were taken after the indicated times.

2.14 Rapamycin sensitizes *rad52* diploids to genotoxic treatment

We have previously provided a link between the cellular nutritional status sensed by the TORC1 pathway and checkpoint adaptation (Klermund et al., 2014). We then wondered if rapamycin could phenocopy the effect of the *cdc5-ad* allele in preventing checkpoint adaptation in repair-defective *rad52* diploids in the presence of CPT. In order to test this, we performed microcolony assays to follow cell cycle progression based on cell morphology (Figure 22 A). Unbudded or small budded *rad52* diploids were micromanipulated onto agar plates containing either rapamycin, CPT or the combination of both. After 4 hours, CPT-induced cell cycle arrest in G2/M phase was confirmed microscopically by the presence of dumbbell-shaped cells (counted as 2 cell bodies, according to (Toczyski et al., 1997)). Of note, a fraction of cells arrested with 4 cell bodies, presumably since they were progressing through S phase and therefore only arrested in the next round of the cell cycle. In contrast, cells incubated on rapamycin-containing plates progressed through the cell cycle in an unperturbed manner. After 24 hours, cell morphology and microcolony formation were assessed again (Figure 22 A). Cells incubated in the presence of CPT were counted as adapted if they had progressed past the dumbbell arrest point, i.e. if the microcolony contained 3, 5 or more than 5 cells. Cells that failed to arrest as dumbbells (for CPT) or were still unbudded (for rapamycin only plates) at the 4 hour time point were excluded from the analysis. As shown in Figure 22 A, more than 90% of HR-defective *rad52* mutants had undergone adaptation and formed microcolonies in the presence of CPT. Rapamycin was able to decrease the occurrence of adaptation approximately two-fold. Indeed, 50% of *rad52* diploids grown in the presence of rapamycin and CPT were still arrested with a dumbbell morphology indicating G2/M arrest. Of note, rapamycin treatment alone decreased adaptation to approximately 80% (Figure 22 A). This could be due to endogenous damage



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occurring in repair-defective cells and suggests an inhibitory role for rapamycin on checkpoint adaptation also under physiological conditions in the absence of genotoxins.

Based on our observations indicating that rapamycin prevents checkpoint adaptation in *rad52* diploids in response to CPT treatment, we wondered if the protective effect of rapamycin required a functional Rad53-mediated DNA damage checkpoint. As shown in Figure 22 B, checkpoint-defective *rad52rad53-11* cells showed decreased survival upon CPT treatment in the presence of rapamycin compared to *rad52rad53-11* cells plated on CPT alone. Although

Figure 22. Rapamycin sensitizes *rad52* mutants to genotoxic treatment in a manner distinct from the *cdc5-ad* allele. (A) Microcolony assay to assess checkpoint adaptation to CPT-induced damage in *rad52* diploids. Unbudded or small-budded cells were manipulated onto agar plates containing either 10 nM rapamycin (provided by a new supplier, the concentration had to be increased to observe the same effect), 2 μM CPT or the combination of both drugs. Cell morphology and microcolony formation were assessed after 4 and 24 hours at 30°C. For details about quantification, see section 4.2.8. The mean of 3 biological replicates is shown and error bars depict the SEM. Statistical analysis was performed using a One-Way ANOVA with ** as $p \leq 0.01$. **(B)** Quantification of cell survival in the presence of rapamycin and/ or CPT. Yeast strains of the indicated genotypes were plated onto YPD agar plates containing 2 nM rapamycin, 2 μM CPT or the combination of rapamycin and CPT. **(C)** Quantification of plating assays using strains of the indicated genotypes after growth on YPD agar plates containing 2 nM rapamycin, after X-ray exposure (45 Gy) or exposure to X-ray in the presence of rapamycin. **(D)** Quantification of plating assays performed as described in (B) using strains of the indicated genotypes. **(E)** Quantification of cell survival using plating assays as described in (C) with yeast strains of the indicated genotypes. Data are represented as mean values and the error bars indicate the SEM of 3 (or 4 in (C)) independent experiments. Statistical analysis was performed using Two-way ANOVA and One-Way ANOVA with * as $p \leq 0.05$, ** as $p \leq 0.01$, *** as $p \leq 0.001$.

rapamycin can prevent checkpoint-adaptation (Figure 22 A), this result suggests a checkpoint- and adaptation- independent function of rapamycin in decreasing the emergence of CPT-resistant *rad52* mutants. We then tested if the rapamycin-mediated effect was also checkpoint-independent in response to X-ray irradiation. To address this question, we irradiated checkpoint-proficient *rad52* diploids and checkpoint-deficient *rad52rad53-11* diploids with 45 Gy in the presence or absence of rapamycin (Figure 22 C). Rapamycin was still able to reduce the survival of *rad52* mutants after irradiation approximately two-fold in the absence of a functional Rad53-mediated checkpoint, however, this decrease was not statistically significant. In summary, these observations are consistent with the notion that TORC1 inhibition by rapamycin induces autophagy (Kamada et al., 2010) and this has been suggested to maintain cell cycle arrest independently of Rad53 in response to DNA damage (Dotiwala et al., 2013). Alternatively, these results could reflect adaptation-independent consequences of rapamycin treatment. Since rapamycin displays a checkpoint-independent effect in preventing genotoxic resistance in *rad52* diploids (Figure 22 B and C) but the *cdc5-ad* allele requires the DNA damage checkpoint (Figure 12 F and G), we tested if both means to prevent resistance would act in a synergistic manner. To address this question, we challenged adaptation-proficient *rad52* cells and adaptation-deficient *rad52cdc5-ad* cells with genotoxic treatment in the presence and absence of rapamycin (Figure 22 D and E). We found that rapamycin was able to further decrease survival in *rad52cdc5-ad* diploids compared to *rad52* diploids upon treatment with CPT (Figure 22 D). We then went on to repeat the experiment using X-ray as a genotoxic treatment. As seen in Figure 22 E, rapamycin was able to further decrease the survival of *rad52cdc5-ad* mutants compared to *rad52* cells. However, this decrease indicating a synergistic effect of rapamycin and the *cdc5-ad* allele in response to X-rays was not statistically significant, presumably since we reached the detection or sensitivity limit of our plating assay. In summary, our analysis of the combined effects of rapamycin and the *cdc5-ad* allele suggest a synergistic effect in decreasing the frequency of resistant and aneuploid repair-defective cells after genotoxic treatment.

3 Discussion

In the work presented here, we have employed two different systems to elucidate the regulation and consequences of checkpoint adaptation. In the first part, we made use of the *cdc13-1* system to elicit DNA damage arising from dysfunctional telomeres upon shifting yeast cells to the non-permissive temperature. Importantly, when *cdc13-1* mutants are shifted back to the permissive temperature, telomere capping is restored and cells can recover from DNA damage. In this experimental set-up, we were able to test the influence of different candidate mutations on the regulation of adaptation in repair-proficient cells.
Using *cdc13-1* mutants, we found that checkpoint adaptation in response to telomere dysfunction (repairable damage) can be prevented by inhibition of TORC1 signalling. In addition, we and others could show that an allele of Cdc5, which is specifically defective for adaptation, preserves cell viability upon prolonged telomere dysfunction. Moreover, our results not only imply an involvement of the Sch9 signalling pathway downstream of TORC1 (Klermund et al., 2014) but also point at a regulatory role of the TORC1 effector Tap42-PP2A during adaptation.

In the second part of the thesis, we modelled irreparable DNA damage by deleting the crucial HR protein Rad52. This enabled us to study the effects of adaptation with respect to genome stability. We employed diploid cells and therefore, NHEJ as the second major DNA repair pathway is suppressed leaving cells only with HR for efficient repair. We found that HR-defective *rad52* mutants treated with different genotoxins were able to form viable colonies after long incubation periods suggesting that cells had acquired genotoxin resistance. Indeed, *rad52* mutants that were able to grow after the first genotoxic treatment had a growth advantage in a second genotoxic treatment confirming that they had become resistant. We show that colony formation is greatly decreased when adaptation is prevented, both genetically using the *cdc5-ad* allele and pharmacologically by treatment with the TORC1 inhibitor rapamycin. However, if adaptation was allowed to occur, *rad52* mutants experienced chromosome loss leading to an aneuploid karyotype of varying degrees. Importantly, this was accompanied by a common aneuploidy-associated phenotype and a well-characterized aneuploidy-induced transcriptional profile. These results provide insights into the relationship between adaptation, the acquisition of genotoxin resistance and an aneuploid karyotype.

3.1 Reasoning for adaptation in unicellular and multicellular organisms

DNA damage has to be detected in a rapid and reliable manner in order to prevent the inheritance of damaged genetic material by daughter cells. This is achieved by the activation of the DNA damage checkpoint, which halts the cell cycle and provides time for damage repair. Therefore, the cell cycle machinery and the DNA damage response are intimately linked. On the other hand, permanent cell cycle arrest due to continuous signalling by the DDC prevents cellular proliferation. Consequently, the physiological relevance to terminate the DDC seems straightforward and reasonable. However, the preservation of cellular viability due to checkpoint termination can come at the cost of lethal genetic alterations due to checkpoint inactivation without successful damage repair. Therefore, the decision between adaptation (i.e.

checkpoint termination despite the presence of DNA damage) and loss of viability due to terminal checkpoint arrest has to be balanced carefully. Nevertheless, unicellular organisms like budding yeast have developed a mechanism allowing them to attempt cell division by overcoming the checkpoint arrest and this has been termed (checkpoint) adaptation. The unicellular model organism *Saccharomyces cerevisiae* has provided a useful system leading to the discovery, and enabling the initial characterization, of adaptation (Sandell and Zakian, 1993; Toczyski et al., 1997). The decision to undergo adaptation seems to be a common response to irreparable damage as it has been described to occur after damage arising from telomere dysfunction, X-ray irradiation and a nuclease-induced DSB (Galgoczy and Toczyski, 2001; Klermund et al., 2014; Sandell and Zakian, 1993; Toczyski et al., 1997). However, also in yeast cells, adaptation does not occur in all instances and the extent of DNA damage appears to play an important role since cells experiencing a single DSB will adapt but the introduction of two DSBs will arrest the cells permanently (Lee et al., 1998; Sandell and Zakian, 1993). In opposition to unicellular organisms, multicellular organisms including human cells seem to bear a higher risk associated with adaptation due to the propagation of potentially harmful genetic alterations that could globally affect organismal fitness. Therefore, adaptation was presumed to be absent in multicellular organisms. Surprisingly, adaptation has been shown to occur in higher eukaryotes: in *Xenopus* egg extracts in response to the replication stress inducing drug aphidicolin and in plant cells and human cancer cells in response to irradiation (Carballo et al., 2006; Syljuasen et al., 2006; Yoo et al., 2004). It has been proposed that adaptation in multicellular organisms could facilitate repair of the damage in a subsequent cell cycle. On the other hand, *in vitro* studies using cancer cells showed that most cells died in mitosis after undergoing adaptation, even though a small number of cells survived. Based on these observations, it has been proposed that cell death in G2 could be impossible or prevented by unknown mechanisms and therefore, cells would have to enter mitosis and die during mitotic catastrophe. In addition, senescence has also been postulated as a potential outcome of adaptation to prevent the proliferation of cells containing damaged genetic material (reviewed in (Roninson et al., 2001)). A third hypothesis has been put forward suggesting that cells have to enter the next G1 phase to undergo apoptosis or experience cell cycle arrest mediated by p53. Again, undergoing adaptation and entering the subsequent G1 phase could enable repair or facilitate cell death (reviewed in (Clemenson and Marsolier-Kergoat, 2009; Syljuasen, 2007)). Indeed, it had been observed that cancer cells underwent several more rounds of cell division after exposure to irradiation although containing damaged DNA (reviewed in (Kalsbeek and Golsteyn, 2017)). Importantly, this was also observed in response to treatment with pharmacological concentrations of CPT (Kubara et al., 2012). Also after etoposide and cisplatin treatment and in response to ionizing radiation, cancer cells were found to undergo adaptation (Swift and Golsteyn, 2016; Syljuasen et al., 2006).

However, little is known about the molecular mechanisms that facilitate adaptation. Studies in budding yeast have led to the identification of Cdc5, the single yeast homolog of the highly conserved Polo kinase family, as a critical factor for adaptation (Toczyski et al., 1997). Cdc5 plays multiple roles during an unperturbed cell cycle including the regulation of mitotic entry and exit and is also involved in the DNA damage response as a target of the DDC machinery. Despite this, the mechanisms leading to adaptation and the exact roles and targets or effectors

of Cdc5 remain to be identified. A critical step towards an understanding of adaptation is to identify how molecular targets of the DDC that are initially inactivated to achieve cell cycle arrest, can become re-activated to facilitate adaptation.
It is conceivable that the nutritional environment could be a determinant in the decision to undergo adaptation. If the cellular environment is nutrient-poor, cells overriding the cell cycle arrest would have to deal with both the presence of damaged DNA and a less favourable environment. Both caveats could have disadvantageous outcomes regarding cell growth and further division thereby making adaptation a poor choice in the absence of nutrients. On the other hand, it is reasonable that the presence of nutrients could be a driver for adaptation as the process allows the resumption of the cell cycle and could permit cell growth. We therefore propose that the nutritional status could serve as a switch to facilitate checkpoint adaptation and we suggest that there is crosstalk between nutrient signalling pathways and the DNA damage response to modulate the decision to undergo adaptation. The existence of such crosstalk between the glucose-sensory PKA pathway and the cell cycle machinery has been reported previously. However, in this case PKA activity was sustained after DNA damage to maintain the cell cycle arrest (Searle et al., 2004). Our attention was drawn to the TOR nutrient signalling pathway by a report showing that rapamycin treatment to inactivate the TOR complex 1 (TORC1) network rescued the viability of yeast mutants with dysfunctional telomeres as a trigger of DNA damage signalling (Qi et al., 2008). Prompted by these observations, we set out to investigate the link between nutrient signalling by TORC1 and adaptation and its impact on genome stability.

3.2 Adaptation in higher eukaryotes: First insights from cancer cells and *Xenopus*

The cellular response to DNA damage is highly conserved throughout eukaryotes. As a first line of defence, the cell cycle is arrested to facilitate repair and prevent the segregation of damaged DNA.
In human cells, the Cdc5 homolog, PLK1, has been shown to be a target of the DNA damage checkpoint mediated by ATM and ATR (van Vugt et al., 2001). DDC signalling is transferred via CHK1, which prevents PLK1 activating phosphorylation events by inhibiting the responsible kinase, Aurora A (Krystyniak et al., 2006). Moreover, ATR signalling leads to the proteasomal degradation of the Aurora A cofactor, Bora, thereby further decreasing PLK1 activity (Qin et al., 2013). In addition to targeting PLK1 directly, DDC activation also results in the inhibition of the CDC25 phosphatase family to inactivate Cyclin B-CDK1 and consequently, prevent entry into mitosis (reviewed in (van Vugt and Medema, 2005)). In order to promote mitotic entry after successful damage repair during checkpoint recovery, Aurora A in conjunction with Bora serves as the activating signal for PLK1 activity (Macurek et al., 2008). Interestingly, while cells recovering from DNA damage seem to be dependent on PLK1 to initiate mitosis, several redundant pathways can lead to the entry into mitosis in unperturbed conditions (van Vugt et al., 2004). In contrast, the mechanisms resulting in checkpoint adaptation in human cells are less well characterized. The interplay between PLK1 and CHK1 seems to be critical as CHK1 is dephosphorylated in adapted cells. However, it is unknown if CHK1 dephosphorylation is a cause or consequence of adaptation and PLK1 itself does not seem to phosphorylate and thereby inactivate CHK1 (Syljuasen et al., 2006). As an alternative

to one factor being upstream of the other to facilitate adaptation, PLK1 and CHK1 could also act in parallel on a common target. As such, CDK1 activity has been proposed and a model was suggested in which the CDK1- promoting activity of PLK1 outcompetes CDK1 inhibition by CHK1 (reviewed in (Syljuasen, 2007)). Indeed, PLK1 targets Wee1, an inhibitor of CDK1, for degradation (van Vugt et al., 2004), while CHK1 inhibits CDC25, a positive CDK1 regulator (reviewed in (van Vugt and Medema, 2005)). It has also been hypothesized that a mitotic regulator could accumulate over time in G2 to promote entry into mitosis. This would suggest a "counting mechanism" instead of an active decision-making process to be the underlying decision to undergo adaptation. Again, PLK1 has been proposed to function as a molecular timer based on observations from *Xenopus* experiments (Yoo et al., 2004). So far, however, experimental evidence is missing to support this hypothesis. Alternatively, Cyclin B levels have been suggested to be critical determinants in this scenario. This is supported by the findings that Cyclin B transcription transiently drop in response to irradiation (Muschel et al., 1992) but subsequently increase during prolonged checkpoint arrest (Maity et al., 1996). Moreover, overproduction of Cyclin B was shown to shorten the time cells spent arrested in G2 after damage (Kao et al., 1997).
Importantly, the majority of studies addressing adaptation in human cells has made use of cancer cells cultured *in vitro*. Nevertheless, it seems reasonable that adaptation also occurs under physiological conditions *in vivo*. This is supported by the notion that p53 deficiency, a common characteristic of human cancers, does not seem to be a prerequisite for adaptation (Syljuasen et al., 2006). In summary, the mechanistic details of adaptation in human cells are still elusive and more experimental work dedicated to the identification of the players involved has to be performed.

Studies using *Xenopus* egg extracts have contributed largely to the characterization of adaptation in higher eukaryotes. In response to replication stress, the *Xenopus* ATR homolog, Xatr, activates Xchk1 (*Xenopus* homolog of CHK1). These activating phosphorylation events are mediated by Claspin, a functional homolog of the yeast S-phase checkpoint mediator Mrc1 (Alcasabas et al., 2001). Like Claspin, yeast Mrc1 was found associated with stalled replication forks and is phosphorylated by Mec1, the yeast ATR homolog (Osborn and Elledge, 2003). In analogy to the checkpoint response described in human cells, activation of Xchk1 results in a cell cycle arrest. However, after prolonged checkpoint arrest in response to the replication stress-inducing drug aphidicolin, Plx1, the *Xenopus* PLK1 homolog, led to the inactivation of the checkpoint mediator Claspin (Yoo et al., 2004). After a priming phosphorylation of chromatin-bound Claspin by Xatr in response to a replication block, Plx1 can bind and phosphorylate Claspin thereby causing its release from chromatin. Consequently, continuous checkpoint-mediated activation of Xchk1 is abolished and entry into mitosis is allowed despite the persistence of replication stress (Yoo et al., 2004).
Prompted by this report demonstrating the involvement of a checkpoint mediator protein in the regulation of adaptation, also other mediators like human MDC1 or 53BP1 have been hypothesized to be targeted by PLK1 or other mitotic kinases to facilitate inactivation of the DDC and hence, allow cell cycle progression. In line with this hypothesis, human Rad9, homologous to yeast Rad17 and part of the 9-1-1 checkpoint clamp, is sequentially phosphorylated by CDK complexes and PLK1 during drug-induced replication stress (Wakida

et al., 2017). The authors suggested a regulation of the damage response via the CDK-PLK1 activity to allow cell cycle progression in the presence of low-level replication stress thereby maximizing cell proliferation. Interestingly, overexpression of the yeast PLK1 homolog, Cdc5, has been reported to interfere with the ability of the yeast checkpoint mediator Rad9 to promote Rad53 (mammalian CHK2) autophosphorylation and thereby could affect checkpoint maintenance (Vidanes et al., 2010). In conclusion, there seem to be many pathways for Polo kinases and their homologs to influence DDC signalling and it will be interesting to determine how the cellular targets are modulated by adaptation-promoting factors. The role of PLK1 is of special interest as it is commonly overexpressed in a variety of cancers (reviewed in (Liu et al., 2017b)).

3.3 Preventing adaptation in response to telomere dysfunction

3.3.1 General regulation of adaptation by rapamycin and Cdc5

3.3.1.1 Inhibition of adaptation by rapamycin

We could show previously that in response to telomere dysfunction, inhibition of TORC1 signalling using rapamycin can prevent checkpoint adaptation (Klermund et al., 2014). These findings implicate the TORC1 nutrient signalling pathway as an important regulator of checkpoint adaptation by coordinating extracellular cues such as nutrient availability with intracellular processes such as the DNA damage response.
Mechanistically, we could show in our previous study that loss of the TORC1 effector Sch9 rescues the viability of *cdc13-1* cells. Similarly to inhibiting TORC1 signalling by rapamycin treatment, *cdc13-1sch9* cells remained arrested in response to telomere dysfunction and maintained Rad53 in an activated state, albeit to a lesser extent (Klermund et al., 2014). The less pronounced effect observed by the deletion of *SCH9* compared to rapamycin can be explained by additional pathways and targets utilized by rapamycin to prevent adaptation (Figure 23). One of these pathways is the TORC1 downstream effector Tap42, best characterized for its regulatory function on the PP2A phosphatase (Di Como and Arndt, 1996; Jiang and Broach, 1999). Although the molecular details regarding how adaptation is prevented in the absence of fully functional Tap42 remain to be addressed, we speculate that PP2A plays an important role, possibly by regulating Cdc5 (see discussion below). Taken together, we hypothesize that TORC1 signalling mediated by Sch9, Tap42 and possibly other effectors converge to facilitate Cdc5 activity. Although Cdc5 is initially inhibited by the DDC in response to damage, we speculate that the nutritional status sensed by TORC1 provides a pro-adaptation signal and allows proliferation despite persistent DNA damage (summarized in Figure 23).

Initial studies that identified *cell division cycle* mutants including *cdc13-1* revealed cell lysis as the cause of cell death in response to prolonged cell cycle arrest (Hartwell et al., 1973). Studies by Qi and colleagues proposed an apoptosis-like cell death in *cdc13-1* cells after long-term incubation at the non-permissive temperature (Qi et al., 2008; Qi et al., 2003). In contrast, an apoptosis-like cell death occurring in *cdc13-1* mutants has been ruled out by others (Wysocki and Kron, 2004). In our live-cell imaging approach we also frequently observed cell lysis in

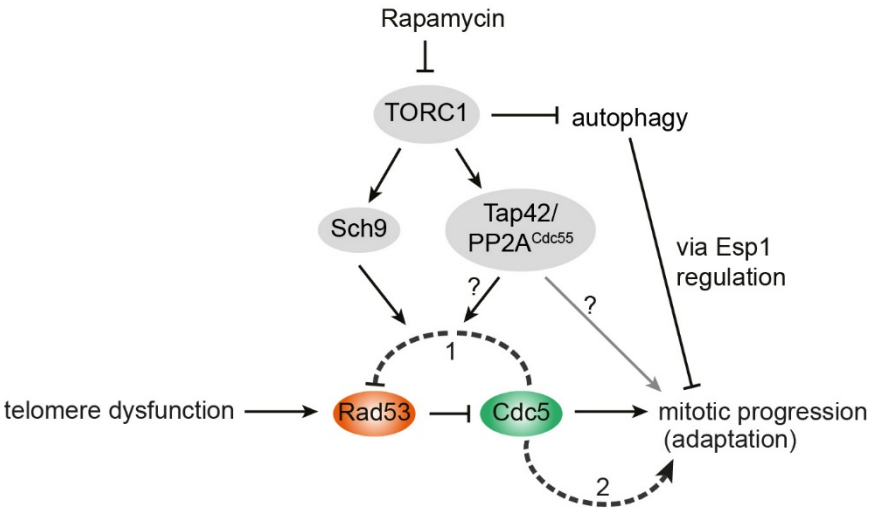

Figure 23. Model of the rapamycin-mediated prevention of adaptation in response to telomere dysfunction. After prolonged telomere dysfunction, Cdc5 inactivates Rad53 to terminate checkpoint signalling (route 1). Consequently, the inhibition of Cdc5 in response to damage is alleviated and Cdc5 can promote mitotic progression (i.e. adaptation) (route 2). By modulating the TORC1 effectors Sch9 and Tap42-PP2A^{Cdc55}, rapamycin inhibits the Cdc5-mediated inactivation of Rad53 (route 1) and therefore, mitotic progression cannot occur (route 2 is prevented). Possible mechanisms (indicated by "?") to counteract adaptation by regulating the Tap42-PP2A axis are discussed in the text. In addition, rapamycin induces autophagy and thereby inhibits mitotic progression in a pathway independent of the DDC as described in (Dotiwala et al., 2013). The model has been modified from (Klermund et al., 2014).

cdc13-1 mutants after prolonged incubation at the non-permissive temperature (data not shown). When we prevented adaptation by rapamycin treatment, we did not observe cell lysis events but only an increase in cell size after prolonged cell cycle arrest. This was observed in the flow cytometry experiments as well as during the microscopy analysis and is consistent with the notion that the vacuole increases in size when autophagy is induced, for example in response to rapamycin (Granot and Snyder, 1991; Heitman et al., 1991a). Therefore, rapamycin could preserve viability in *cdc13-1* cells not by inhibiting adaptation but by preventing cell lysis. However, several observations argue against the conclusion that rapamycin simply prevents cell lysis. Firstly, rapamycin in general did not seem to act by restricting the cell size and keeping the cells arrested in G1 to prevent cell lysis. This can be inferred from our observation of an increase in cell size upon rapamycin treatment during prolonged cell cycle arrest. An increase in cell size might not only be caused by vacuole swelling due to autophagy induction as rapamycin can rescue the viability of *cdc13-1* mutants independently of autophagy (Figure 10, (Klermund et al., 2014)). A likely explanation for the increased cell size could be an arrest in G2/M phase of the cell cycle due to DNA damage checkpoint activity. In line with this, rapamycin preserved checkpoint activity by maintaining Rad53 phosphorylation and prevented the entry into the subsequent G1 phase and not simply the occurrence of dead cells (Figure 8, (Klermund et al., 2014)). Lastly, the presence of damaged DNA in (attempted) subsequent cell cycles is a reasonable explanation for the failure of checkpoint-deficient cells to regrow at the permissive temperature (for example *cdc13-1rad53-11* double mutants, Figure 9). This scenario is prevented in the presence of rapamycin due to the inhibition of adaptation and not by rapamycin counteracting cell lysis.

We could show previously that treatment with rapamycin improved the cell viability and recovery of many *cdc* mutants that arrest in G2/M at the non-permissive temperature including temperature-sensitive alleles of *CDC14, CDC15, CDC16, CDC17* and *CDC20*. Use of the *cdc5-ad* allele did not have a beneficial effect in these mutants indicating that preventing adaptation could have a specific role after telomere dysfunction or DNA damage in general (Klermund et al., 2014) Rather, inducing autophagy using an *ATG13* allele that is refractory to TORC1-mediated inhibition (Kamada et al., 2010) was sufficient for the rescue of viability. In addition, also *cdc* mutants arresting in G1, at the G1/S transition or in early S such as *cdc2-2, cdc4-1, cdc34-1* and *cdc7-4* show partially increased recovery after incubation at the restrictive temperature in the presence of rapamycin (Klermund et al., 2014) suggesting that lowering TORC1 activity might be a generally beneficial response in adverse conditions at various time points during the cell cycle.

3.3.1.2 Prevention of adaptation by interfering with Cdc5

Checkpoint adaptation occurs in response to various types of DNA damage, including telomere dysfunction, treatment with DNA damaging drugs and a nuclease-induced DSB. During adaptation, Rad53 phosphorylation is lost and this has been described to mark the onset of the adaptation process (Pellicioli et al., 2001). We wanted to follow the adaptation process to get insights into its timing using the *cdc13-1* model system. Telomere uncapping after a temperature shift of *cdc13-1* mutants activates the DDC and has been used for the characterization of adaptation regulators in yeast (Toczyski et al., 1997). Consistently with previous results, we observed a downregulation of the DDC evident from loss of Rad53 phosphorylation in *cdc13-1* cells grown for a prolonged period at the non-permissive temperature. Checkpoint inactivation was accompanied by cell cycle resumption monitored by flow cytometry and Western Blot analysis (this thesis and (Klermund et al., 2014). From bulk analysis of adaptation timing as well as following single cells during a live-cell imaging experiment we found that adaptation begins around 8 hours after checkpoint activation. This is in the range of adaptation onset reported previously in response to a single irreparable DSB (Lee et al., 1998). Adaptation of *cdc13-1* mutants to persistent telomere dysfunction leads to cell death (Klermund et al., 2014; Qi et al., 2008). Therefore, the prevention of adaptation using the *cdc5-ad* allele preserves cell viability which is achieved by maintaining an active DDC and therefore continued cell cycle arrest in G2/M, as previously shown by us and others (Klermund et al., 2014; Rawal et al., 2016). The adaptation-deficient *cdc5-ad* mutant contains a leucine to tryptophan substitution at amino acid position 251 (Toczyski et al., 1997). Overall kinase activity seems unaffected in *cdc5-ad* mutants suggesting that it is not a general loss-of-function mutant (Charles et al., 1998; Rawal et al., 2016). However, these studies have used heterologous substrates such as casein to analyse the kinase activity. In fact, others have described the *cdc5-ad* mutation as a gain-of-function allele and hypothesized that *cdc5-ad*-mediated checkpoint hyperactivation causes the adaptation defect (Schleker et al., 2010). The prevalent hypothesis to explain the *cdc5-ad* impairment during adaptation suggests a partial defect in substrate recognition and/or APC activation (Cheng et al., 1998; Toczyski et al., 1997).
Cellular targets of Cdc5 that could influence checkpoint adaptation will be discussed in more detail in the following section.

3.3.2 Possible targets of Cdc5 to drive adaptation

Upon DDC activation in the presence of DNA damage, Cdc5 is inhibited by Mec1- and Rad53-mediated phosphorylation. In combination with the targeting of Pds1 and other factors, this inactivation of Cdc5 ensures cell cycle arrest (Cheng et al., 1998; Sanchez et al., 1999). Damage-induced phosphorylation of Cdc5 by the DDC components results in a decrease in Cdc5 activity (Zhang et al., 2009). In order for a cell to continue cell cycle progression after an arrest, damage signalling has to be terminated: either because the signal is removed (i.e. repair/ recovery) or because the signalling cascade is overridden (i.e. adaptation). Indeed, using irreparable DNA damage to study adaptation revealed a pivotal role for Cdc5 (Toczyski et al., 1997).

As we proposed previously, rapamycin regulates adaptation via the Sch9 and Tap42 branches of TORC1 (Klermund et al., 2014) and also autophagy induction is involved in preventing adaptation (Dotiwala et al., 2013). We could show that rapamycin treatment results in maintenance of Rad53 in a highly phosphorylated state and requires a functional DDC to prevent adaptation (Figures 8, 9 and 23 and (Klermund et al., 2014)). In agreement with this assumption, the rapamycin-mediated rescue is largely diminished but not abolished in Rad53-compromised cells (Figure 9). Therefore, it is reasonable that rapamycin also prevents adaptation by affecting the status of the DDC. The remaining positive effect of rapamycin could be due to checkpoint-independent pathways to prevent mitotic progression in the presence of damage, such as autophagy (Figure 23). Moreover, in addition to being regulated by the DDC in response to damage, Cdc5 also regulates checkpoint signalling at multiple steps. Taking all these observations into account, we decided to focus on Cdc5 as a possible rapamycin-regulated target to prevent adaptation.

Studies addressing the role of Cdc5 after DNA damage have not been fully elusive as there are opposing results regarding Cdc5 activity. A report by Hu and colleagues demonstrated that Bfa1, a target of Cdc5 to prevent mitotic exit, remains phosphorylated upon DNA damage (Hu et al., 2001). However, in its phosphorylated state, Bfa1 is inactive and cannot inhibit Tem1, which in turn is a positive regulator of MEN (reviewed in (Weiss, 2012)). Hu and colleagues therefore postulated two hypotheses: (1) DNA damage does not restrain Cdc5 activity as evident from subsequent Bfa1 phosphorylation but rather (2) Rad53 and Dun1 collaborate to add an additional, non-inhibitory phosphorylation onto Bfa1 that supports Bfa1's role as a negative regulator of MEN to ensure cell cycle arrest. Hu *et al* hypothesized that the additional checkpoint-mediated modification counteracts or prevents the inhibitory Cdc5 activity on Bfa1 (Hu et al., 2001). Based on these results, the authors ruled out the previously proposed inhibition of Cdc5 by Rad53 activation in response to DNA damage (Sanchez et al., 1999) (Figure 24). In this second but alternative scenario, activation of the DDC leads to the phosphorylation of Cdc5 in a manner dependent on Mec1, Rad53 and Rad9 (Cheng et al., 1998; Sanchez et al., 1999). Consequently, Cdc5 activity is inhibited leading to a hypo-phosphorylation of Bfa1 and therefore, retaining Bfa1 in an active state (i.e. MEN is prevented, Figure 24) (Valerio-Santiago et al., 2013). This model is supported by the finding that Rad53-mediated inhibition of Cdc5 allows the sustained activity of the APC co-factor Cdh1, which ultimately prevents the extension of the mitotic spindle and thereby prevents mitotic exit (Zhang et al., 2009). During adaptation, the levels of Cdc5 appear to be dose-dependent (Vidanes et al., 2010) and Rad53 phosphorylation is lost when cells adapt (Pellicioli et al., 2001). These findings suggest that

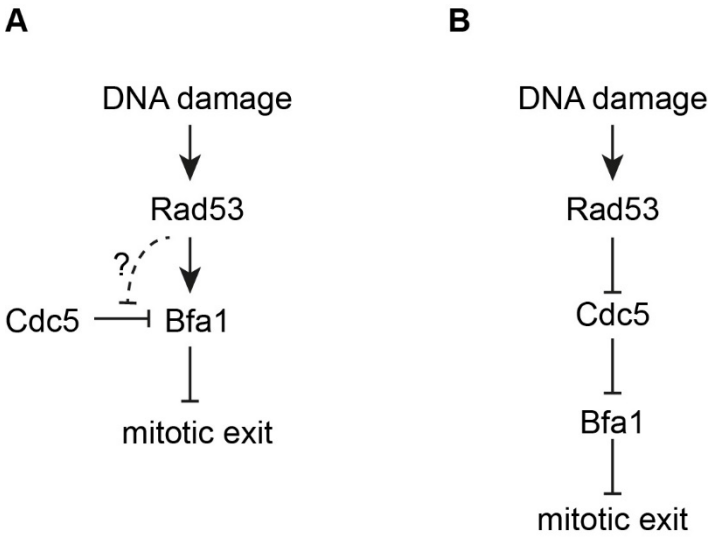

Figure 24. Simplified schemes depicting different models for the prevention of mitotic exit in the presence of damage. (**A**) Based on (Hu et al., 2001), DNA damage induces Rad53 which in turn keeps Bfa1 in an active state. Bfa1 is inactivated after phosphorylation by Cdc5, therefore the Rad53-mediated phosphorylation could prevent modification of Bfa1 by Cdc5. When Bfa1 remains active due to checkpoint signalling, the mitotic exit network is prevented and cells stay arrested. (**B**) Alternatively and as suggested by (Valerio-Santiago et al., 2013) and others, checkpoint activation leads to the inhibition of Cdc5. Consequently, Cdc5-mediated inactivation of Bfa1 is prevented and cell cycle arrest is maintained. See text for more details and references.

Cdc5 could in turn inactivate Rad53 to terminate DDC signalling and allow cell cycle progression. Indeed, overproduction of Cdc5 results in the complete loss of Rad53 phosphorylation and accelerates adaptation (Donnianni et al., 2010; Pellicioli et al., 2001; Vidanes et al., 2010). We therefore hypothesized that rapamycin prevents adaptation by reinforcing the checkpoint and keeping Cdc5 in an inactive state. In support of this idea, we could show that rapamycin suppresses the phosphorylation of Bfa1 upon telomere dysfunction indicating that Cdc5 is inactivated in this scenario. Further, rapamycin treatment maintains Rad53 phosphorylation indicative for an active DDC but this effect is abolished in *CDC5* overexpressing cells (Klermund et al., 2014). Taken together, these findings favour our hypothesis that rapamycin acts on Cdc5 to prevent adaptation and maintaining an active checkpoint in response to telomere dysfunction. Consequently, telomere dysfunction mutants remain arrested at the metaphase-to anaphase transition and therefore retain viability.

The identification of the adaptation-defective *cdc5-ad* allele has fuelled the investigation of adaptation in response to various types of DNA damage. Nevertheless, the exact defect of the *cdc5-ad* allele remains to be elucidated. In agreement with the idea that adaptation occurs by shutting off the DDC, the absence of a functional checkpoint in *rad53-11* or *mec1* (in absence of *SML1*) mutants abolishes the protective effect of both rapamycin and the *cdc5-ad* allele on cell viability (Figure 9). However, the lack of the central checkpoint kinases Rad53 and Mec1 renders cells genetically unstable due to increased mutation rates. This phenotype is not only due to an impairment of the DDC but also caused by de-regulated origin firing during DNA replication causing higher mutation frequencies (Craven et al., 2002; Lang and Murray, 2011). This might be a plausible explanation for the only partially observed rescue by the *cdc5-ad* allele in *cdc13-1* mutants derived from a checkpoint-compromised diploid cell and the

acquisition of suppressor mutations in *cdc13-1* mutants (Figure 9 A). In any case, these results suggest that the prevention of adaptation by the *cdc5-ad* allele requires a functional DDC. Consistently, checkpoint signalling must be continuous for a permanent cell cycle arrest (i.e. adaptation defect or the prevention of adaptation). This has been concluded from the finding that adaptation-defective *cdc5-ad* mutants resumed cell cycle progression in the presence of damage when Mec1 was prematurely degraded using degron alleles (Pellicioli et al., 2001). Since loss of Mec1 signalling results in the inactivation of Rad53 and this was still observed in a *cdc5-ad* mutant, this experiment also suggests the adaptation defect in *cdc5-ad* cells is not due to a defect in Rad53 dephosphorylation (Pellicioli et al., 2001). To add another layer of complexity, Cdc5 has been shown to phosphorylate Rad53 in a cell cycle-dependent manner in association with Cdc28. Although these phosphorylation events occurred during an unperturbed mitosis independently of DNA damage, their absence caused the premature loss of Rad53 phosphorylation during the DNA damage response, i.e. accelerated adaptation (Schleker et al., 2010). This suggested positive role for Cdc5 in checkpoint maintenance seems contradictory to the adaptation-defect observed in *cdc5-ad* mutants and it has been hypothesized that Rad53 is continuously active in *cdc5-ad* mutants causing the inability to override the checkpoint (Schleker et al., 2010). In line with this, a kinase-deficiency due to the amino acid substitution at position 251 (a leucine to tryptophan mutation) does not seem to be the cause of the *cdc5-ad* adaptation defect (Charles et al., 1998; Rawal et al., 2016). Rather, *cdc5-ad* cells showed a hyperactive kinase activity (Charles et al., 1998) and furthermore, Cdc5 kinase activity was required to cause Rad53 dephosphorylation (Vidanes et al., 2010). Likewise, *cdc5-ad* does not appear to be regulated differently on the protein level during the cell cycle (Rawal et al., 2016). Moreover, generation of ssDNA as a trigger for checkpoint activation was not altered in *cdc5- ad* mutants supporting the idea that the adaptation defect does not stem from a failure to activate the checkpoint in the first place (Pellicioli et al., 2001). As mentioned before, a defect in recognizing certain substrates has been suggested as the underlying cause for the adaptation defect in *cdc5-ad* cells as APC activity was stimulated less efficiently in the mutant (Charles et al., 1998). This could cause an inability to degrade Clb2 by the APC^{Cdh1} complex and therefore, hamper mitotic exit (Schwab et al., 1997). An independent report has also suggested that *cdc5- ad* mutants fail to adapt due to a defect in mitotic exit. Consistently, the authors showed that also mutants with defects in the FEAR pathway that initiates mitotic exit, display an adaptation defect (Jin and Wang, 2006).Taking all these observations into account, the exact defect of *cdc5-ad* mutants still remains uncharacterized. Based on our results presented in Figure 9, we hypothesize that rapamycin prevents adaptation largely by modulating the Cdc5- Rad53 interplay, although we note the checkpoint-independent effects of rapamycin such as autophagy and possibly additional pathways (Figure 23).

In agreement with the observation of a cell-cycle dependent modification of Rad53 by Cdc5 (Schleker et al., 2010), a second study reported an interaction of Cdc5 and Rad53 in the absence of damage (Vidanes et al., 2010). In support of this, a cell cycle- and DNA damage-regulated interaction has been observed between human CHK2 (homolog to yeast Rad53) and PLK1 (homolog to yeast Cdc5) (Tsvetkov et al., 2005). Interestingly, in addition to loss of Rad53 phosphorylation, also Chk1 is inactivated during adaptation and this was prevented in a *cdc5- ad* mutant background suggesting that downregulation of the checkpoint involves both branches of Mec1 signalling (Pellicioli et al., 2001).

Irrespective of our complete understanding of the mechanisms used by Cdc5 to inactivate the DDC and alleviate cell cycle arrest in the presence of DNA damage, the question remains how Cdc5 is regulated to become active (again). We hypothesize that TORC1 activity serves as the promoting signal in such a scenario (this thesis, (Klermund et al., 2014)). According to this model, active TORC1 provides the activating signal for Cdc5 to inactivate Rad53 and shut-off the DDC cascade. In contrast, decreasing TORC1 signalling by rapamycin impacts on Cdc5 via several possible mechanisms (see below) and prevents its reactivation. Hence, DDC signalling via Rad53 is maintained and cells stay arrested. Interestingly, Tel2, a protein initially identified due to its role in telomere biology (Runge and Zakian, 1996), was then shown to be a regulator of PIKK assembly and stability (reviewed in (Boulton, 2008)), the conserved kinase family that also comprises Mec1, Tel1 and the Tor proteins. If and how Tel2 also plays a role in adaptation remains to be addressed experimentally and could provide interesting insights into the regulation of adaptation.

3.3.3 Possible targets for PP2A in preventing adaptation

As described above, Cdc5 is a critical player in the adaptation process. On the other hand, nutrient signalling by TORC1, the target of rapamycin, has been shown to influence adaptation (Klermund et al., 2014) and is involved in Cdc5 regulation (Nakashima et al., 2008). Therefore, we hypothesized that, in addition to other targets, rapamycin treatment resulting in the inhibition of TORC1 prevents adaptation by acting on Cdc5. We could previously provide mechanistic insights into the involvement of Sch9 in the adaptation process (Klermund et al., 2014). Therefore, we decided to focus on $PP2A^{Cdc55}$ as a TORC1 downstream effector and possible mediator of rapamycin to prevent adaptation. In the section below, I will present several possible mechanisms how the effect of rapamycin could be mediated in budding yeast.

3.3.3.1 A model for the direct regulation of Cdc5 by PP2A

An important unanswered question in studying checkpoint adaptation is its temporal regulation. We and others could show that adaptation starts to occur approximately 6 to 8 hours after the checkpoint arrest has been elicited (Figure 8, (Lee et al., 1998; Toczyski et al., 1997)). This would suggest that one or more critical factors responsible for initiating and executing adaptation become active or re-activated at this time point. Since Cdc5 is a main driver of adaptation (Toczyski et al., 1997) and becomes inactivated by DDC proteins (Donnianni et al., 2010; Pellicioli et al., 2001) after damage has been detected, Cdc5 constitutes a reasonable candidate. Furthermore, activity of Cdc5 is governed by its phosphorylation status (reviewed in (Botchkarev and Haber, 2017)) with phosphorylation being one of the most versatile and important regulatory tools for protein activity (Salazar and Hofer, 2007). However, it is mechanistically unknown how Cdc5 overcomes inhibition by the DDC to downregulate checkpoint players itself and thereby facilitate cell cycle progression. Although initially phosphorylated by the DDC in response to DNA damage to inactivate its kinase activity (Cheng et al., 1998; Zhang et al., 2009), Cdc5 is released from this block to drive adaptation. We have proposed previously that nutrients could serve as a trigger to allow the regaining of Cdc5 activity (Klermund et al., 2014) and therefore adaptation. Nutrient availability sensed by

TORC1 has been linked to Cdc5 regulation in several studies. Active TORC1 signalling allows the nuclear accumulation of Cdc5 via the Tap42-PP2A effector branch and thereby promotes mitotic entry (Nakashima et al., 2008). Intriguingly, genotoxic treatment of *Xenopus laevis* egg extracts led to an increased interaction between PP2A and Plx1, the *Xenopus* Plk1 homolog. This interaction was mediated by B55α, which is the homolog of yeast Cdc55, and resulted in dephosphorlyation of the activating phosphorylation site within Plx1 (Wang et al., 2015). These observations revealed a DNA damage-induced downregulation of Plk1 activity via the PP2A phosphatase and could implicate PP2A in regulating checkpoint adaptation. As in yeast, also mammalian PP2A activity is regulated by mTOR signalling via a Tap42 homolog, alpha4 (Murata et al., 1997; Nanahoshi et al., 1998). Since DNA damage downregulates TOR signalling (Budanov and Karin, 2008) this results in PP2A activation. We observed a rescue of cell viability in *cdc13-1* mutants lacking Tap42, a negative regulator of PP2A, suggesting that PP2A activation could prevent adaptation (Figure 10). Taking these observations into account, we hypothesized that PP2A dephosphorylates Cdc5, thereby retaining it in an inactive state, similarly to what has been shown in the *Xenopus* system (Wang et al., 2015). This could be a possible mechanism to prevent Cdc5 from regaining activity and driving adaptation in the presence of persistent DNA damage. In agreement with these findings, we observed an interaction between Cdc5 and Pph21, one of the two redundant catalytic subunits of PP2A. However, the Cdc5-PP2A interaction seemed independent of DNA damage but rather cell cycle-regulated since it was also observed in nocodazole-arrested cells (Figure 10). This is consistent with PP2A being a regulator of Cdc5 localization during the cell cycle (Nakashima et al., 2008). We were therefore interested if inhibition of TORC1 would increase the interaction between PP2A and its possible target Cdc5. We hypothesized that downregulation of TOR signalling by rapamycin would strengthen or prolong the binding between phosphatase complex and Cdc5 and therefore inactivate Cdc5. Instead of increased binding, we observed a weakened interaction between Cdc5 and Pph21 in the presence of rapamycin and DNA damage (in a *cdc13-1* background). However, future experimental work is required to investigate the regulation of this interaction. A possible influence on total Cdc5 protein levels upon rapamycin treatment is discussed below (see section 3.3.4).

Taken together, we could provide evidence for a physical interaction between the PP2A catalytic subunit Pph21 and the Polo-like kinase Cdc5 during unperturbed conditions and after DNA damage induced by telomere dysfunction. It has been suggested that TORC1 only impacts Cdc5 during entry into mitosis (Nakashima et al., 2008), however, rapamycin treatment is able to prevent checkpoint adaptation in cells arrested at the metaphase-to-anaphase transition after DDC activation (Klermund et al., 2014) suggesting that there is also an involvement of TORC1 signalling at later time points. These two conflicting observations could be explained by a Cdc5-independent rescue effect of rapamycin, for example via autophagy induction. Alternatively, rapamycin could sustain PP2A activity, possibly by preventing the re-binding of Tap42. This would maintain a cell cycle arrest prior to sister chromatid segregation as downregulation of PP2A is necessary to facilitate mitotic exit during an unperturbed cell cycle. This inhibition is mediated by separase at the onset of anaphase (Queralt et al., 2006).

The importance of correct PP2A regulation is highlighted by its tumour suppressor function (reviewed in (Ruvolo, 2016)). Downregulated PP2A activity has been found in many cancers and could be linked to increased mTOR signalling. Importantly, although proposed to act

downstream of mTORC1, PP2A has also been shown to signal upstream of mTOR (reviewed in (Wlodarchak and Xing, 2016)).

3.3.3.2 Additional mitotic factors as PP2A targets

In addition to a possible regulation of Cdc5 by $PP2A^{Cdc55}$ after rapamycin treatment, the PP2A phosphatase complex could also influence adaptation by regulating other factors critical for mitosis. Such a regulatory role for $PP2A^{Cdc55}$ has been shown during adaptation to the spindle assembly checkpoint (SAC). This checkpoint operates in metaphase and monitors the correct attachment of kinetochores to the mitotic spindle to ensure proper chromosome segregation. In order to allow adaptation to the SAC, Clb2 levels are gradually increasing during a prolonged metaphase arrest. This allows the activation of the APC^{Cdc20} complex which in turn targets Clb2 for proteasomal degradation evidenced by a rapid drop in Clb2 levels. However, the activating phosphorylation of APC^{Cdc20} complexes by Clb2/Cdc28 is counteracted by the $PP2A^{Cdc55}$ phosphatase. This leaves the question how Clb2/Cdc28 can phosphorylate enough APC^{Cdc20} complexes before being degraded by APC and while APC phosphorylation is actively counteracted. A model involving both a negative and positive feedback loop has been proposed (Vernieri et al., 2013): In a negative feedback loop, Clb2/Cdc28 promotes APC^{Cdc20} activation due to increasing Clb2 levels thereby leading to the rapid degradation of Clb2 as APC^{Cdc20} activity increases. At the same time, increasing activity of APC^{Cdc20} targets $PP2A^{Cdc55}$ and thereby creates a positive feedback loop since $PP2A^{Cdc55}$ acts as an inhibitor of APC^{Cdc20}. This bistable switch between opposing APC and PP2A activity states has been hypothesized to regulate the metaphase-to-anaphase transition during adaptation to the SAC (Vernieri et al., 2013).

Another study has implicated $PP2A^{Cdc55}$ as a functional link between the FEAR and MEN pathway. When $PP2A^{Cdc55}$ is downregulated at anaphase onset in a separase-dependent manner during the FEAR pathway to trigger a first wave of Cdc14 activity ((Queralt et al., 2006), reviewed in (Weiss, 2012)), this allows Cdc5 to phosphorylate and thereby inhibit Bfa1 leading to the activation of the MEN (Baro et al., 2013; Queralt et al., 2006). However, in the absence of $PP2A^{Cdc55}$ activity, MEN was not initiated prematurely suggesting that this first and incomplete Cdc14 release from the nucleolus is not sufficient to trigger mitotic exit. It has been proposed that MEN activity is restrained due to the inhibitory effect of Clb2-Cdc28 complexes on core MEN components Cdc15 and Dbf2-Mob1 (Baro et al., 2013). Taken together, $PP2A^{Cdc55}$ activity seems required for the activation of both FEAR and MEN. Since Bfa1 appears to be a key player in the regulation of mitotic exit, it is possible that rapamycin prevents adaptation by keeping Bfa1 in an active state to prevent MEN. This would be consistent with the finding that rapamycin prevented the Cdc5-dependent phosphorylation of Bfa1 in response to telomere dysfunction (Klermund et al., 2014). In the absence of *BFA1*, *cdc13-1* mutants had already lost viability after short-term telomere dysfunction (J. Klermund, personal communication), a condition in which *cdc13-1* viability is usually not compromised even in the absence of rapamycin (Figure 8, (Klermund et al., 2014; Qi et al., 2008)). In support of our observation, also others have found that damage arising from dysfunctional telomeres requires Bfa1 to ensure metaphase arrest (Valerio-Santiago et al., 2013). Strikingly, *cdc13-1bfa1* double mutants could still be rescued by rapamycin treatment (J. Klermund, personal communication).

There are at least two different possible explanations for these results: First, rapamycin could prevent adaptation by acting upstream of Bfa1, for example by maintaining Rad53 phosphorylation. Alternatively, it is conceivable that rapamycin does not prevent adaptation via Bfa1 but by regulating a parallel pathway to achieve cell cycle arrest. Indeed, $PP2A^{Cdc55}$ also acts as a negative regulator of MEN by dephosphorylating Tem1 (Wang and Ng, 2006), a critical positive regulator of mitotic exit that is inhibited by Bfa1/Bub2. Further experiments taking into account the phosphorylation status of Tem1 in the presence of rapamycin could provide insights into the role of MEN regulation during adaptation.

3.3.4 Other Cdc5 regulatory mechanisms to counteract adaptation

In addition to a possible regulation via PP2A (discussed above), also other TORC1 signalling pathways modulated by rapamycin could result in the prevention of adaptation by acting on Cdc5.

As described above, preliminary results led us to hypothesize that rapamycin treatment prevents checkpoint adaptation in response to telomere dysfunction by decreasing Cdc5 protein levels (Figure 10). This could be explained by the induction of an ubiquitin ligase in response to TORC1 inhibition which targets Cdc5 for degradation. During unperturbed conditions, Cdc5 is degraded by the proteasome after ubiquitination by APC^{Cdh1}. However, this E3 ligase complex can only form after Cdc14 has been released in anaphase and is still inactive at the *cdc13-1* arrest point at the non-permissive temperature (reviewed in (Weiss, 2012)). Therefore, it is highly unlikely that Cdc5 is degraded by APC^{Cdh1} triggered by rapamycin treatment. Rather, APC in conjunction with Cdc20 as a specificity factor could target Cdc5 to decrease protein levels and thereby prevent checkpoint adaptation. APC^{Cdc20} targets include Pds1 (Visintin et al., 1997) and the mitotic cyclin Clb5 (Shirayama et al., 1999) and its activity can be regulated by $PP2A^{Cdc55}$, although this has been shown to occur in response to activation of the spindle assembly checkpoint (Vernieri et al., 2013). Recently, mammalian TORC1 has been reported to activate the E3 ubiquitin ligase ZNRF2, the putative homolog of yeast Pib1 (Hoxhaj et al., 2016). However, yeast Pib1 has been suggested to target endosomal and lysosomal proteins (Shin et al., 2001) and therefore, most likely does not represent a candidate E3 ubiquitin ligase regulating Cdc5 protein levels. It will be interesting to address a possible rapamycin-induced degradation of Cdc5 as a mechanism to prevent adaptation. Future experiments aiming at the identification of candidate E3 ubiquitin ligases will hopefully provide experimental evidence for this hypothesis. Intriguingly, the regulation of the transcription factor Stp1 at the protein level has been described in response to TORC1 inactivation and has been attributed to the PP2A-like phosphatase, Sit4. Although the mechanistic details remain elusive, decreased TORC1 signalling was shown to lead to the disappearance of Stp1 from the nucleus (Shin et al., 2009). This report indicates the potential of cytoplasmic TORC1 to regulate the abundance of specific proteins in the nucleus although further experiments are required to provide further details.

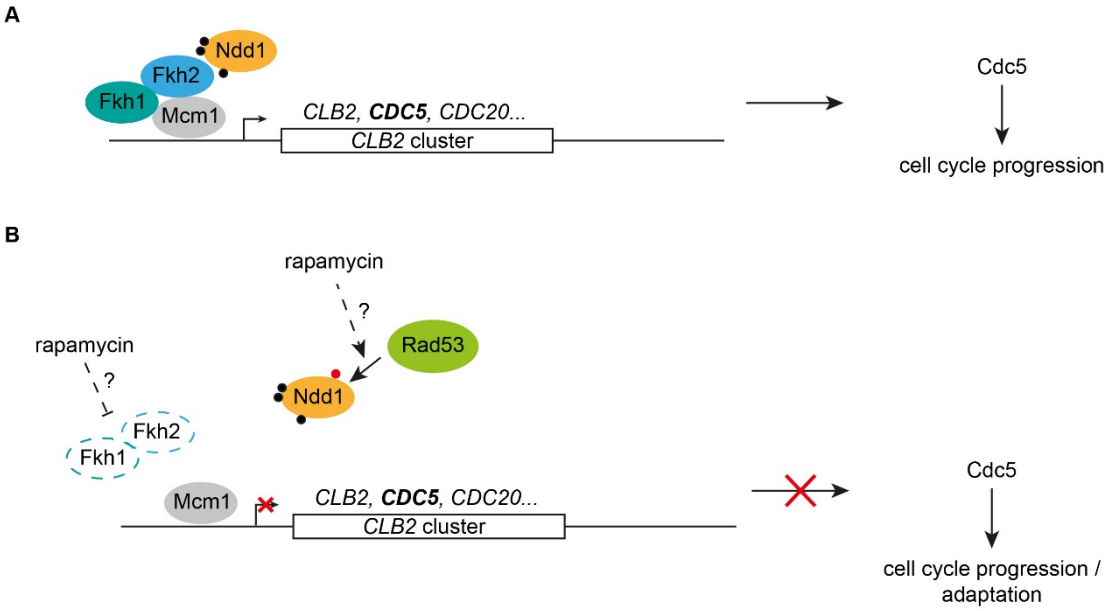

Figure 25. Possible regulatory roles of rapamycin on the transcription of Cdc5 mRNA to prevent adaptation. (**A**) Scheme representing transcription from the CLB2 cluster in an unperturbed cell cycle. Assembly of the transcription factor complex with the positive regulator Ndd1 allows Cdc5 mRNA and protein production allowing progression through mitosis. (**B**) Upon DNA damage, Ndd1 is phosphorylated by Rad53 which impairs CLB2 cluster transcription resulting in cell cycle arrest due to the lack of critical mitosis-promoting factors such as Cdc5. Rapamycin could reinforce G2/M arrest induced by DNA damage by indirectly maintaining the inhibitory Ndd1 phosphorylation. Alternatively, rapamycin could lead to the translocation of one or both Fkh transcription factors to the cytoplasm thereby negatively regulating CLB2 cluster transcription and resulting in continued cell cycle arrest (i.e. prevention of adaptation).

Preliminary results using the proteasome inhibitor MG132 indicated that the decrease of Cdc5 protein levels in response to rapamycin treatment is independent of proteasomal function (data not shown). However, TORC1 inhibition, for example by rapamycin treatment, was shown to induce proteasome abundance by inducing Adc17, a chaperone that couples stress conditions to proteasome assembly (Rousseau and Bertolotti, 2016). Therefore, proteasome inhibition in this experimental set-up could have been incomplete. To address the hypothesis of proteasome-dependent degradation of Cdc5 in response to rapamycin treatment, the experiment should be performed in an *adc17* mutant background and proteasome inactivation should be monitored by an accumulation of total ubiquitin signal.

Assessing the stability of Cdc5 protein in response to rapamycin treatment using a classical cycloheximide-chase experiment is difficult to interpret due to the observation that the increase in free amino acids triggered by cycloheximide serves as an activating signal for TORC1 (Beugnet et al., 2003; Urban et al., 2007). However, the low rapamycin concentrations used in this study and in our previous report (Klermund et al., 2014) are substantially lower than the concentrations used for full TORC1 inhibition resulting in G1 cell cycle arrest (Barbet et al., 1996). To circumvent this complications, a promoter shut-off experiment could be employed. However, since *CDC5* is an essential gene, such an experiment is only feasible within a short time range to avoid cell death.

Taken together, these experiments could provide further evidence for a rapamycin-mediated degradation of Cdc5 protein to prevent adaptation in response to telomere dysfunction.

In addition to preventing adaptation by regulating Cdc5 on the protein level, rapamycin could also exert its effect by acting at the transcriptional level. One possibility to regulate *Cdc5* mRNA is by affecting the forkhead transcription factors, Fkh1 and Fkh2. Together with a third transcription factor, Mcm1, the Fkh proteins mediate transcription of the *CLB2* cluster including *CLB2* itself but also *CDC5* (Kumar et al., 2000; Zhu et al., 2000). As outlined in section 1.1.3, Fkh2 interacts with Ndd1 (Koranda et al., 2000), a positive regulator of the *CLB2* transcription cluster. This interaction is abrogated upon DNA damage due to Rad53-mediated phosphorylation of Ndd1 thereby contributing to the cell cycle arrest (Edenberg et al., 2014). In metazoans, the highly conserved PI3K-Akt-mTOR signalling axis negatively regulates the forkhead box O class of transcription factors, termed FOXO (reviewed in (Hay, 2011)). Inhibition of FOXO transcription factors results in a pro-survival signal and consistently, FOXO transcription factors are thought to act as tumour suppressors (reviewed in (Dansen and Burgering, 2008)). In agreement with the idea that FOXO family members must be inhibited to allow proliferation, PLK1 has been shown to be a negative regulator of FOXO3 (Bucur et al., 2014), another member of the FOXO transcription factor family, thereby counteracting its tumour-suppressive activity. Although the PI3K/Akt/mTOR signalling node is often aberrantly active in cancer cells and acts as an oncogenic signal, inhibition of mTOR signalling by rapamycin as the sole therapeutic agent was ineffective (reviewed in (Xie et al., 2016)). Rather, rapamycin treatment was reported to cause the phosphorylation of FOXO1 resulting in the nuclear export of the transcription factor rendering it inactive as a tumour suppressor (Abdelnour-Berchtold et al., 2010). This provided one explanation for the less promising efficacy of rapamycin in cancer therapy. Nevertheless, the study showed a direct implication of TOR signalling in the regulation of forkhead transcription factors. Given the high conservation of the signalling pathways amongst eukaryotes, a possible mode of action for rapamycin to prevent checkpoint adaptation in budding yeast could be by regulating Cdc5 on the transcriptional level via the Fkh transcription factors. In this scenario, rapamycin treatment would lead to a change in the subcellular localization of one or both Fkh transcription factor paralogues resulting in decreased transcription of the *CLB2* cluster and thereby lowering *Cdc5* mRNA levels to prevent checkpoint adaptation. Alternatively, one could speculate that rapamycin regulates *CLB2* cluster transcription by impinging on the positive regulator Ndd1, for example by maintaining its inhibitory Rad53-mediated phosphorylation to reinforce damage-induced cell cycle arrest (Figure 25).

3.3.5 The influence of autophagy on adaptation

3.3.5.1 The role of macroautophagy

The induction of autophagy is one of the best characterized effects in response to TORC1 inhibition. The molecular mechanisms leading to autophagic degradation when TORC1 is inactivated have been addressed by many different labs. However, also other nutrient sensory pathways were shown to influence autophagy, for example the Ras/PKA (protein kinase A) network responding to glucose availability. Importantly, simultaneous inhibition of PKA and

Sch9 signalling leads to the activation of autophagy independently of TORC1 activity (Yorimitsu et al., 2007). This observation could not unambiguously place PKA signalling in parallel or downstream of TORC1. Another study analysing the interplay between PKA and TORC1 signalling found TORC1 responsible for the localization of PKA subunits and therefore, it was hypothesized that PKA signalling occurs downstream of the TORC1 signalling node (Schmelzle et al., 2004).

Independent of the exact molecular pathway causing its induction, autophagy has been proposed as a determinant factor for genome stability (Matsui et al., 2013). Acute nutrient deprivation or rapamycin treatment leading to TORC1 inactivation arrests yeast cells in G1 before they enter stationary phase (Barbet et al., 1996; Johnston et al., 1977). However, malfunction of TORC1 also affects cell cycle progression at the G2/M transition due to the mislocalization of Cdc5 (Nakashima et al., 2008). Further experiments led to the conclusion that autophagy is required to provide the building blocks which are needed to complete the cell cycle and arrest in the subsequent G1 phase in response to nutrient starvation (Matsui et al., 2013). In autophagy-deficient cells, nutrient limitation caused an increased occurrence of aneuploidy by facilitating pre-mature mitosis thereby suggesting autophagy as positive regulator of genome stability. The critical role of autophagy in this scenario has been explained by the hypothesized benefits to re-initiate cell growth and division from G1 phase when nutrients are favourable again (Matsui et al., 2013). The observation that autophagy can protect genome integrity has also been made in mammalian cells (Mathew et al., 2007).

In our experiments using *cdc13-1* mutants, rapamycin led to an induction of autophagy, both at the early time point (6 h) and late time point (24 h of telomere dysfunction) (Klermund et al., 2014). However, we did not observe a requirement for autophagy to prevent adaptation by rapamycin treatment (Figure 10 and (Klermund et al., 2014)). This suggests that rapamycin can regulate adaptation independently of autophagic degradation, for example by modulating post-translational modifications of positive regulators of checkpoint adaptation. On the other hand, induction of macroautophagy using an allele of *ATG13* that is refractory to the inhibition by active TORC1 (Atg13-8SA, (Kamada et al., 2010)) is sufficient to rescue cell viability in *cdc13-1* mutants (Klermund et al., 2014). Taken together these results indicate that upon telomere dysfunction, autophagy is not necessary but sufficient to prevent checkpoint adaptation by rapamycin.

3.3.5.2 A link between selective autophagy and adaptation

Consistent with a general protective role for genome stability, autophagy is directly induced in response to DNA damage. The checkpoint kinases Mec1, Tel1 and Rad53 mediate the induction of autophagy in response to DNA damaging agents in a process termed genotoxin-induced targeted autophagy (GTA) (Eapen et al., 2017). Importantly, GTA utilizes proteins involved in the selective Cvt pathway in addition to the core autophagic machinery and is different from starvation- or rapamycin-induced autophagy which highlights the specificity of the response (Eapen et al., 2017).

Autophagy induction in response to DNA damage seems to be temporally regulated. An acute and transient upregulation of autophagy mediates cell cycle arrest via the partial destruction of Pds1 through the Cvt pathway (Dotiwala et al., 2013). Pds1 functions not only as an inhibitor

of Esp1 but is also important for Esp1's nuclear localization (Agarwal and Cohen-Fix, 2002). Therefore, autophagy-induced Pds1 degradation in response to damage causes the mislocalization of Esp1 and thus prevents sister chromatid separation. This process seems to be enhanced in cells lacking *VPS51* that have a defect in the Cvt pathway and that suffer from permanent cell cycle arrest (which is the failure to undergo checkpoint adaptation) (Dotiwala et al., 2013). While Dotiwala and colleagues argue that *vps51* mutants display hyperactive autophagy, others have proposed that loss of *VPS51* impairs autophagy (Reggiori et al., 2003). In support of their hypothesis that hyperactive and prolonged autophagy after DNA damage in *vps51* cells causes an adaptation defect by completely excluding Pds1 from the nucleus and thereby preventing sister chromatid separation, Dotiwala *et al* report the restoration of adaptation by blocking autophagy in *vps51atg5* double mutants (Dotiwala et al., 2013). Taken together, these results imply that a transient induction of autophagy in response to DNA damage is important to establish cell cycle arrest in G2/M. Eventually, autophagy has to decrease to normal physiological levels to allow checkpoint adaptation.

Utilizing *cdc13-1* mutants in our study, we observed that loss of *VPS51* was sufficient to rescue cell viability upon telomere dysfunction (Figure 10). In contrast to the observation by Dotiwala and colleagues, impairment of non-selective autophagy by deleting *ATG5* did not abolish the *vps51*-mediated rescue of *cdc13-1* mutants. Moreover, the absence of a functional DDC in *mec1sml1* mutants did not compromise the rescue of cell viability in *cdc13-1vps51* double mutants (Figure 10). This is consistent with the study by Dotiwala and colleagues where the adaptation defect in *vps51* cells persisted even in the absence of an active Rad53-mediated DDC (Dotiwala et al., 2013). One could hypothesize that in our experimental set-up, loss of *VPS51* leads to the degradation, mislocalization or inactivation of one or more yet uncharacterized factor(s) independently of non-selective autophagy (via Atg5) and the DDC (via Mec1). Consequently checkpoint adaptation is prevented and therefore cell viability upon telomere dysfunction is preserved. A likely explanation for the discrepancy between our analysis and the report by Dotiwala *et al* could be given by the tools to induce DNA damage.

Further experiments including the assessment of the cell cycle profile in *cdc13-1vps51* double mutants will be required to investigate the involvement of the Cvt pathway in preventing adaptation. Since Vps51 is not only part of the Cvt pathway, also crosstalk to other selective autophagic processes or protein sorting pathways could account for the positive effect observed in *vps51* mutants. Another interesting observation is the rapamycin sensitivity of *vps51* mutants irrespective of DNA damage (Figure 10). This has been observed previously in genetic screens (Parsons et al., 2004; Xie et al., 2005) but the underlying mechanism remains elusive. In accordance with the hypothesis that loss of Vps51 induces autophagy (Dotiwala et al., 2013), one could speculate that further excessive induction of autophagic degradation by rapamycin causes lethality.

In addition to the nuclear exclusion of Pds1 after DNA damage-induced autophagy, also Rnr1 protein levels have been shown to be regulated via autophagic degradation and the degradation was exacerbated upon TORC1 inhibition (Dyavaiah et al., 2011). As hypothesized by the authors, these findings highlight that crosstalk between TORC1 signalling leading to autophagy induction and the DDC is required to fine-tune the cellular response.

3.3.6 An involvement of other nutrient sensory pathways in regulating adaptation

The yeast TOR signalling pathway is mainly responding to nitrogen availability. Glucose levels are primarily sensed and integrated by the Ras/ PKA (Protein Kinase A) signalling network. However, extensive crosstalk between nutrient sensory cascades exists to achieve optimal growth in the nutritional environment (reviewed in (Zaman et al., 2008)). For example, activity of the Sch9 kinase, a well-characterized downstream effector of TORC1 and the closest yeast homolog to mammalian Akt, is also coupled to glucose levels ((Urban et al., 2007), reviewed in (Zaman et al., 2008)).

Ras1 and Ras2 are small GTPases that trigger rapid intracellular accumulation of cyclic adenosine monophosphate (cAMP) which in turn serves as an activating signal for PKA. Eventually, PKA promotes ribosome biogenesis and actively prevents expression of stress-responsive and stationary phase genes (reviewed in (Conrad et al., 2014)). The Ras GTPase family is highly conserved throughout eukaryotic cells as demonstrated by the ability of mammalian Ras to complement yeast Ras mutants (Kataoka et al., 1985). The impact of the Ras signalling cascade is highlighted by the identification of hyperactivating Ras mutations as the first oncogenic alterations in cancers (reviewed in (Fernandez-Medarde and Santos, 2011)). However, despite the remarkable conservation of Ras proteins, the signalling networks and target proteins differ in yeast and mammalian cells.

A yeast mutant allele of Ras2, containing a single amino acid substitution to create *RAS2 G19V* (Kataoka et al., 1984), has been shown to constitutively activate PKA signalling (Toda et al., 1985). Consistently, comparable hyperactivating mutations in the N-terminus of a mammalian Ras isoform were described to increase its tumorigenic potential (Tabin et al., 1982). Given its growth-promoting role, we hypothesized that also the yeast Ras signalling pathway could play a role in promoting adaptation in *cdc13-1* telomere dysfunction mutants. However, disrupting the signalling cascade by deletion of *RAS2* did not rescue the cell viability of *cdc13-1* mutants (Klermund et al., 2014). Moreover, expressing the hyperactive *RAS2 G19V* mutant did not compromise the rapamycin-mediated rescue of cell viability (data not shown). This is consistent with the notion that also other pathways like the Gpa2 network regulate the cellular cAMP levels which activate PKA (reviewed in (Zaman et al., 2008)). Indeed, high PKA activity after DNA damage has been reported to help maintain a stable checkpoint arrest by phosphorylating the APC co-factor, Cdc20. In addition to a damage-induced phosphorylation of Cdc20 by Chk1, the PKA-mediated Cdc20 modification decreases APC activity towards Pds1 and Clb2. However, degradation of both Pds1 and Clb2 are required for mitotic progression and thereby link PKA signalling and the DDC (Searle et al., 2004). A follow-up study revealed that PKA activity in response to damage is due to the checkpoint-mediated phosphorylation and re-localization of the inhibitory regulatory subunit of PKA, Bcy1 (Searle et al., 2011). Furthermore, hyperactivation of Ras signalling by deletion of the GTPase-activating proteins (GAPs) Ira1 and Ira2 led to a recovery defect after transient incubation of *cdc13-1* cells at the non-permissive temperature (Wood and Sanchez, 2010). In this study, it was suggested that PKA activity needs to be downregulated when the checkpoint is terminated in order to allow recovery. However, this cannot be achieved in cells lacking *IRA1* and *IRA2* since these mutants display continuous Ras activity leading to high cAMP levels that ultimately stimulate PKA activity (Wood and Sanchez, 2010). In contrast, we did not observe a recovery defect in *cdc13-1* cells expressing the hyperactive *RAS2 G19V* allele (data not shown). A more careful analysis

of the checkpoint status and cell cycle distribution of the *RAS2* mutant expressing *cdc13-1* cells will reveal an influence of the Ras/PKA signalling pathways on adaptation.

3.4 Consequences of adaptation

3.4.1 Adaptation, DNA damage repair and tolerance

In the previous sections I have outlined mechanisms to influence the cellular decision to undergo adaptation. Together with the results obtained during our previous study (Klermund et al., 2014), the findings presented here have led to the hypothesis that TORC1 activity provides the growth-promoting signal which ultimately allows Cdc5 to override the DDC and drive adaptation (Figure 23). In addition, we set out to address the cellular consequences of adaptation. It seems obvious that continued cell division in the presence of unrepaired DNA damage could be lethal for the cell. Nevertheless, checkpoint adaptation has been observed in eukaryotic model organisms ranging from yeast to cancer cells. Possible explanations for the existence of such a potentially detrimental process have been outlined before (see section 3.1).

Initial studies in yeast had already indicated the consequences of adaptation. In these experiments, preventing adaptation using the adaptation-deficient *cdc5-ad* allele decreased the frequency of translocations and chromosome loss induced by X-ray treatment (Galgoczy and Toczyski, 2001) suggesting adaptation as a major source of genome instability. On the other hand, this study also clearly pointed out that the ability to undergo adaptation was required to achieve maximal cell viability in response to irreparable DNA damage. In other words, adaptation allows cell to acquire damage resistance at the expense of genomic aberrations. Already in this initial study, the authors reasoned that there might be no beneficial effect of checkpoint adaptation on damage resistance (i.e. the survival after damage) in haploid cells. They proposed this was due to the fact that adaptation causes chromosomal rearrangements and loss of genetic information that cannot be tolerated by haploid cells. Rather, they used diploid yeast cells to study the consequences of adaption with respect to genome stability and therefore, mimic the genetic situation in diploid cells of higher eukaryotes.
Employing a similar experimental set-up, we were able to recapitulate the observation that diploid yeast cells harbouring a homozygous deletion of the *RAD52* gene were eventually able to recover from DNA damage induced either by CPT treatment or X-ray exposure (Figure 12 and (Galgoczy and Toczyski, 2001)). Strikingly, preventing adaptation using the *cdc5-ad* allele lead to a decrease in colony formation after genotoxin exposure (either CPT or X-ray, Figure 12 and (Galgoczy and Toczyski, 2001)). In the study that identified the *cdc5-ad* allele, approximately 90% of *rad52cdc5-ad* cells experiencing an irreparable nuclease-induced DSB remained arrested (Toczyski et al., 1997). In our experiments, we observed an approximate reduction of cell survival (i.e. adaptation) of 2.5-fold in the case of CPT and 5.5-fold for X-ray treatment (Figure 12) which is likely to be explained by differences in the damage source. In agreement with the hypothesis that it is indeed checkpoint adaptation that allows *rad52* mutants to grow in this situation, we found that abolishment of a functional Rad53-mediated checkpoint abolished the effect of the *cdc5-ad* allele (Figure 12). Using the *cdc13-1* mutation to elicit telomere dysfunction, we also observed a failure of *cdc5-ad* to rescue the viability in Rad53-

compromised cells (Figure 9 and discussion above). Taken together with our observations in *rad52* cells, this suggests that the effect of *cdc5-ad* is mediated by active Rad53 signalling.
Having observed that adaptation allows *rad52* mutants to form colonies after genotoxic treatment, we set out to investigate these adapted mutants. When we challenged them with a second genotoxic treatment, we observed that adapted HR-defective *rad52* cells had a growth advantage compared to the parental strain that had never been challenged before (Figure 13). Strikingly, *rad52* mutants that had adapted to CPT-induced DNA damage showed this improved growth in response to a broader variety of damaging agents, including the DSB-inducing radiomimetic drug bleomycin and UV irradiation, which mainly induces bulky DNA adducts. This difference in the response to CPT and X-ray treatment could be due to different modes of actions, the cellular response in terms of repair pathway choices and treatment regimes. CPT is present continuously in our experiments and its toxicity requires cell proliferation as it causes the stabilization of the transient Top1-cc involving the Top1 protein and the DNA. Consequently, this stabilized complex leads to collisions with the replication and transcription machinery ultimately resulting in DSBs (reviewed in (Pommier et al., 2010)). On the other hand, X-rays are used as a single-dose damaging agent and due to their ionizing potential induce a variety of lesions affecting both bases and the DNA backbone. In order to address if this difference comes from the exposure duration, the delivery of X-rays in fractionated doses could be employed. During radiotherapy, fractionation of irradiation has been shown to promote the occurrence of radioresistant cancer cells (reviewed in (Kim et al., 2015)). These findings suggest that indeed, the amount of damage delivered at a given time point largely affects the cell`s probability to survive. With adaptation being the cause for cell survival in the presence of damage, this again would argue that the decision to undergo adaptation is determined by the damage load, as it has been suggested for yeast (Lee et al., 1998). However, also the second possibility to explain the difference between CPT and X-rays, which is the complexity of the induced damage, might influence adaptation. In support of this idea, we observe approximately 80% survival in *rad52* cells after CPT treatment while less than 10% of mutants are able to form colonies after X-ray exposure (Figure 12).

An important open question concerns the repair pathways employed in *rad52* mutants. We observe that *rad52* cells still harbour DNA damage after forming a colony and further passages in unchallenged conditions (Figure 19). This observation could have different explanations: either repair has been successful and the observed damage is due to spontaneous and yet unrepaired damage or damage has only been repaired partially (and presumably faulty). It has been shown that although *rad52* mutants undergo adaptation, they repair DNA damage, at least to an extent (Galgoczy and Toczyski, 2001). One explanation for this could be that cells attempt to lower the DNA damage load to allow adaptation. This idea would be consistent with the hypothesis that the decision to undergo adaptation is influenced by the amount of damage present in the cell. We were therefore interested in understanding possible repair pathways used in *rad52* mutants to be able to form colonies after genotoxic treatment.
Rad52 is a crucial mediator of homology-directed repair (HDR) as it is required to remove the ssDNA binding protein RPA from the newly generated ssDNA in the early steps of DSB site processing (Game and Mortimer, 1974; Sung, 1997a). RPA removal is the necessary step to facilitate binding of Rad51 resulting in the formation of a nucleoprotein filament that scans the

genome searching for a homologous repair template (reviewed in (Symington et al., 2014)). However, Rad52-independent repair pathways have been documented in yeast. For example, in the presence of extensive homology around the break site as found at the rDNA locus, Rad52 is dispensable for HR-mediated repair (Ozenberger and Roeder, 1991). Still, yeast cells lacking *RAD52* display an approximately 500-fold decrease in repair efficiency by homologous recombination (Bartsch et al., 2000) and therefore, deletion of *RAD52* in yeast can be used as a tool to study a defect in HDR. This raises the question if there are other repair pathways used in *rad52* mutants to allow colony formation after genotoxic treatment. NHEJ could represent a reasonable candidate to facilitate repair in the absence of HR. However, diploid cells actively suppress the error-prone NHEJ pathway through the a1-α2 heterodimer encoded by the heterozygous mating loci. Thus, HR is used as the preferred repair mechanism in all cell cycle stages due to the availability of a homologous repair template (Frank-Vaillant and Marcand, 2001; Kegel et al., 2001; Valencia et al., 2001). Nevertheless, we wanted to address the possibility that diploid *rad52* mutants aberrantly succeeded in NHEJ and therefore were able to repair CPT- and X-ray- induced damage. Deletion of *DNL4*, which encodes DNA Ligase IV required during NHEJ, did not affect cell survival after genotoxic treatment compared to NHEJ-proficient *rad52* mutants (Figure 18). Therefore, we conclude that HR-defective yeast cells do not form colonies upon genotoxic treatment after NHEJ-mediated repair based on three different observations: (1) The ligation step is essential for successful NHEJ, (2) Dnl4 is the only ligase implicated in this process so far and (3) NHEJ is suppressed in diploid yeast cells.

In an attempt to gain further insight into the possible involvement of other repair pathways used in *rad52* mutants, we analysed their transcriptional response to genotoxic treatment (both CPT and X-ray, Figure 20). Strikingly, we observed already a dramatic change on the transcriptional level in response to *RAD52* deletion. Nevertheless, analysing significantly different expressed genes in *rad52* mutants after genotoxic treatment compared to the parental *rad52* strain revealed a common transcriptional pattern. Of note, this pattern was different from the changed expression behaviour induced by loss of Rad52 *per se* indicating that the pattern reflects the response to genotoxic treatment. Another interesting observation is the number of significantly different expressed genes in our comparisons (Figure 20). While we found 119 genes differentially expressed in CPT-treated cells, only 4 were found altered in X-ray treated *rad52* mutants. This could be due to the different natures of the genotoxic agents (as discussed above) or reflect different consequences on the cellular physiology, for example on the ploidy (discussed below). Break-induced replication (BIR), an error-prone and mutagenic repair pathway, has been reported to play an important role during adaptation (Galgoczy and Toczyski, 2001). This study utilized *rad51* mutants to investigate the role of BIR during adaptation. In the absence of *RAD51*, cells can still perform BIR, however, this is not the case for loss of *RAD52* (reviewed in (Symington et al., 2014)). Because we are employing *rad52* mutants, we hypothesize that BIR is not a relevant repair pathway to allow the formation of genotoxin resistant colonies in our experimental set-up.

In addition to HR and classical NHEJ as the major DSB repair pathways, an alternative NHEJ mechanism using microhomology-mediated end joining (MMEJ) has been described. During alternative NHEJ, a DSB is repaired using 2 to 20 bp stretches of overlapping sequence (microhomology) and does not require classical repair proteins such as Rad52 or the Ku complex. This process is considered highly error-prone and therefore, could contribute to

genome instability due to its propensity to cause deletions and genomic rearrangements (reviewed in (Seol et al., 2017)). It is tempting to speculate that survival of *rad52* mutants after genotoxic treatment could be mediated by this alternative repair mechanism and future experimental work will focus on a potential role of alternative NHEJ using microhomologies in this context.

It could also be possible for *rad52* mutants to somehow re-establish HR. Plasmid-based assays to monitor a cell's ability to use HR to repair genotoxin-induced damage to the plasmid are commonly used to address this question. However, depending on the experimental set-up, these assays differ in the exact HR pathway and genetic requirements necessary to repair the reporter construct and therefore appeared not suitable in our situation. A second possibility allowing genotoxin-treated *rad52* cells to perform HDR could be given by their diploidy which is still manifested by the heterozygous mating locus although cells might have experienced extensive chromosome loss. Therefore, a possible explanation for *rad52* survival in the presence of genotoxins could be the use or upregulation of recombination proteins normally used during meiosis, for example the meiosis-specific recombinase Dcm1 (Bishop et al., 1992). However, we did not observe the differential expression of such meiosis-specific factors after genotoxic treatment in our transcriptional analysis. Alternatively, one could speculate that diploid *rad52* mutants could decrease the damage load by meiotic division and thus, display a haploid karyotype observed in our analysis. We addressed this possibility by deleting *SPO11*, which encodes a meiosis-specific endonuclease that is required to induce DSBs in order to initiate meiotic recombination. Therefore, *spo11* mutants fail to produce viable spores during meiosis (Bhuiyan and Schmekel, 2004), reviewed in (Lam and Keeney, 2014)). The absence of *SPO11* did not change the survival rate of *rad52* mutants in the presence of CPT suggesting that chromosome loss as a mean to decrease the amount of DNA damage is not the case for the observed growth of *rad52* mutants after genotoxic treatment. Moreover, loss of *RAD52* as a central factor for recombination renders cells meiosis-defective (reviewed in (Symington, 2002)) providing additional support for our conclusion. Furthermore, one would expect that regular meiosis yields a euploid karyotype, yet we consistently observe aneuploid *rad52* mutants monosomic for chromosome III suggesting that our observations are not due to meiosis.

As outlined briefly in section 1.4, the localization of damaged DNA within the nuclear space appears as an important determinant of repair pathways and outcomes. Damage sites that are more challenging to repair or irreparable relocalize to nuclear pores or the inner nuclear membrane. The differential localization seems to depend on the cell cycle stage since relocalization to the inner nuclear membrane was observed during G2 and S phase while association of damaged DNA with nuclear pore complexes was found throughout the cell cycle. The mechanisms facilitating the relocalization steps are not yet fully understood but SUMOylation seems to play an important role (reviewed in (Freudenreich and Su, 2016)). In addition, also the DDC factors Mec1 and Rad9 as well as recombination proteins seem to be involved in relocalization of damaged DNA, possibly due to their role in facilitating mobility of a DSB (see section 1.4.2.1, (Dion et al., 2012)). The insights provided so far suggest that relocalization of damaged DNA could allow alternative repair mechanisms. It has been hypothesized that this occurs to prevent cell death and gross chromosomal rearrangements (GCR) although alternative, less preferred repair pathways are usually associated with the risk of mutations (reviewed in (Freudenreich and Su, 2016)). Consistently, artificial tethering of a

damaged locus, in this case a subtelomeric DSB, to the nuclear pores was shown to increase BIR (Chung et al., 2015). BIR is proposed to increase genome instability by producing GCRs (reviewed in (Anand et al., 2013)). However, the increase in BIR in such a scenario seemed dependent on Rad52 as well as other factors previously linked to BIR ((Chung et al., 2015), reviewed in (Anand et al., 2013)). Therefore, it seems unlikely that the irreparable damage created in our experiments in *rad52* cells trigger such ectopic BIR events. Importantly, Rad52 itself does not seem to be required for the relocalization of damaged DNA to the nuclear periphery *per se,* at least based on observations using an endonuclease-induced DSB (Horigome et al., 2014). The positioning of damaged DNA within the nuclear space might play an important role in understanding possible repair mechanisms in *rad52* cells leading to genotoxin resistance and future studies will try to gain insights into this.

3.4.2 Rapamycin and its influence on DNA repair

Our findings demonstrate a role for rapamycin in preventing adaptation (this thesis and (Klermund et al., 2014)). Importantly, in our experiments using the *cdc13-1* mutant to elicit telomere dysfunction that results in checkpoint activation, we could confirm that rapamycin did not affect recovery from the checkpoint as rapamycin-treated *cdc13-1* mutants were fully viable and proficient in colony formation after shifting them back to the permissive temperature (Figure 8). Likewise, recovery-defective mutants did not interfere with the rapamycin-mediated rescue of *cdc13-1* cells (J. Klermund, personal communication) suggesting that rapamycin did not affect repair in our experiments. In general, studies in human cells have demonstrated a negative effect of rapamycin on HR: rapamycin treatment caused the transcriptional suppression of SUV39H1 (Mo et al., 2016), a chromatin compaction factor with an emerging role during the DNA damage response and HR-mediated repair (Ayrapetov et al., 2014). In addition, rapamycin impairs BRCA1 and RAD51 recruitment and thereby negatively effects HR efficiency (Chen et al., 2011a).

It has been established that rapamycin treatment leads to the induction of autophagy and this anabolic pathway is emerging as an important player in the DNA damage response as well as in DSB repair pathway choice. In yeast, autophagy is induced by DNA damage to facilitate cell cycle arrest (Dotiwala et al., 2013; Eapen and Haber, 2013), and a specific autophagic subpathway termed genotoxin-induced targeted autophagy (GTA) depending on checkpoint proteins has been identified recently (Eapen et al., 2017). Also in mammalian cells, DNA damage has been shown to cause autophagy induction, however, this has been described to function both as a pro- and anti-survival signal. The consequences of autophagy induction seem to be dependent on p53 signalling which has a bi-functional role as an inducer and inhibitor of autophagy in a damage situation (reviewed in (Rodriguez-Rocha et al., 2011)). Moreover, a recent report has suggested autophagy as a protective mechanism for cells against the adverse effects of aneuploidy (Ariyoshi et al., 2016). In summary, rapamycin can modulate damage repair at least via 3 different mechanisms: via the induction of autophagy downstream of TOR signalling and in a transcription-dependent manner as in the case of SUV39H1. Moreover, the fact that proteins involved in the DNA damage checkpoint such as the MRX complex are crucial factors for DNA repair already indicates cross-talk between these two fundamental processes. Thus, it is not surprising that checkpoint factors determine repair efficiency and outcomes

(Haghnazari and Heyer, 2004). Therefore, as a third possibility, rapamycin could modulate DNA repair also indirectly by maintenance of the checkpoint to prevent adaptation.

Rapamycin has been identified as a specific inhibitor of TORC1 signalling by forming a complex with the cellular FKBP12 protein and eventually, forming a ternary complex consisting of rapamycin-FKBP12-TORC1 (reviewed in (Loewith and Hall, 2011)). The binding interface of the rapamycin-FKBP12 complex is adjacent to the TOR kinase domain, however, it remains unclear how TOR signalling is inhibited mechanistically in the presence of rapamycin. It seems unlikely that rapamycin directly inhibits TOR kinase activity since rapamycin treatment arrests cells in G1 while abolishing Tor2 kinase activity (which participates in both TORC1 and 2) by mutation is lethal (reviewed in (Raught et al., 2001)). The observation that rapamycin requires FKBP12 to have an inhibitory effect while loss of *FPR1*, which encodes the yeast FKBP12, does not have an obvious phenotype, led to a new idea of drug activity where the drug requires a cellular component and only then can exert its function (reviewed in (Loewith and Hall, 2011)). Despite the incompletely characterized mode of action, rapamycin is considered a specific inhibitor for TORC1 in all model organisms. This has been recently confirmed by the finding that Avo3, a subunit unique to TORC2, masks the binding site of the rapamycin-FKBP12 complex in Tor2 when it is engaged in TORC2 (Gaubitz et al., 2015). In addition to a link between the DNA damage response and TORC1, also TORC2 is involved in ensuring genome stability. This mainly has been attributed to TORC2`s role in actin cytoskeleton organization (Shimada et al., 2013).

3.4.3 A connection between adaptation and aneuploidy

It has been noted a long time ago that *rad52* mutants lose chromosomes at a higher frequency than wild-type cells and these chromosome loss events were found exacerbated after DNA damage (Mortimer et al., 1981). In addition, adaptation was shown to cause and precede different kinds of genetic aberrations including translocations and chromosome loss (Galgoczy and Toczyski, 2001). Although the majority of human cancer cells undergoing adaptation were found to die, surviving adapted cells could pass on damaged genetic material thereby contributing to cancerogenesis. This hypothesis is supported by experiments using plant cells: in this set-up, adapted cells entered mitosis harbouring broken or acentric chromosomes as well as chromosome bridges (Carballo et al., 2006). Similarly, a connection between adaptation in human cancer cells and the incidence of micronuclei has been described (Chang et al., 1999). Micronuclei frequently contain damaged or broken DNA and are associated with errors during mitosis, aneuploidy and genome instability (reviewed in (Kalsbeek and Golsteyn, 2017)). Indeed, DNA content analysis of *rad52* mutants that formed a colony after genotoxic treatment revealed chromosome losses to varying degrees (Figure 14 and 15) suggesting that adaptation was accompanied by chromosome loss events. However, based on a flow cytometry read-out we could not determine the exact extent of chromosome loss and if the loss events affected whole chromosomes. Therefore, we used whole genome sequencing to assess the karyotype of adapted *rad52* mutants. This in-depth analysis revealed extensive whole chromosome loss events resulting in a nearly haploid DNA content after CPT treatment and a mix of different ploidies in response to X-ray exposure (Figure 15). The different karyotypes characterized by

more or less pronounced loss of chromosomes in response to X-rays seemed to be unstable as *rad52* mutants continued to lose genetic material in subsequent passages (Figure 16). This could be due to subsequent adaptation events, however, we cannot conclude this from our experiments. Interestingly, we observed a preferred maintenance of chromosome III (Figure 15). This chromosome is the second smallest in *S. cerevisiae* with 184 open reading frames (covering 75% of the sequence). A striking feature of chromosome III is that it harbours the mating type locus. As the strains used in our studies have been created by mating isogenic haploid strains of mating type a and α, the resulting diploid yeast cells are homozygous except for the mating locus. Heterozygosity at the mating type locus has long been known to influence sensitivity to DNA damage (Heude and Fabre, 1993). It has been reported that several genes under the control of the mating type locus could suppress the sensitivity of recombination mutants including cells with *RAD52* truncations to DNA damaging agents. Interestingly, loss of the prolyl isomerase Fpr3 rescued the CPT sensitivity of Rad52 truncation mutants, however, the *FPR3* gene itself did not appear to be regulated directly by the mating type locus (Valencia-Burton et al., 2006). Fpr3 belongs to a family of prolyl isomerases that also includes Fpr1, the yeast rapamycin-binding protein required for the rapamycin-mediated inhibition of TORC1 (reviewed in (Loewith and Hall, 2011)). Thus, Fpr3 was described as a novel rapamycin-binding protein localizing to the nucleolus. However, the authors proposed that despite the formation of a rapamycin-Fpr3 complex, this did not cause a growth defect upon rapamycin treatment due to TORC1 inhibition because of the limited accessibility of the cytoplasmic TOR complex by the nucleolar Fpr3-rapamycin complex (Benton et al., 1994). Taking these observations into account, TOR signalling is presumably not involved in this phenomenon but rather TOR-independent functions of Fpr3.

A study interested in the effect of aneuploidy on heterochromatin silencing found that especially chromosome III and X were gained in randomly created aneuploid yeast strains with a silencing defect (Mulla et al., 2017). The authors further commented that gain of chromosome III alone seem insufficient to cause a silencing defect and argued that an abnormal silencing pattern might not necessarily be a consequence of aneuploidy but rather, aberrant copy numbers of a set of chromosomes contribute to this phenotype.

Studies of aneuploidy in yeast have often used sets of disomic yeast strains, i.e. cells that only harbour one additional copy of a specific chromosome. Strikingly, strains disomic for chromosome III or VI are usually not included in these studies. In the case of chromosome VI, this is due to the detrimental effects caused by the overexpression of some genes, including the *TUB2* gene. Simultaneous overexpression of *TUB1* allows the generation of cells disomic for chromosome VI. In the case of chromosome III, the presence of the mating locus has been suggested as an inhibitory factor for the creation of chromosome III disomic strains. However, disomic strains are usually obtained due to the stochastic and spontaneous failure of chromosome segregation. In contrast, another method based on the specific inhibition of the segregation of a target chromosome yielded yeast cells disomic for chromosome III and VI (Anders et al., 2009).

A beneficial effect of a "selectively aneuploid" karyotype, in this case a monosomy for chromosome VIII, has been reported in the context of telomerase insufficiency in yeast (Millet et al., 2015). In this case, growth of haploid yeast cells at high temperature resulted in telomere shortening caused by limited telomerase activity. Yeast survivors emerged that exhibited a near-

diploid karyotype with duplicated chromosome copy number for all chromosomes except chromosome VIII. Further analysis revealed that chromosome VIII encodes the TORC1 subunit Kog1. The authors suggested that by decreasing *KOG1* expression, TOR complex 1 assembly was hampered leading to decreased activity. Ultimately, this resulted in a decrease in ribosome biogenesis and shifted the capacity of the cellular translation machinery towards less abundant proteins including telomerase holoenzyme components (Millet et al., 2015). These findings highlight that modulation of the copy number of a single chromosome can have fundamental impact on cell physiology and allow an adaptation to a changing environment.
In summary, the reason why initially diploid *rad52* cells that experience DNA damage and chromosome loss leading to aneuploidy have a propensity to maintain chromosome III in two copies remains elusive. Further, future studies will have to address if this selective retention of chromosome III is related to the influence of the mating type locus or caused by other unknown factors.

Independently of the mating locus influence, the ploidy status itself has been linked to damage resistance and the stability of the cellular genome. For example, a study using tetraploid yeast cells (increased ploidy but not aneuploid) identified genes that caused "ploidy-specific lethality" and further analysis of these genes revealed their involvement in ensuring genome stability (Storchova et al., 2006). In agreement with the idea that increased ploidy is a challenge for genome stability, tetraploidization in human cells appears to promote chromosomal instability and render the cells more tolerant to mitotic errors and genotoxic treatment (Kuznetsova et al., 2015). However, studies in yeast also suggested that an increased base ploidy can partially buffer adverse aneuploidy-associated effects, i.e. a tetraploid cell is more tolerant to an additional chromosome than a diploid cell (Beach et al., 2017).
In our experiments, we observed a growth advantage of haploid cells compared to diploid cells in response to both CPT and X-ray exposure (Figure 16). A likely explanation for this is the ability of haploid cells to repair damage via NHEJ, which is prevented in diploid cells (as discussed above). Therefore, the improved survival of haploid cells should be abolished in *rad52dnl4* double mutants compromised in both NHEJ and HR pathways. However, these findings seem to be in contrast to previous findings (for example in (Galgoczy and Toczyski, 2001)) which show that diploid yeast cells have an increased tolerance to damage compared to haploids. These different findings could be due to the time frame and set-up of the experiments. While the previous study quantified colony formation after genotoxic treatment in a plating assay (Galgoczy and Toczyski, 2001), we used a more qualitative approach in our spotting assay (Figure 16). Therefore, it is possible that after longer incubation or in a plating assay to quantify colony numbers, we would also eventually observe the expected growth advantage in diploid *rad52* mutants. While adapted diploid cells should be able to form colonies, adapted haploid *rad52* mutants would probably fail to do so due to lethal rearrangements or chromosome loss, as reasoned by previous studies.
It could be possible that near-haploid *rad52* mutants observed after CPT treatment switch between a near-haploid and diploid state by mating with each other and thereby are more resistant to genotoxic treatment, as expected for a diploid cell. We speculate that such a switch between haploid and diploid phase is not the cause for our observations since neither our flow cytometry-based DNA content analysis nor the sequencing-based karyotyping indicated such a

behaviour. However, when we tested the ability of adapted and aneuploid *rad52* mutants created by CPT treatment, we observed a bipolar mating pattern (J. Klermund, personal communication). To rule out the possibility that *rad52* mutants in our experimental set-up transit from a nearly-haploid state to a diploid state by mating, we will employ sterile *rad52* mutants that are unable to mate with each other. In support of our hypothesis that mating most likely does not occur, one would expect that the diploids resulting from mating between near-haploid *rad52* mutants would have to undergo meiosis to exhibit the (near-) haploid karyotype that we observe in our analysis (flow cytometry and DNA sequencing). However, we could already demonstrate that the decreased chromosome number in *rad52* mutants is most likely not due to meiosis (as discussed above).

3.4.4 The relationship between adaptation, aneuploidy and genome instability

Studies in yeast cells were crucial to get insights into the connection between adaptation and genome instability. Adaptation has been identified as the causative event preceding multiple forms of genomic rearrangements that compromised genomic stability (Galgoczy and Toczyski, 2001). At the same time, aneuploidy has been described as a driver for genome instability including increased mutation rates, sensitivity to genotoxic treatment and further instability affecting whole chromosomes (Sheltzer et al., 2011). However, the relationship between adaptation, aneuploidy and genome instability has not been fully understood. Using HR-defective *rad52* yeast cells, we could show that adaptation is linked to the development of an aneuploid karyotype as the vast majority of adaptation-proficient *rad52* mutants that was able to form a colony after genotoxic treatment showed chromosome loss events to varying degrees. When adaptation is prevented, spontaneous and X-ray-induced chromosome loss rates in *rad52* cells are decreased (Galgoczy and Toczyski, 2001) and also the number of colonies formed by *rad52* mutants after genotoxic treatment is diminished (Figure 12). Nevertheless, all resistant *rad52cdc5-ad* mutants that we analysed showed the same characteristics as adaptation-proficient cells. The ability of these cells to adapt despite the *cdc5-ad* allele could be due to other mechanisms influencing adaptation or an incomplete prevention of adaptation by the *cdc5-ad* allele. The idea that also other factors could be regulating adaptation in *rad52* cells is supported by the finding that rapamycin and the *cdc5-ad* allele are partially synergistic (significant synergy in response to CPT treatment but not X-ray exposure, Figure 22). *Vice versa*, these findings also indicate that rapamycin has a checkpoint-independent function. This is in agreement with our studies on the regulation of adaptation using the *cdc13-1* model system which revealed a checkpoint-dependency for the *cdc5-ad* allele (Figure 9) thereby placing Cdc5-ad and Rad53 in the same genetic pathway. Indeed, while rapamycin was able to phenocopy the effect of the *cdc5-ad* allele in preventing the survival of *rad52* mutants after genotoxic treatment, this was independent of a Rad53-mediated checkpoint. This observation raised the possibility that rapamycin could prevent resistant and aneuploid *rad52* mutants by a mechanism other than preventing checkpoint adaptation. To address this concern, we performed a microcolony assay, a classical experimental set-up to study adaptation. Indeed, rapamycin was able to maintain the checkpoint-induced cell cycle arrest in *rad52* cells in the presence of CPT (Figure 22). This is consistent with our previous findings using *cdc13-1* mutants to investigate mechanisms involved in the regulation of adaptation and demonstrates the

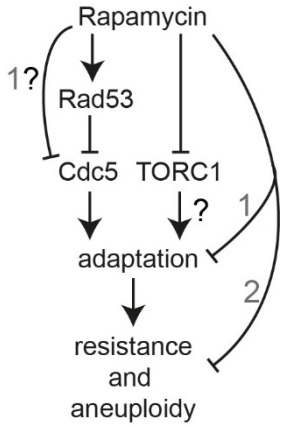

Figure 26. A model for the dual function of rapamycin to prevent adaptation and resistance associated with aneuploidy. Rapamycin prevents adaptation directly by inhibiting TORC1 and possibly by regulating Cdc5 levels (described as pathway 1). In addition, rapamycin sensitizes aneuploid cells that are resistant to genotoxic treatment (pathway 2).

involvement of the nutrient signalling TORC1 pathway in preserving genome stability. Interestingly, aneuploid cells, both in yeast and mammalians, are sensitive to compounds inducing metabolic stress, such as rapamycin (Tang et al., 2011; Torres et al., 2007). This is also the case in aneuploid *rad52* mutants after X-ray exposure as rapamycin significantly increases the doubling time even in nutrient-rich conditions (O. Vydzhak, personal communication). As aneuploid cells exhibit elevated levels of proteotoxic stress due to protein imbalances (see 1.6.1) (Donnelly et al., 2014; Oromendia et al., 2012; Tang et al., 2011; Torres et al., 2007), these cells have an increased energy demand thereby likely explaining their hypersensitivity to starvation-mimetic compounds. As rapamycin treatment leads to the induction of autophagy and since autophagy is up-regulated in aneuploid cells (Ariyoshi et al., 2016; Stingele et al., 2012), rapamycin could also be toxic to aneuploid cells by further increasing autophagy. It has become clear that sustained autophagy is linked to cell death, however, the causal relationship is not fully understood (reviewed in (Kroemer and Levine, 2008)). We suspect that the checkpoint-independent effect of rapamycin in decreasing the survival of *rad52* mutants after genotoxic treatment could be due to the induction of autophagy. However, we have not yet addressed this possibility in our experiments. A recent study has suggested that aneuploidy-induced imbalances in protein homeostasis that lead to proteotoxic stress, cause the upregulation of master regulators of autophagic and lysosomal degradation (Santaguida et al., 2015). Consistently, aneuploid cells are not only sensitive to inhibitors of autophagy but also to compounds that interfere with lysosomal function (Tang et al., 2011). It is tempting to speculate that the aneuploidy-associated increase of degradation products in the vacuole, which is the yeast equivalent of mammalian lysosomes, could trigger the activity of TORC1 via the EGO complex, which responds to intravacuolar nutrients. Thereby, aneuploidy could ultimately facilitate further adaptation by increasing TORC1 activity. However, this hypothesis lacks experimental proof and requires validation. Such a scenario where TORC1 is hyperactivated is reminiscent of the situation in many cancers that show aberrant high mTOR signalling accompanied by a failure to downregulate this activity in response to DNA damage ((Zhou et al., 2017), reviewed in (Xie et al., 2016)).

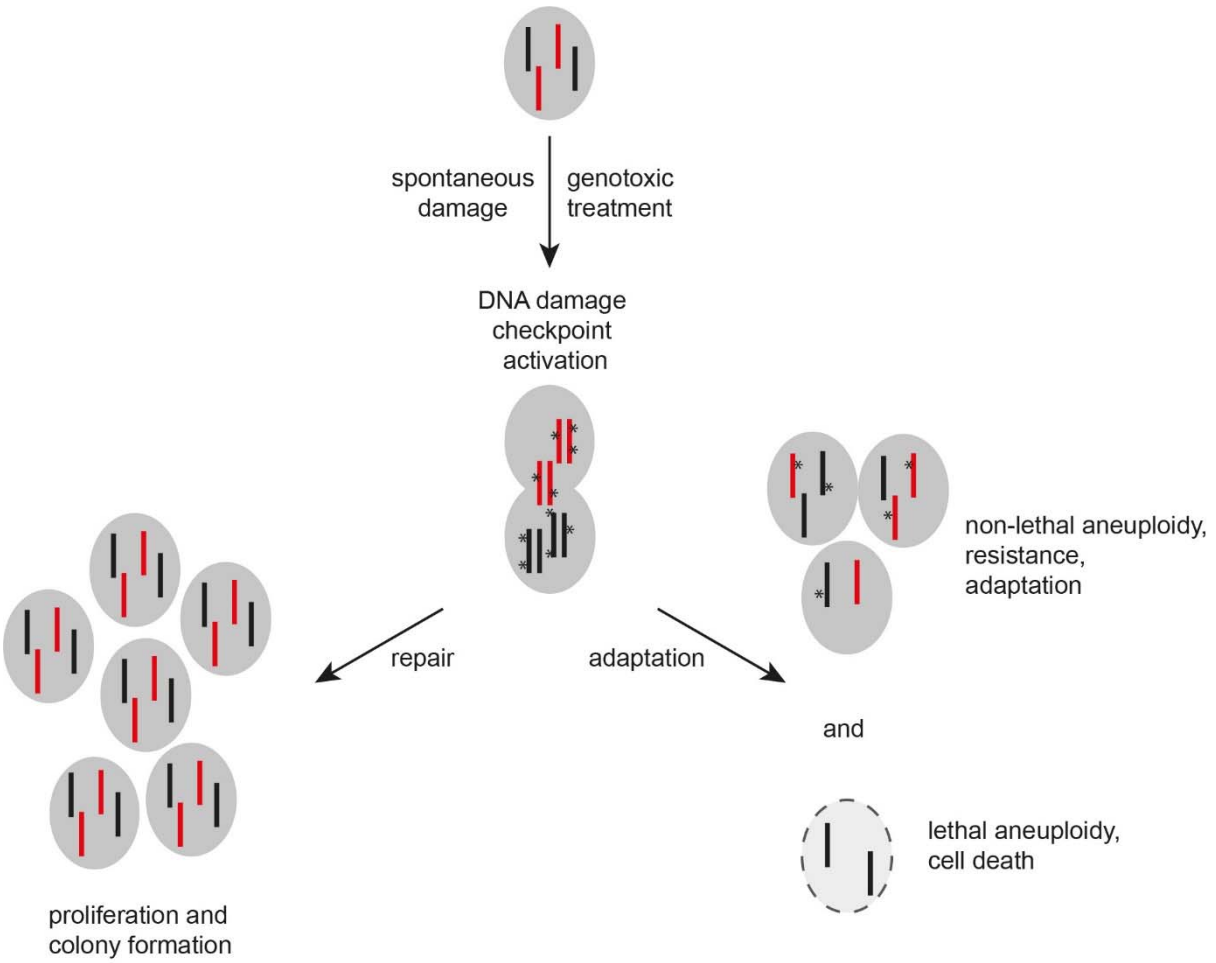

Figure 27. Repair and adaptation as fundamentally different consequences of DNA damage checkpoint activation. In the absence of repair, adaptation is the only possible route to attempt cell survival. However, also adaptation can have two possible outcomes: it can lead to further adaptation events in subsequent cell cycles, non-lethal aneuploidy and possibly other genome instabilities and resistance to genotoxic treatment. Alternatively, adaptation can result in cell death due to multiple reasons including loss of essential genetic information caused by lethal aneuploidy.

In addition to the previously reported rapamycin sensitivity of aneuploid cells (see above), we could also show that aneuploid *rad52* cells exhibited proteotoxic stress. Transcriptome analysis of cells that were able to form colonies after CPT treatment and had acquired an aneuploid karyotype showed an upregulation of transcripts that encode for factors involved in protein folding and refolding (Figure 20). This suggests that also aneuploid *rad52* mutants after genotoxic treatment suffer from proteotoxic stress, a well-established and characteristic trait of aneuploidy (reviewed in (Oromendia and Amon, 2014)).

Taken together, we speculate that in *rad52* mutants treated with genotoxins, rapamycin could have a dual function: in agreement with our previous results, rapamycin can prevent adaptation and thereby decrease the number of damage-resistant and aneuploid *rad52* mutants. However, in case these cells adapt, rapamycin can be used as a sensitizing agent in the aftermath of adaptation (Figure 26). The role of autophagy induction for the prevention of adaptation and the sensitization of aneuploid *rad52* mutants will be addressed in future studies. We envision that the checkpoint-independent effect of rapamycin, indicated by its ability to prevent the

survival of *rad52* cells after genotoxic treatment in a checkpoint-compromised genetic background (Figure 12, 22 and 26), is due to its role in autophagy induction and sensitization of aneuploid cells.

Based on our findings and previous studies conducted by many others, we hypothesize that in the absence of faithful and error-free DNA damage repair, cells undergo adaptation as a means to maximize cell viability. In order to allow adaptation or as a result, cells experience whole chromosome loss events and acquire an aneuploid karyotype. These genetic alterations allow them to survive in the presence of further genotoxic agents, possibly acting as a trailblazer for further rounds of adaptation (Figure 27). An indication that genome instability can also act as a driver for adaptation is given by the observation that a fusion oncogene, resulting from a frequently found translocation in a subtype of rhabdomyosarcomas, drives the transcription of crucial adaptation-promoting factors including PLK1 (Kikuchi et al., 2014). However, our results cannot establish the exact causal relationships between adaptation, aneuploidy and genome instability. One possibility would be that adaptation causes genome instability by promoting aneuploidy (scenario A, Figure 28) or in conjunction with an aberrant karyotype (scenario B, Figure 28). However, it is also reasonable that aneuploidy, facilitated by the inherently unstable karyotype of repair-defective mutants, allows adaptation. In the second scenario, adaptation could be the initial cause for the inherently unstable and altered karyotype (scenario C, Figure 28). In order to get insights into the interconnections between adaptation and aneuploidy, we overexpressed Cdc5 in repair-proficient wild type yeast to investigate if accelerating adaptation could lead to aneuploidy (Figure 17). Overexpression of *CDC5* has been shown to override the checkpoint and accelerate the adaptation process (Donnianni et al., 2010; Pellicioli et al., 2001; Vidanes et al., 2010), however, we could not observe indications for aneuploidy in response to X-ray treatment in this experiment. Importantly, in this experimental set-up, adaptation is not the only possibility for cell survival as cells were repair-proficient. Another caveat is the time required to develop an aneuploid karyotype. It could also be possible that multiple successive adaptation events are required to become aneuploid. We cannot exclude this possibility based on our experimental data as we usually passage *rad52* mutants that had formed a colony after genotoxic treatment once more in the absence of exogenous damage before subsequent analysis (to obtain more cells). Therefore, it is possible that further adaptation events occur as cells proliferate and only these lead to aneuploidy. Nevertheless, the results described in Figure 17 might suggest that accelerated adaptation alone, caused by increased levels of Cdc5, is not sufficient to induce aneuploidy. In contrast, a recent report revealed that PLK1 overexpression in combination with irradiation was sufficient to promote tumorigenesis accompanied by aneuploidy in mice. The authors suggested that this was due to decreased checkpoint signalling resulting from PLK1-mediated transcriptional repression of DDC factors and reduced phosphorylation of critical DDC players (Li et al., 2017). These observations suggest a causative role for adaptation in the development of an aneuploid karyotype. Further experimental work will be required to improve our understanding of this relationship.

Genome instability has already been recognized as an enabling characteristic of cancer cells (reviewed in (Hanahan and Weinberg, 2011)) and also adaptation and aneuploidy are emerging players in cancerogenesis. Interestingly, p53, together with ATM, has been proposed as an aneuploidy-counteracting factor in human cells and by doing so, limit aneuploidy-induced

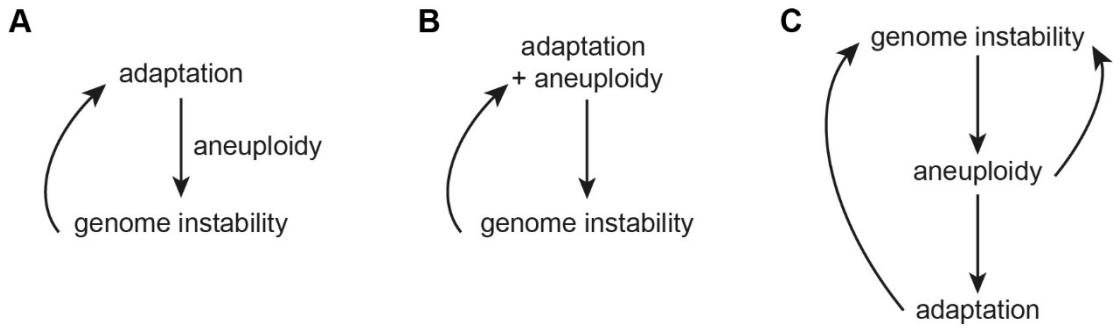

Figure 28. Models for the relationships between adaptation, aneuploidy and genome instability. Panel A describes adaptation as a cause for genome instability thereby promoting aneuploidy. Panel B depicts a scenario in which adaptation and aneuploidy together give rise to genome instability. Alternatively, genome instability could lead to aneuploidy and further facilitate adaptation. Aneuploidy could thereby also cause the initial genome instability (depicted in panel C).

tumorigenesis (Li et al., 2010; Thompson and Compton, 2010). At the same time, p53 is listed as one of the most common mutations across all cancers at this point (Kandoth et al., 2013) and ATM belongs to the group of genes that are most likely to carry a cancer-driving mutation (Greenman et al., 2007). These findings indicate that cells have not only evolved elaborate pathways to counteract aneuploidy, they also do so by using the same mechanisms that deal with DNA damage which highlights the detrimental effects of aneuploidy on genome stability.

3.4.5 Adaptation, aneuploidy and drug resistance

Even though the initial causes and ultimate consequences of adaptation, aneuploidy and genome instability are not yet fully established, their importance especially with regards to cancer has become clear. Based on our observations, we hypothesize that adaptation is the cause for the acquisition of genotoxin resistance in *rad52* mutants and we speculate that the resistance to damage is accompanied by aneuploidy (Figure 27). Although we observe that colonies formed by adapted *rad52* cells in response to genotoxic treatment are initially metabolically compromised as evident from the accumulation of Phloxine B (Minois et al., 2005) and even contain a large fraction of dead cells (Figure 12 and 16), cellular fitness seems to improve over time with successive passaging in the absence of exogenous damage. This could be explained by our observation that karyotypically unstable cells that show a DNA content intermediate between haploid and diploid (after X-ray treatment) continuously lose chromosomes until they reach a seemingly more advantageous near-haploid DNA content (Figure 16). Moreover, this could also be supported by the idea that aneuploid cells have the ability to adjust faster to a changing environment and thus have a growth advantage although they exhibit various growth defects during cultivation (reviewed in (Giam and Rancati, 2015)). These growth defects have been largely attributed to the presence of unrepaired DNA damage (Blank et al., 2015; Passerini et al., 2016). Consistently, we could also show that aneuploid *rad52* mutants displayed chronic DNA damage as evident from phosphorylated Rad53 and an upregulation or Rnr3 (Figure 19). Together with the finding that the same cells are still checkpoint-competent (Figure 19) and the observation that colony formation after genotoxic treatment is prevented by the adaptation-defective *cdc5-ad* allele (Figure 12) we suggest that cell survival after CPT or X-ray exposure

of *rad52* mutants is indeed facilitated by adaptation (Figure 27). In agreement, we could provide direct evidence that rapamycin treatment prevents adaptation in *rad52* mutants treated with CPT (Figure 22), consistent with our report using telomere dysfunctional mutants (Klermund et al., 2014). In addition, we observed a downregulation of Mcm5, a helicase involved in DNA replication, in our transcriptional analysis of aneuploid *rad52* mutants emerging from CPT treatment. This finding is consistent with a previous report in human cells demonstrating that a single supernumerous chromosome was sufficient to trigger genome instability by decreasing the levels of MCM helicase complex members on the transcriptional level (Passerini et al., 2016). Why components of the DNA replication machinery such as the MCM complex are consistently found to be transcriptionally downregulated in aneuploid cells is not yet fully understood.

Adaptation has been proposed as a cellular response to persistent DNA damage in an attempt to maximize survival and thereby promotes tolerance (or resistance) to genotoxins. Based on this and our observations, we hypothesize that the emergence of aneuploid *rad52* mutants that are genotoxin-resistant is a consequence of adaptation (Figure 12 and 27). Strikingly, *rad52* mutants that had adapted and were thus able to form a colony in the presence of CPT, subsequently displayed a growth advantage in the presence of various other genotoxic agents (Figure 13). This suggests that adaptation had caused the acquisition of resistance. Alternatively, it could be possible that *rad52* cells are less sensitive to genotoxic treatment as they had become aneuploid and therefore show aneuploidy-associated growth defects (as discussed above). However, slowing down the proliferation rate by incubation at lower temperatures did not cause an increased resistance to CPT (Figure 21). This indicates that a growth defect due to aneuploidy is most likely not the cause for the observed resistance to CPT and further suggests that aneuploidy could be a consequence of adaptation and not the cause in *rad52* cells.

Our transcriptome analysis of adapted and aneuploid *rad52* mutants (Figure 20, corrected for the varying chromosome copy numbers based on DNA sequencing in Figure 15) revealed an upregulation of factors involved in protein homeostasis. This is consistent with previous reports focusing on the physiological consequences of aneuploidy in both yeast and mammalian cells (see 1.6.1). Our results suggest that the development of an aneuploid karyotype during the acquisition of genotoxin resistance in repair-defective cells could provide helpful insights into de-regulated pathways that can be exploited to prevent such resistance. Investigating the sensitivity of aneuploid *rad52* mutants arising after genotoxic treatment as possible targeting strategies will be the subject of future studies. Importantly, preventing adaptation (in this case genetically with the *cdc5-ad* allele) in repair-proficient cells did not cause a hypersensitivity to X-ray treatment (Figure 17). This observation indicates that inhibition of adaptation can be specifically used to target repair-defective cells by inducing DNA damage. With regard to a pathophysiological situation, this finding suggests that repair-defective cells forming a tumour would be hypersensitive to genotoxic treatment when adaptation is prevented pharmacologically, however, repair-proficient surrounding tissue would not be affected (Figure 27, left side *versus* right side).

In cancer therapy, the acquisition of resistance is a major obstacle to curative treatment. In fact, radiotherapy constitutes one of the major approaches to treat various types of cancer and radioresistance leading to tumour relapse is an emerging complication. The repair status of a

cell (e.g. *BRCA*-deficiency) and the tumour microenvironment (e.g. tumour hypoxia) have proven to be two critical determinants of radioresistance (reviewed in (Huber et al., 2013)). Inhibitors of mTOR as well as compounds targeting the upstream PI3K signalling pathway have been shown to sensitize radioresistant cancer cells (Gottschalk et al., 2005; Miyasaka et al., 2015). In light of the results described here, we speculate that this could at least partially be due to the prevention of adaptation in response to decreased TOR signalling as well as a growth-inhibitory effect of low TOR activity on adapted and possibly aneuploid cells. Thus, future experiments will aim at investigating the TORC1 activity status in *rad52* cells after genotoxic treatment.

A prominent example of chemoresistance is given by the resistance of HR-defective *BRCA*-deficient cancer cells to PARP inhibitors. As described in section 1.4.2.1.2, PARP inhibition concomitant to a DNA damaging agent channels repair in *BRCA*-deficient cells towards error-prone repair pathways which drive cells into cell cycle arrest or apoptosis (reviewed in (Ohmoto and Yachida, 2017)). However, by forcing cells to use error-prone repair pathways, the acquisition of secondary mutations that restore BRCA2 function and thus the ability to perform HDR has been shown to be a major cause of resistance in this scenario (Edwards et al., 2008; Sakai et al., 2008). Mammalian BRCA2 plays a mediator function during homologous recombination and this role is taken over by Rad52 in budding yeast (reviewed in (Liu and Heyer, 2011)). Therefore, our experiments performed in *rad52* cells to understand the consequences of adaptation could provide useful insights for the acquisition of resistance in *BRCA2*-deficient cancers. By investigating which repair pathways are utilized in yeast *rad52* mutants to form colonies after genotoxic treatment, we could speculate which pathways facilitate repair in *BRCA2*-deficient cancers treated with PARP inhibitors. Moreover, the regulation of adaptation by modulating TOR activity as well as the inhibitory effect of rapamycin could be useful tools to prevent resistance in this cancer model. Importantly, resistance to chemotherapeutic agents has not only been observed in response to *BRCA2*-deficiency but also in cancers lacking *BRCA1*. A recent report showed that HR can be restored leading to resistance to PARP inhibition in these cells by concomitant loss of REV7 (Xu et al., 2015), a subunit of the translesion polymerase complex zeta (reviewed in (Gan et al., 2008)). Already some time ago, mTOR signalling was found induced in response to ionizing radiation exposure (Sunavala-Dossabhoy et al., 2004) indicating that mTOR activity functions as a pro-survival signal. Consistently, several studies could show that rapamycin had a radiosensitizing effects in various cancer models including breast and prostate cancer cell lines (Albert et al., 2006; Cao et al., 2006). Indeed, a rapalog was even found more efficient in radiosensitization than the previously used cisplatin but showed less negative side effects (Ekshyyan et al., 2009). Moreover, inhibition of mTOR signalling seems to be beneficial also during fractionated radiotherapy (Eshleman et al., 2002), even though this approach has been shown to increase the emergence of radioresistant cells (reviewed in (Kim et al., 2015)). Molecular insights into the beneficial effect of rapamycin in preventing resistance associated with PARP1 inhibitors came from a study in 2014. The authors reported that the ribosomal protein S6, a target of mTOR, showed increased phosphorylation in *BRCA1*-deficient cells that had acquired PARP1 inhibitor resistance. This observation gave a rationale for the prevention of resistance by co-treatment with rapamycin (Sun et al., 2014). Importantly, rapamycin can act synergistically with PARP1 inhibitors to inhibit the proliferation of *BRCA1*-defective breast

and lung cancer cells (Osoegawa et al., 2017). This report together with previous studies indicates that also in cancer cells, rapamycin could have a dual effect: it affects repair pathways and their outcomes (therefore, can act synergistically with PARP1 inhibitors) and sensitizes cancer cells that have acquired resistance. This would be consistent with our observations in HR-defective yeast cells (see Figure 26) and highlights the relevance of our findings.

3.5 Future perspectives

Together with our previous study (Klermund et al., 2014), the experiments presented here describe the regulation of adaptation in response to telomere dysfunction. This can be achieved by modulating Cdc5 function directly as seen from our results using the adaptation-defective *cdc5-ad* allele. Moreover, we could show that TORC1 signalling fine-tunes the cellular decision to undergo adaptation. However, the experimental system used here was telomere dysfunction. It has been established that cells activate the DNA damage checkpoint in response to eroded telomeres, nevertheless the damage signalling pathways and their cellular consequences might be regulated differently than in response to genotoxin-induced damage or an intrachromosomal DSB.

Future work will investigate the role of TORC1 downstream effectors such as the phosphatase PP2A in preventing adaptation. We hypothesize that this regulation might be achieved by impinging on Cdc5 and we plan to further investigate this possibility. In addition, it will be interesting to address the role of CKII, a second important regulator of adaptation (Toczyski et al., 1997) and possible target of TORC1 signalling (Sanchez-Casalongue et al., 2015). Moreover, we could provide insights into the mechanisms that lead to the acquisition of genotoxin resistance in mutants that are deficient in homologous recombination. We could show that the resistance to DNA damage is tightly linked with adaptation and aneuploidy, both acting as critical determinants of genome instability. On the other hand, genome instability is known to influence the response to DNA damage and thus, also adaptation. Moreover, compromised genome stability is intimately linked to aneuploidy. Therefore, investigating the interplay between adaptation, aneuploidy and genome instability in the model organism *Saccharomyces cerevisiae* will provide useful insights in the mechanisms leading to the acquisition of resistance, an emerging obstacle in cancer treatment.

4 Materials and Methods

4.1 Materials

4.1.1 Reagents and Kits

Reagents and Kits	Supplier
1 kb DNA ladder	New England Biolabs
100 bp DNA ladder	New England Biolabs
Acetone	Sigma Aldrich
Agar	Sigma Aldrich
Agarose	Sigma Aldrich
alpha-factor (α-Factor)	Zymo Research
Ampure XP beads	Beckman Coulter
Bacto Yeast	BD Biosciences
Bleomycin	AppliChem
Boric acid	Sigma Aldrich
Bradford Solution	AppliChem
Bromphenol blue	Sigma Aldrich
Camptothecin (CPT)	Sigma Aldrich
cOmplete Mini EDTA-free Protease Inhibitor Cocktail Tablets	Roche
Concanavalin A	Sigma Aldrich
Dimethylsulfoxide (DMSO)	Sigma Aldrich
D-sorbitol	Sigma Aldrich
Ethanol, absolute	Sigma Aldrich
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	AppliChem
G418 disulfate solution (Kanamycin)	AppliChem
Glucose (D+)	AppliChem, Merck Millipore
Glycerol	Grüssing
Hydroxyurea	Sigma Aldrich
Hygromycin B	InvivoGen
Isopropanol	Sigma Aldrich
Lyticase	Sigma Aldrich
Magnesium chloride ($MgCl_2$)	AppliChem
Mini-Protean TGX stain free precast gels	BioRad
Nitrocellulose membrane 0.45 µm pore size	GE Healthcare Amersham
Nocodazole	Sigma Aldrich
Nonidet P40 (NP40)	AppliChem
Nourseothricin-dihydrogen sulfate (ClonNaT)	WERNER BioAgents
Peptone	Sigma Aldrich
Phenol (solid)	Fluka
Phenol:chloroform:isoamylalcohol (25:24:1)	AppliChem
Phenylmethansulfoylflouride (PMSF)	AppliChem
Phloxine B	Sigma Aldrich
Phusion HF mastermix 2x	New England Biolabs

Poly(ethylenglycol) 400 (PEG)	Sigma Aldrich
Ponceau S	Sigma Aldrich
Potassium chloride (KCl)	Sigma Aldrich
Potassium phosphate monobasic (KH_2PO_4)	Sigma Aldrich
Prestained protein marker, broad range (11-190 kDa)	New England Biolabs
Prestained protein marker, broad range 7-175 kDa)	New England Biolabs
Proteinase K	Qiagen
QiaPrep Spin Miniprep Kit	Qiagen
Qubit dsDNA HS Assay Kit	Life Technologies
Raffinose x 5 H_2O (D+)	AppliChem
Rapamycin	AppliChem, Sigma Aldrich, Selleckchem
RedSafe Nucleic Acid Stain	iNtRON
Ribo-Zero rRNA Removal Kit (Yeast)	Illumina
RNase A	Qiagen
RNase-Free DNase Set including DNase I, buffer and water	Qiagen
RNaseOUT recombinant ribonuclease inhibitor	Invitrogen
RNeasy MinElute	Qiagen
Skim milk powder	Sigma Aldrich
Sodium acetate (Na-acetate)	Sigma Aldrich
Sodium chloride (NaCl)	Sigma Aldrich
Sodium dodecyl sulfate (SDS)	AppliChem
Sodium hydroxide (NaOH)	Sigma Aldrich
Sodium phosphate dibasic (Na_2HPO_4 x 2 H_2O)	Sigma Aldrich
ß-Mercaptoethanol	Sigma Aldrich
SuperSignal West Dura Extended Duration Substrate	Thermo Scientific
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific
Sytox Green	Thermo Scientific
Taq Mastermix 2x	New England Biolabs
Trichloroacetic acid (TCA)	Sigma Aldrich
Tris (Trizma base)	Sigma Aldrich
TruSeq stranded Total RNA LT Sample Prep Kit	Illumina
Tween-20	Sigma Aldrich
Urea	Sigma Aldrich
Yeast Marker Carrier DNA	Clontech
Yeast Nitrogen Base without amino acids	Sigma Aldrich
Yeast Synthetic Dropout Medium Supplement without amino acid (SC or SD)	MP Biomedicals
β-Mercaptoethanol	Sigma Aldrich

4.1.2 Buffers and solutions

Buffers and solutions	Composition
1 x PBST	1 x PBS (diluted in ddH_2O), 0.1 % Tween-20
10 x PBS	1.37 M NaCl, 0.03 M KCl, 0.08 M Na_2HPO_4 x 2 H_2O, 0.02 M KH_2PO_4, adjusted to pH 7.4 with HCl, autoclaved
10 x SDS Running buffer	25 mM Tris, 192 mM glycine, 0.1 % SDS, adjusted to pH 8.3, autoclaved
10 x TBE	0.89 M Tris base, 0.89 M boric acid, 0.02 M EDTA pH 8.0, autoclaved
10 x TE	0.1 M Tris base pH 7.5, 0.01 M EDTA pH 8.0
10 x Transfer buffer	25 mM Tris, 192 mM glycine, 0.45 µm filter-sterilized
6 x DNA loading buffer	15% Ficoll, 10 mM EDTA pH 8.0, Orange G
AE buffer	50 mM sodium acetate pH 5.3, 10 mM EDTA, adjust to pH 5.3 with NaOH
Blocking buffer	5% (w/v) skim milk powder in 1x PBST
EDTA pH 7.5 or 8.0	0.5 M disodium EDTA x $2H_2O$, adjusted to pH 7.5 or 8.0 with NaOH
gDNA buffer 1	0.9 M sorbitol, 0.1 M EDTA pH 7.5
gDNA buffer 2	0.27 M EDTA pH 8.5, 0.44 M Tris, 2.2 % SDS
IP buffer	50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 4 protease inhibitor cocktail tablets
LiAc mix	0.1 M lithium acetate, 1 x TE, ddH_2O ad final volume
PEG mix	40 % (w/v) PEG 400, dissolved in LiAc mix, autoclaved
Solution 1	1.85 M NaOH, 1.09 M β-mercaptoethanol
Solution 2	50 % TCA
Urea buffer	120 mM Tris-HCl pH 6.8, 5 % glycerol, 8 M urea, 143 mM β-mercaptoethanol, 8 % SDS, bromphenol blue

If indicated, solutions were sterilized by autoclaving at 121°C for 20 minutes.

4.1.3 Technical equipment, software and additional materials

Technical equipment, software and additional materials	Supplier
2100 Bioanalyzer	Agilent Technologies
Agarose gel chambers	Custom made
Analytical balance ED224S	Sartorius
BD FACSVerse	Becton Dickinson (BD)
Benchtop Shaker Excella E24	Eppendorf
CellRad X-ray cell irradiator	Faxitron
Centrifuge 5430 R	Eppendorf

Centrifuge 5810 R	Eppendorf
ChemiDoc Touch Imaging System	BioRad
Dissection Microscope MSM 400	Singer Instruments
DNA 1000 chip	Agilent Technologies
EndNote X5	Thomson Reuters
Excel 2013	Microsoft
FACSuite 1.0.5 and FACSDiva™	Becton Dickinson
Fast Prep-25	MP Biomedicals
Fiji 1.51d	ImageJ
FileMaker Pro 10	FileMaker Inc
FlowJo	Miltenyi Biotec
Heraeus Pico 17 Centrifuge	Thermo Scientific
HiSeq 2500	Illumina
IKA VORTEX genius 3	Sigma Aldrich
Illustrator CC 2017 and CS5	Adobe
ImageLab 5.2	BioRad
Incubator Heratherm IMC18	Thermo Scientific
Incubator Heratherm IMH60	Thermo Scientific
Leica DM1000 LED	Leica
Microscopy chambers 8 wells	Lab-Tek
Mini Centrifuge CD1008	Phoenix Instrument
NanoDrop 2000	Thermo Scientific
Neubauer counting chamber	Assistent Germany
NextSeq 500	Illumina
Ovation Ultralow System V2 (2014-2017)	NuGEN
pH-Meter PB-11	Sartorius
PowerPac Basic	BioRad
Prism 7.02	GraphPad
Qubit 2.0 Fluorometer	Life Technologies
S2 ultrasonicator	Covaris
Shaker Duomax 1030	Heidolph
Shaker Multitron Standard	Infors HT
Sonifier	Branson
Spectrophotometer Ultrospec 2100 pro	Biochrom
Test tube rotator	Labinco
Thermal Cycler C1000 Touch	BioRad
UV Stratalinker 2400	Stratagene
Western Blot equipment	BioRad
Word 2013	Microsoft

4.1.4 Antibodies

Antibodies	Dilution	Source	Figure
Primary antibodies			
mouse anti-Rad53	1:16	Gift from M. Foiani	8A, 8C, 8E, 19A-B
rabbit anti-Clb2	1:1000	Santa Cruz, #y-180	8C, 8E
rabbit anti-Sic1	1:2000	Gift from E. Schiebel	8C, 8E
mouse anti-GFP	1:1000	Roche, #11 814 460 001	10J
mouse anti-HA	1:2000	Covance, #MMS-101P	10J, 11B, 17B

mouse anti-Pgk1	1:400 000	Invitrogen, #459250	11B
rabbit anti-Rnr3	1:300	Agrisera, #AS09 574	19A-B
mouse anti-actin	1:2000	Millipore, MAB1501R	17B
Secondary antibodies			
goat anti-mouse (HRP conjugate)	1:3000	BioRad, #170-5047	8A, 8C, 8E, 10J, 19A-B
goat anti-rabbit (HRP conjugate)	1:3000	BioRad, #170-5046	8A, 8C, 8E, 19A-B
goat-anti mouse IRDye 680RD	1:10 000	LI-COR, #926-68070	11B
Antibody-coupled beads			
anti-GFP V_HH coupled to agarose beads	25 µL/ sample	Chromotek, #gta-10	10J

4.1.5 Oligonucleotides

Oligo-nucleotide	Database name	sequence (5`- 3`)	Comments
Cdc5-3xHA tagging fw	oKB19	TGGTAATTT CGTATTCGTATTTCTTTCT ACTTTAATATTGGTTTAAT CGATGAATTCGAGCTCG	based on S2 primer design published in (Janke et al., 2004)
Cdc5-3xHA tagging rev	oKB20	ATAAAGGAAGGTTTGAAG CAGAAGTCCACAATTGTT ACCGTAGATCGTACGCTG CAGGTCGAC	based on S3 primer design published in (Janke et al., 2004)
Cdc5-3xHA confirmation fw	oKB21	TGGCAGATGCAGAAGAGT TTTG	Forward primer to confirm C-terminal Cdc5-3xHA tagging
Cdc5-3xHA confirmation rev	oBL29	CTGCAGCGAGGAGCCGTA AT	Reverse primer to confirm C-terminal Cdc5-3xHA tagging

4.1.6 Yeast strains

All yeast strains used in this study were originally derived from the BY4741 background with the genotype *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*. For spotting experiments in Figures 8-10, one representative experiment is shown and the diploid strain from which the individual haploid mutant sets are derived is listed. Genotoxin-treated diploids from Figures 12-22 were usually not frozen down except for the strains used in sequencing experiments. Strains are partially published in (Klermund et al., 2014). All yeast strains were generated using yeast genetic protocols described previously (Guthrie and Fink, 1991).

Database name	Genotype	Figure
YBL7	MATa	8B-E, 16C-F, 20A-B
YJK710	MATa/MATalpha *CDC13/cdc13::cdc13-1 (KAN) CDC5/cdc5::cdc5-ad (URA) ATG5/atg5::NAT*	8C
YKB239	MATa *CDC13::cdc13-1 (KAN) bar1::NAT CDC5::Cdc5-3xHA (HYG)*	8A
YJK628	MATa *CDC13::cdc13-1 (KAN) CDC5::cdc5-ad (URA)*	8B, 8F
YJK625	MATa *CDC5::cdc5-ad (URA)*	8B
YJK31	MATa *CDC13::cdc13-1 (KAN)*	8B-F
YJK511	MATalpha *CDC13:: cdc13-1 (KAN) TUB1::Tub1-GFP (HIS)*	8G
YKB99	MATa/MATalpha *CDC13/cdc13::cdc13-1 (KAN) CDC5/cdc5::cdc5-ad (URA) RAD53/rad53::rad53-11 (NAT)*	9A
YJK458	MATa/MATalpha *CDC13/Cdc13::cdc13-1(HIS) RAD53/Rad53::rad53-11(URA)*	9B
YJK456	MATa/MATalpha *CDC13/Cdc13::cdc13-1(NAT) MEC1/mec1::HIS SML1/sml1::KAN*	9C
YJK664	MATa/MATalpha *CDC13/cdc13::cdc13-1 (HIS)ATG5/atg5::KAN*	10A
YJK813	MATa/MATalpha *CDC13/cdc13::cdc13-1(HIS) VPS51/vps51::KAN*	10B
YKB98	MATa/MATalpha *CDC13/cdc13::cdc13-1 (HIS) VPS51/vps51::KAN ATG5/atg5::NAT*	10C
YJK854	MATa/MATalpha *CDC13/cdc13::cdc13-1(NAT) MEC1/mec1::HIS SML1/sml1::KAN VPS51/vps51::HYG*	10D
YKB89	MATa/MATalpha *CDC13/cdc13::cdc13-1 (HIS) NVJ1/nvj1::KAN*	10E
YKB90	MATa/MATalpha *CDC13/cdc13::cdc13-1 (HIS) VAC8/vac8::KAN*	10F
YJK986	MATa/MATalpha *CDC13/cdc13::cdc13-1 (HIS) TAP42/tap42::tap42-11 (KAN)*	10H
YJK987	MATa/MATalpha *CDC13/cdc13::cdc13-1 (HIS) TIP41/tip41::KAN*	10I

YKB114	MATa/MATalpha *CDC13/CDC13::cdc13-1 (HIS) CDC5/CDC5::CDC5-3xHA (HYG) CDC55/CDC55::CDC55-GFP (HIS)*	10J
YKB115	MATa/MATalpha *CDC13/CDC13::cdc13-1 (HIS) CDC5/CDC5::CDC5-3xHA (HYG) PPH21/PPH21::PPH21-GFP (HIS)*	10J
YKB238	MATa *CDC13::cdc13-1 (KAN) CDC5::Cdc5-3xHA (HYG) bar1::NAT*	11A-B
YJK1049	MATa/MATalpha	12A-E, 13A, 14A-B, 16C-F, 17C, 19A, 19C, 22D-E
YJK1050	MATa/MATalpha	12A-E, 16B, 17C, 22D-E
YJK1051	MATa/MATalpha *CDC5::cdc5-ad (URA)/ CDC5::cdc5-ad (URA)*	12A-E, 13A, 14A-B, 17C, 22D-E
YJK1052	MATa/MATalpha *CDC5::cdc5-ad (URA)/ CDC5::cdc5-ad (URA)*	12A-E, 17C, 22D-E
YJK1053	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	12A-E, 13A, 14A-B, 19A, 19C, 22D-E
YJK1054	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	12A-E, 16B, 22D-E
YJK1055	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	12A-E, 13A, 14A, 14C-D, 19A, 19C, 22D-E
YJK1056	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	12A-E, 22D-E
YKB258	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	12F-G, 22A-E
YKB262	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	12F-G, 21, 22D-E
YKB264	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) RAD53::rad53-11 (NAT) / RAD52::KAN CDC5::cdc5-ad (URA) RAD53::rad53-11 (NAT)*	12F-G, 22B
YKB269	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	12F-G, 22B-C
YKB273	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	12F-G
YKB275	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) RAD53::rad53-11 (NAT) / RAD52::KAN CDC5::cdc5-ad (URA) RAD53::rad53-11 (NAT)*	12F-G
YJK1252	MATa/MATalpha	13B, 15A-B, 20A-B
YJK1253	MATa/MATalpha after X-ray	13B, 15A-B, 19B, 19D, 20A - B
YJK1254	MATa/MATalpha *CDC5::cdc5-ad (URA)/ CDC5::cdc5-ad (URA)*	13B
YJK1255	MATa/MATalpha *CDC5::cdc5-ad (URA)/ CDC5::cdc5-ad (URA)* after X-ray	13B

YJK1256	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	13B, 19B, 19D, 20A-B
YJK1267	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	13B
YKB405	MATa/MATalpha	14E-F, 16A, 16E-F
YKB406	MATa/MATalpha *SPO11::KAN / SPO11::KAN*	14E-F
YKB407	MATa/MATalpha *RAD52::NAT / RAD52::NAT*	14E-F, 16A, 16E-F, 21
YKB408	MATa/MATalpha *RAD52::NAT SPO11::KAN / RAD52::NAT SPO11::KAN*	14E-F
YJK1156	MATa/MATalpha after CPT treatment	15A-B, 20A-B
YJK1256	Derived from YJK1053	15A-B
YJK1158	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after CPT treatment	15A-B, 20A-B
YJK1160	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after CPT treatment	15A-B, 19B, 19D, 20A-B
YJK1262	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after X-ray treatment	15A-B, 19B, 19D
YJK1259	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after X-ray treatment	15A-B, 16E-F, 19B, 19D, 20A-B
YJK1261	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after X-ray treatment	15A-B, 16E-F, 20A-B
YJK1026	MATa *RAD52::KAN*	16C-F
YJK1262	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after X-ray treatment	16E-F, 20A-B
YKB257	MATa/MATalpha	17A-C, 22B-E
YKB260	MATa/MATalpha *CDC5::cdc5-ad (URA)/ CDC5::cdc5-ad (URA)*	17C, 22D-E
YKB317	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	18A-B
YKB318	MATa/MATalpha *DNL4::NAT / DNL4::NAT*	18A-B
YKB319	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	18A-B, 21
YKB320	MATa/MATalpha *RAD52::KAN DNL::NAT / RAD52::KAN DNL4::NAT*	18A-B
YKB321	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	18A-B
YKB322	MATa/MATalpha *DNL4::NAT / DNL4::NAT*	18A-B
YKB323	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	18A-B
YKB324	MATa/MATalpha *RAD52::KAN DNL::NAT / RAD52::KAN DNL4::NAT*	18A-B
YKB333	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	18A-B, 22A

YKB334	MATa/MATalpha *DNL4::NAT / DNL4::NAT*	18A-B
YKB335	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	18A-B
YKB336	MATa/MATalpha *RAD52::KAN DNL::NAT / RAD52::KAN DNL4::NAT*	18A-B
YKB337	MATa/MATalpha *RAD52::KAN / RAD52::KAN*	18A-B, 22A
YKB338	MATa/MATalpha *DNL4::NAT / DNL4::NAT*	18A-B
YKB339	MATa/MATalpha *RAD52::KAN CDC5::cdc5-ad (URA) / RAD52::KAN CDC5::cdc5-ad (URA)*	18A-B
YKB340	MATa/MATalpha *RAD52::KAN DNL::NAT / RAD52::KAN DNL4::NAT*	18A-B
YJK1265	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after X-ray treatment	19B, 19D, 20A-B
YJK1157	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after CPT treatment	20A-B
YJK1159	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after CPT treatment	20A-B
YJK1161	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after CPT treatment	20A-B
YJK1162	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after CPT treatment	20A-B
YJK1260	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after X-ray treatment	20A-B
YJK1264	MATa/MATalpha *RAD52::KAN / RAD52::KAN* after X-ray treatment	20A-B
YKB368	MATa/MATalpha	21
YKB369	MATa/MATalpha	21
YKB353	MATa/MATalpha *RAD52:: KAN / RAD52::KAN*	21
YKB372	MATa/MATalpha *RAD52:: KAN / RAD52::KAN*	21
YKB375	MATa/MATalpha *RAD52:: KAN / RAD52::KAN*	21
YKB259	MATa/MATalpha *RAD53::rad53-11 (NAT) / RAD53::rad53-11(NAT)*	22B-C
YKB261	MATa/MATalpha *RAD52::KAN RAD53::rad53-11 (NAT) / RAD52::KAN RAD53::rad53-11 (NAT)*	22B-C
YKB268	MATa/MATalpha	22B-C
YKB270	MATa/MATalpha *RAD53::rad53-11 (NAT) / RAD53::rad53-11(NAT)*	22B-C
YKB272	MATa/MATalpha *RAD52::KAN RAD53::rad53-11 (NAT) / RAD52::KAN RAD53::rad53-11 (NAT)*	22B-C

4.1.7 Plasmids

Name	Description	Source
pBL183	BG1766 Gal ORF control vector	B. Greyhack
pBL186	pRS423-GAL-*HIS3*, empty vector	M. Peter
pBL211	pRS425-Gal-*LEU2*, empty vector	M. Peter
pBL299	pRS41-hygB, empty vector	M. Knop
pBL303	pRS42-KAN, empty vector	M. Knop
pBL304	pRS42-NAT, empty vector	M. Knop
pBL328	pYM24, for C-terminal 3x HA tagging	Yanke et al., 2004
pBL504	BG1805-Gal-CDC5	ORF collection, Dharmacon

4.1.8 Yeast media

YP liquid medium 2.5x stock	**for 1 liter**
Peptone	44 g
Bacto Yeast	22 g
H_2O	ad 1 L
autoclave 20 minutes at 121°C	
to prepare 1 x YP + 2% glucose (1 liter)	
2.5 x YP medium stock	400 mL
20 % glucose	100 mL
H_2O	ad 1 liter

YPD agar plates	**for 1 liter**
YPD agar	65 g
H_2O	ad 1 L
autoclave 20 minutes at 121°C	

final concentration of antibiotics for YPD drug plates:

Nourseothricin-dihydrogen sulfate (ClonNaT)	100 µg/mL
Hygromycin B	300 µg/mL
G418 disulfate solution (Kanamycin)	250 µg/mL

Combinations of antibiotics are possible.

S-Gal/Raff-Ura liquid medium (2% galactose/2% raffinose)	**for 1 liter**
Yeast synthetic dropout medium supplement without uracil	1.92 g
Yeast nitrogen base without amino acids	6.7 g
Raffinose x 5 H_2O	23.6 g

H_2O	900 mL
autoclave 20 minutes at 121°C	
20 % galactose	100 mL
To prepare complete medium, the amino acid lacking in the dropout medium supplement was added.	

S-Gal/Raff-Ura plates (2% galactose/2% raffinose)	**for 1 liter**
Yeast synthetic dropout medium supplement without uracil	1.92 g
Yeast nitrogen base without amino acids	6.7 g
agar	20 g
Raffinose x 5 H_2O	23.6 g
H_2O	900 mL
autoclave 20 minutes at 121°C	
20 % galactose	100 mL
To prepare complete medium, the amino acid lacking in the dropout medium supplement was added.	

SC/SD liquid medium (2% glucose)	**for 1 liter**
Yeast synthetic dropout medium supplement without amino acid	1.92 g
Yeast nitrogen base without amino acids	6.7 g
H_2O	900 mL
autoclave 20 minutes at 121°C	
20 % glucose	100 mL

SC-Trp liquid medium (2% glucose)	**for 1 liter**
Yeast synthetic dropout medium supplement without tryptophan	1.92 g
Yeast nitrogen base without amino acids	6.7 g
H_2O	900 mL
autoclave 20 minutes at 121°C	
20 % glucose	100 mL
To prepare complete medium, the amino acid lacking in the dropout medium supplement was added.	

4.2 Methods

4.2.1 Spotting assay

4.2.1.1 For *cdc13-1* experiments

Yeast strains were grown in 5 mL overnight cultures in YPD at the permissive temperature of 23°C. Cells were then diluted to OD_{600} 0.3 in 5 mL YPD and treated with 5 nM rapamycin dissolved in DMSO or the appropriate volume of DMSO as vehicle control if indicated. Subsequently, cells were shifted to the indicated restrictive temperatures. At the indicated time points of 6 and 24 h after the temperature shift, cells were spotted in tenfold serial dilutions starting with OD_{600} 0.5 in the first dilution. To allow cell recovery visualized as colony formation, plates were incubated at the permissive temperature of 23°C. Plates were imaged using the ChemiDoc™ Touch Imaging System (BioRad) using the settings for "Blot colorimetric" and a "small" field of view (9.5 cm x 7.6 cm). Images created by the BioRad software were exported as TIFFs with a 600 dpi resolution and imported into Illustrator CC 2017 (Adobe) for labelling and arrangement.

4.2.1.2 For *rad52* experiments

Cells were grown in 5 mL overnight cultures in YPD at 30°C. Subsequently, cells were spotted in tenfold serial dilutions starting with OD_{600} 0.5 in the first dilution onto plates containing the indicated drugs or onto YPD plates that were either treated with the indicated genotoxic agent or left untreated (control plates). Plates were imaged after 3 to 4 days (unless indicated otherwise) as described for spotting assays using *cdc13-1* strains. YPD agar plates contained the vital dye Phloxine B at a final concentration of 8 µg/mL.

4.2.2 Western Blot

4.2.2.1 Protein extraction

For the preparation of total cell lysates, 2 OD units of culture were harvested by centrifugation at 13 000 rpm for 2 minutes. Cell pellets were either stored at -20°C until further processing or proteins were extracted immediately after harvesting. For extraction, cell pellets were resuspended in 150 µL of solution 1 and incubated on ice for 10 minutes. Subsequently, 150 µL of solution 2 were added and the suspension was mixed briefly by vortexing. Following another incubation on ice for 10 minutes, samples were cleared by centrifugation at 13 000 rpm for 2 minutes at 4°C. The pellet was washed in 1 mL acetone and centrifuged at 13 000 rpm for 2 minutes at 4°C. Subsequently, the pellet was resuspended in 100 µL urea buffer and incubated at 75°C (or 50°C when analysing Rad53 phosphorylation) for 5 minutes. If the sample turned yellow due to an acidic pH, 1 to 2 µL of Tris-HCl at pH 7.5 were added. Samples were centrifuged at 13 000 rpm for 1 minute to precipitate insoluble material and either processed further or stored at -20°C.

4.2.2.2 SDS-PAGE, transfer and signal detection

Samples were loaded onto Mini-PROTEAN® TGX Stain-Free™ precast gels (BioRad) using 15-well gels with 7.5% polyacrylamide for Rad53 detection or 15-well gels with a gradient of 4-15% polyacrylamide for all other proteins (as indicated in the figure legends). All gels were run in Mini-PROTEAN® electrophoresis chambers (BioRad). For Rad53 detection, gels were run at 100 V for 2 hours in 1x SDS running buffer, while all other proteins were separated by

running gels at 200 to 250 V for approximately 30 minutes. Subsequently, proteins were transferred to nitrocellulose membranes with 0.45 µm pore size (GE Healthcare) either by wet blot in 1x transfer buffer using Mini Trans-Blot® Cell chambers (BioRad) for detection of Rad53 or by semi-dry blot using the Trans-Blot® Turbo™ Transfer System (BioRad) with the pre-set 10 minutes blotting program for high molecular weight proteins. Transfer efficiency was visualized by staining the membrane with Ponceau S followed by rinsing in water. Blocking was performed for at least 1 hour at room temperature in blocking buffer followed by incubation with the primary antibodies diluted in blocking buffer over night at 4°C. After washing the membrane four times in 1x PBS + 0.1 % Tween-20, the secondary antibody diluted in blocking buffer was added and incubated for 1 hour at room temperature. After three washing steps in 1x PBS + 0.1 % Tween-20 followed by a last wash in 1x PBS, proteins were visualized by chemiluminescence using ECL Western Blotting substrate (Pierce). Alternatively, secondary antibodies coupled to fluorophores were used and protein detection was performed on an Odyssey CLx Imaging system (LI-COR). All antibodies used are described in section 4.1.4.

4.2.3 DNA content analysis using flow cytometry

Cell pellets equivalent to 0.18 OD_{600nm} units were harvested by centrifugation at 13 000 rpm for 1 minute. Afterwards, pellets were washed once in 1 mL water to remove medium and resuspended in 300 µL water. After the addition of 700 µL 100% ethanol, cells were fixed at 4°C and stored until further processing or fixed for 1 hour at room temperature on a rotating wheel. Subsequently, cells were centrifuged at 3000 rpm for 5 minutes to remove ethanol and washed in 1 mL water. After centrifugation at 3000 rpm for 5 minutes, pellets were resuspended in 500 µL 50 mM Tris-HCl pH 7.5 containing 10 mg/mL RNase A and incubated at 37°C for 3 hours or overnight. After centrifugation at 3000 rpm for 5 minutes, pellets were resuspended in 500 µL 50 mM Tris-HCl pH 7.5 containing 1 mg/mL Proteinase K and incubated at 50°C for 45 minutes. Cells were then centrifuged at 3000 rpm for 5 minutes and resuspended in 500 µL 50 mM Tris-HCl pH 7.5. Sonification was performed for 10 seconds at level 1 using "constant" mode with a 3 mm sonification tip (Branson). Subsequently, 500µL 50 mM Tris-HCl pH 7.5 containing 2µM Sytox Green were added (final concentration 1 µM), cells were transferred to FACS tubes (Falcon) and cells were analysed immediately on a FACSVerse flow cytometer (BD) using FACS Suite software (BD).

4.2.4 Yeast transformation for creation of homozygous diploids

In order to create homozygous diploids, isogenic haploids of mating type a or α, respectively, were transformed with different plasmids and after mating (standard procedure as described in Guthrie and Fink, 1991), diploid selection was performed using the plasmid-borne markers. For transformation, cells were grown to exponential phase (OD_{600nm} 0.6-0.8) in 25 mL YPD at 30°C and then harvested by centrifugation at 3000 rpm for 2 minutes. Cells were washed in 5 mL LiAc mix, centrifuged again at 3000 rpm for 2 minutes and then resuspended in 250 µL LiAc mix. 100 µL of competent cells were incubated together with 150 ng of the respective plasmids, 10 µL Yeast Marker Carrier DNA and 700 µL PEG mix at 30°C for 30 minutes. After a heat shock at 42°C for 15 minutes, cells were centrifuged at 3000 rpm for 1 minute and resuspended in 300 µL YPD to allow plasmid expression. For antibiotic selection, cells were incubated for 2 hours at 30°C, for auxotrophy-based selection for only 30 minutes. Subsequently, 100 µL cells were plated on appropriate selection plates and single colonies were used for mating. Newly created diploids were confirmed again on selective plates and then propagated on non-selective plates allowing plasmid loss.

Plasmids were purified from competent DH5α bacteria using QiaPrep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

4.2.5 Co-immunoprecipitation

Cell pellets from 100 mL exponential cultures were harvested by centrifugation at 3000 rpm for 5 minutes (all steps were performed at 4°C), resuspended in 200 µL IP buffer and transferred to Lysing Matrix C tubes containing 1 mm silica spheres (MP Biomedicals). Cells were lysed using two 30 seconds cycles at full power (setting 6.5) with a one minute break on ice between runs using a FastPrep (MP Biomedicals). After addition of 800 µL IP buffer supplemented with 0.2 % NP40, samples were vortexed briefly at high speed and transferred to reaction tubes for clearance by centrifugation at 13 000 rpm for 5 minutes. Cell lysates were transferred to a new reaction tube and cleared in a second centrifugation step. Subsequently, protein content was measured with a Bradford assay. In brief, 1 µL sample was diluted in 99 µL water and 1 mL filtered Bradford solution was added. Using a standard curve, protein content was estimated after measuring the absorption at OD_{595nm}. For immunoprecipitation, 2 mg protein were incubated in 1 mL final volume with 25 µL agarose beads coupled to GFP antibody fragments (ChromoTek) at 4°C for 2 hours in the presence of 4 µL DNase I (2000 U/ mL, NEB). An input sample (50 µg, equals 1/40) was taken from the remaining lysate as a control and diluted in 25 µL urea buffer. Immunoprecipitated samples were washed four times in 1 mL IP buffer supplemented with 0.2 % NP40 while rotating on a wheel at 4°C followed by centrifugation at 3000 rpm for 1 minute. After the last washing step, wash buffer was removed completely and immunoprecipitates were resuspended in 50 µL urea buffer, boiled at 75°C for 5 minutes and 15 µL were subjected to SDS-PAGE followed by detection of (co-)immunoprecipitated proteins using chemiluminescence (see section 4.1.4).

4.2.6 X-ray irradiation

Cells were irradiated with the indicated doses after plating on YPD agar plates as described using a 0.5 mm aluminium filter to remove low-energy X-rays. The dose rate was kept constant and was set to approximately 0.9 Gy/ min at 130 kV and 5 mA.

4.2.7 Plating assay and statistical analysis of cell survival

Cells were taken from 5 mL overnight cultures in YPD at 30°C and cell numbers per mL were assessed by counting in 1:100 or 1:50 dilutions in a Neubauer counting chamber. Unless indicated otherwise, 300 cells were plated in 2 technical replicates on YPD plates as control, on drug-containing plates or on plates that were subjected to X-ray or UV irradiation afterwards. Plates were incubated at 30°C for the indicated times. Colonies were counted manually taking all colonies visible by eye into account and the average of the 2 technical replicates was used for further quantification. Plates with *rad52* cells were incubated for 3 to 4 days after X-ray treatment and 7 to 8 days in the case of CPT treatment. Plates with wild type strains were usually counted after 2 or 3 days (in the presence of rapamycin). Colony numbers on plates with genotoxic treatment were normalized to number of colonies formed on control YPD plates and expressed as % survival. If not indicated otherwise, every quantification represents three biological replicates. Statistical analysis was performed using One-way ANOVA after estimating normal data distribution (Shapiro-Wilk test, alpha = 0.05) and testing the heterogeneity of variances (Brown-Forsythe test, $P < 0.05$) as built-in analyses in the Prism 7.02 software package (GraphPad). In the case of plating assays where genotype and treatment

represented two different factors, normal data distribution and variance heterogeneity were tested as before. Subsequently, a Two-way ANOVA was used to confirm statistically significant interaction between the two factors. Finally, One-Way ANOVA was performed on data sub-groups taking into consideration either the genotype or the treatment. For Figure 17 C, Two-way ANOVA revealed no significant interaction between genotype and treatment. Therefore, data were pooled either depending on genotype (regardless of treatment) or depending on treatment (regardless of genotype) and analysed using an unpaired two-tailed t-test.

4.2.8 Microcolony assay

Cells were grown to saturation overnight in YPD medium at 30°C. Unbudded or small budded cells were manipulated onto YPD agar plates that contained 10 nM rapamycin (supplier switch required an adjustment of the concentration), 2 µM CPT or the combination of both. Based on a similar experiment performed in (Toczyski et al., 1997), microcolony formation was assessed by quantifying cell bodies, i.e. a cell arrest in G2/M evident as a dumbbell shape was counted as 2 cell bodies. Microcolonies that contained only one cell body at the 4 hour time point were excluded from further analysis. After 24 hours, cell bodies present in every microcolony were counted. Cells were counted as adapted if the microcolony contained 3, 5 or more than 5 cell bodies. Four cell bodies were still considered arrested since a fraction of cells did not arrest with only 2 but 4 cell bodies at 4 hours. This is due to a CPT-induced arrest in the next cell cycle if cells had already transitioned through S phase at the start of the experiment. For every condition, more than 90 individual cells have been analysed and mean values with the SEM are depicted. Normal data distribution was assessed using the Shapiro-Wilk test (alpha = 0.05) and homogeneity of variances was confirmed using the Brown-Forsythe test ($P < 0.05$) as a built-in tool in the Prism software. Subsequently, a One-way ANOVA was performed.

4.2.9 Live-cell microscopy

Cells were grown overnight at 23°C in SC-Trp medium and then diluted to OD_{600nm} 0.2 in 10 mL fresh SC-Trp followed by a temperature shift to 37°C for 6 hrs to arrest *cdc13-1* mutants. Subsequently, 0.1 OD_{600nm} units were harvested, resuspended in 200 µL pre-warmed SC-Trp supplemented either with 5 nM rapamycin or the appropriate volume of DMSO. Cells were placed in 8-well microscopy chambers (Lab-Tek) that had been coated with 200 µL 2 mg/mL Concanavalin A for 15 minutes. Afterwards, images were acquired on a TCS STED CW super resolution microscope (Leica) every 30 minutes for 13 hours (total time at the non-permissive temperature 19 hours) in a 37°C humidified chamber using a 63x oil objective. At every time point, 3 regions of interest were imaged per well and with 8 z-stacks (2.9 µm step size). In total, < than 90 cells per condition were followed over the whole time course and quantified manually for re-budding events using Fiji image analysis software (Fiji 1.51d) and displayed in Prism 7.02 (GraphPad).

4.2.10 Genomic DNA extraction

Cells were harvested from 20 mL exponential culture (OD_{600nm} 0.7) by centrifugation at 3000 rom for 3 minutes. Subsequently, cells were resuspended in 1 mL gDNA buffer 1, transferred to a reaction tube and centrifuged at 13 000 rpm for 1 minute. After resuspension in 400 µL of gDNA buffer 1 supplemented with 14 mM β-Mercaptoethanol, cell walls were digested by adding 20 µL of lyticase (2.5 mg/mL) for 45 minutes at 37°C. Successful creation of

spheroblasts was monitored with a light microscope. Subsequently, spheroblasted cells were centrifuged at 13 000 rpm for 1 minute and resuspended in 400 µL 1x TE buffer. After addition of 90 µL gDNA buffer 2, samples were vortexed briefly and incubated at 65°C for 30 minutes. Subsequently, 80 µL of 5 M potassium acetate were added and samples were incubated at 4°C for at least 1 hour. Cell residues were then eliminated by centrifugation at 14 000 rpm for 15 minutes at 4°C. Afterwards, DNA contained in the supernatant was precipitated by addition of 750 µL cold 100 % ethanol. To facilitate precipitation, samples were incubated at -20°C for 30 minutes and subsequently centrifuged at 14 000 rpm for 5 minutes at 4°C. After discarding the supernatant, DNA contained in the pellet was washed with 1 mL cold 70% ethanol followed by air-drying the pellet at room temperature. DNA was resuspended gently in 500 µL 1x TE and incubated at 37°C for 60 minutes in the presence of 2.5 µL RNase A (10 mg/mL). Subsequently, 500 µL isopropanol were added, the sample was mixed and centrifuged at 14 000 rpm for 15 minutes at 4°C. After washing the pellet with 1 mL 70% ethanol, DNA was air-dried at room temperature and resuspended gently in 50 µL 1xTE. Insoluble material was precipitated and removed following centrifugation at 14 000 rpm for 10 minutes at 4°C.

4.2.11 DNA copy number analysis by whole genome sequencing (WGS)

4.2.11.1 Library preparation and sequencing

1 µg of genomic DNA was brought to a final volume of 50 µL and sheared using a Covaris S2 ultrasonicator system in a microTUBE AFA Fiber 6x16mm, applying the following shearing parameters: duty factor 10%, intensity 5, 200 cycles per curst, 45 seconds, 7°C water bath temperature. After DNA shearing, a double-size selection was performed using Ampure XP beads with ratios of 0.6:1 (beads:DNA) to exclude larger fragments and 1:1 (beads:DNA) to remove smaller fragments. This procedure enriched the DNA for the 100-500 bp fragments. Purified DNA was quantified using the Qubit dsDNA HS Assay Kit in a Qubit 2.0 fluorometer (Life Technologies) and the DNA size distribution was profiled in a High Sensitivity DNA chip on a 2100 Bioanalyzer (Agilent Technologies).
DNA sequencing library preparation was performed using NuGEN´s Ovation Ultralow System V2 (2014-2017), following the manufacturer's recommendations. Libraries were prepared with a starting amount of 80 ng of sheared DNA (100-500 bp in size) and were amplified in 8 PCR cycles. Libraries were profiled on a DNA 1000 chip on a 2100 Bioanalyzer (Agilent Technologies) and quantified using the Qubit dsDNA HS Assay Kit in a Qubit 2.0 fluorometer (Life Technologies). All samples were pooled in equimolar ratio and sequenced on one NextSeq 500 Midoutput FC (Illumina), PE for 2x 79 cycles plus 8 cycles for the index read.
DNA sequencing (DNA-seq) libraries were sequenced on Illumina NextSeq 500 (75-nt paired-end, with an effective read length of 79 nt), yielding 10-13 million read pairs per sample.

4.2.11.2 DNA sequencing analysis

Sequencing qualities were checked using FastQC (version 0.11.5) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All reads were mapped to the yeast genome (Ensembl genome assembly version R64) with the Burrows-Wheeler Aligner BWA (version 0.7.15) (Li and Durbin, 2010). Reads not mapped in a proper pair as well as secondary alignments were removed using Samtools (version 1.3.1) (Li et al., 2009). Furthermore, duplicate reads were removed using MarkDuplicates of the Picard tool package (version 2.6.0) (http://broadinstitute.github.io/picard/). Genome coverage depth and breadth were determined using Samtools as well as a custom made bash script. Due to very high and unequal coverage, the mitochondrial chromosome was removed from all subsequent analyses.

Reads were counted and summarized in 1000bp bins using readCounter of the HMMcopy package (version 0.1.1) ((Ha et al., 2012) and (https://github.com/shahcompbio/hmmcopy_utils)). Resulting wig files were transformed to bedgraph files using wigToBedGraph of the kentUtils package (version v302) without collapsing adjacent windows (https://github.com/ENCODE-DCC/kentUtils) and normalized to reads per million (PRM). Using a custom made R script, RPMs of each bin were then normalized to the sample-wide median RPM and chromosome copy numbers were estimated for each sample based on the chromosome median of this bin-wise RPM ratio (log2). A cutoff of 0.6 or -0.6 was chosen to determine a higher or lower chromosome copy number than the other chromosomes in the same sample.

4.2.12 Total RNA extraction

Cell pellets corresponding to 8 OD_{600nm} units from exponential cultures with an OD_{600nm} of 0.7 were harvested by centrifugation at 3000 rpm for 5 minutes. Pellets were then resuspended in 400 µL AE buffer, 20 µL 20% SDS were added and the samples were vortexed. Subsequently, 500 µL phenol (pre-equilibrated in AE buffer) were added, the sample was vortexed again and incubated at 65°C for 5 minutes. After cooling the samples on ice for 5 minutes, they were centrifuged at 13 000 rpm for 3 minutes at 4°C and the upper aqueous phase was transferred to a new reaction tube. 500µL phenol:chloroform:isoamylalcohol (25:24:1) were added and samples were incubated at room temperature for 5 minutes. After centrifugation at 13 000 rpm for 3 minutes, the upper aqueous phase was transferred to a new reaction tube and 40 µL of 3M sodium acetate were added. After mixing, 1 mL cold 100% ethanol were added and samples were inverted several times. After centrifugation at 13 000 rpm for 3 minutes, the supernatant was discarded and nucleic acids contained in the pellet were washed with 1 mL 80% ethanol. Pellets were then air-dried at room temperature and residual ethanol was removed using a vacuum pump. To digest DNA, every pellet was resuspended in 100 µL DNase I mix containing 87 µL RNase-free water, 10 µL DNase I buffer RDD and 3 µL DNase I in the presence of 1 µL recombinant RNase inhibitor RNaseOut. After incubation at 37°C for 45 minutes, samples were purified using the RNeasy MinElute kit (Qiagen) according to the manufacturer's instructions and eluted in 38 µL RNase-free water. Subsequently, cells were subjected to a second DNase I treatment by adding 63 µL DNase I mix containing 50 µL RNase-free water, 10 µL DNase I buffer RDD, 3 µL DNase I and 1 µL RNaseOut. Combining the DNase I mix with the eluted RNA again yielded a 100 µL sample that was incubated at 37°C for 45 minutes. As before, sample was purified using the RNeasy MinElute kit but RNA was eluted in 20 µL RNase-free water and stored at -80°C until further processing.

4.2.13 RNA sequencing

4.2.13.1 Library preparation and sequencing

Ribosomal RNA was depleted from 1 µg of total RNA using Illumina's Ribo-Zero rRNA Removal Kit (Yeast), following the manufacturer's instructions. After purification of rRNA-depleted RNA, library preparation was continued by adding the Elute-Prime-Fragment buffer from the TruSeq stranded Total RNA LT Sample Prep Kit, and the standard protocol was followed until the end of library prep (Part # 15031048 Rev. E). For library amplification, 10 PCR cycles were used.
The RNA sequencing libraries were profiled in a DNA 1000 chip on a 2100 Bioanalyzer (Agilent Technologies) and quantified using the Qubit dsDNA HS Assay Kit in a Qubit 2.0 fluorometer (Life Technologies).

The resulting RNA sequencing libraries were pooled in equimolar ratios in 2 pools (one pool containing 13 libraries and the second pool 14 libraries) and each pool was sequenced on one lane of a HiSeq 2500 rapid flow cell, single read for 68 cycles plus 7 cycles for the index read. RNA sequencing (RNA-seq) libraries were sequenced on Illumina HiSeq 2500 (50-nt single-end, with an effective read length of 68 nt), yielding 10-12 million reads per sample.

4.2.13.2 RNA sequencing and GO term enrichment analysis

Sequencing qualities were checked using FastQC (version 0.11.3) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All reads were mapped to the yeast genome (Ensembl genome assembly version R64, gene annotation version 86) with the splice-aware aligner STAR (version 2.5.2b) (Dobin et al., 2013) with two allowed mismatches and an overhang of at most 67 base pairs at each splice junction. Uniquely mapped reads were summarized per gene using featureCounts from the subread package (version 1.5.0) (Liao et al., 2014). Read counts per gene were corrected based on the chromosome copy number estimated based on the DNA sequencing (see 4.2.10.2). Differential gene expression analysis was done on corrected read counts using DESeq2 (Love et al., 2014). All genes with a p-value corrected for multiple testing of < 0.01 were considered significantly differentially expressed. A gene ontology (GO) term analysis was performed on significantly up- and down-regulated genes, respectively, using clusterProfiler (Yu et al., 2012). Here, up- and down-regulated genes were compare to the background of all expressed genes and the minimal number of genes annotated by an ontology term for testing was set to 5. A GO term was considered significantly enriched, if its adjusted p-value was < 0.01. Similar GO terms were combined using the simplify function of the clusterProfiler package

4.2.14 C-terminal tagging of Cdc5-3xHA

To tag Cdc5 under its endogenous promoter C-terminally with a 3xHA-hphNT1 cassette, the tagging cassette was amplified from a plasmid (pBL328) in a PCR using oKB19 as a forward and oKB20 as a reverse primer at a concentration of 60 nM each and using 2x Phusion HF master mix in a 50 µL PCR reaction. Cycling parameters were as follows: denaturation at 98°C for 30 seconds, 98°C for 10 seconds, 72°C for 30 seconds, 72°C for 1 minute, repeat step 2-4 34 times, final elongation at 72°C for 10 minutes followed by incubation at 12°C until further processing. Correct amplification of the tagging cassette was verified by electrophoresis on a 1.2% agarose gel run in 1x TBE buffer and subsequent staining and detection using RedSafe nucleic acid stain. The tagging cassette was subsequently transformed into wild type cells (YBL7) and positive clones selected on YPD plates containing 300 µg/mL hygromycin were used in a second PCR to verify the tagging. For the confirmation PCR, 2x Taq master mix was used and oKB21 and oBL29 served as forward or reverse primer, respectively, at a concentration of 10 µM. Cycling parameters were as follows: denaturation at 95°C for 30 seconds, 95°C for 30 seconds, 50°C for 1 minute, 68°C for 1 minute, repeat steps 2-4 34 times, final elongation at 72°C for 5 minutes followed by incubation at 12°C until further processing. Correct amplification of the tagging cassette was visualized on 1.4% agarose gel and successfully tagged clones were send for sequencing and tested by Western Blot for expression of the HA peptide for final confirmation.

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