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Adaptation to the DNA damage checkpoint requires the rewiring of the cell cycle machinery

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List of Abbreviations

5-FU	5-Fluorouracil
8-oxoG	8-oxoguanine
9-1-1 checkpoint clamp	Ddc1-Rad17-Mec3
AID	Auxin-inducible degron
AP	Apyrimidinic/apurinic
APC/C	Anaphase promoting complex
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BER	Base excision repair
BIR	Break-induced replication
cdc5-ad	cdc5-adaptation defective
CDK	Cyclin-dependent kinase
CPD	Cyclobutane pyrimidine dimer
DDC	DNA damage checkpoint.
DDR	DNA damage response
dHJ	Double Holliday junction
D-loop	Displacement loop
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	Nucleoside triphosphate
FEAR	Cdc fourteen early anaphase release
G_1 phase	Gap phase 1
G_2 phase	Gap phase 2

GAP	GTPase activating protein
GEF	Guanine-nucleotide exchange factor
GG-NER	Global-genome NER
GTA	Genotoxin-induced targeted autophagy
Ho	HOmothallic
HR	Homologous recombination
HU	Hydroxyurea
M phase	Mitotic phase
MCC	Mitotic checkpoint complex
MEN	Mitotic exit network
MMEJ	Microhomology-mediated end joining
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MRN complex	Mre11-Rad50-Nbs1
MRX complex	Mre11-Rad50-Xrs2
NAA	1-Naphthaleneacetic acid
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
PBD	Polo-box domain
PCNA	Proliferating cell nuclear antigen
PIKK	Phosphoinositide 3-kinase-related kinase
PLK	Polo-like kinase
Pol α	DNA polymerase α
Pol ε	DNA polymerase ε

RENT	Regulator of nucleolar silencing and telophase
RNR	Ribonucleotide reductase
rNTP	Ribonucleoside triphosphate
ROS	Reactive oxygen species
S phase	DNA Synthesis phase
S. cerevisiae	Saccharomyces cerevisiae
SAC	Spindle assembly checkpoint
sad	S phase arrest defective
SAM	S-adenosylmethionine
SBF	SCB-binding factor
SDSA	Synthesis-dependent strand annealing
SGA	Synthetic Genetic Array
SPB	Spindle pole body
SSB	Single strand break
STR complex	Sgs1-Top3-Rmi1
TC-NER	Transcription-coupled NER
TPE	Telomere position effect
WT	Wild type

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Abstract

The DNA damage checkpoint is a surveillance mechanism evolved to preserve genome integrity in response to DNA damaging agents. The DNA damage checkpoint senses DNA insults and halts the cell cycle providing time and conditions to repair the lesion(s). If the damage is successfully repaired, cells reenter in the cell cycle in a process known as recovery to the DNA damage checkpoint. If the damage is not repaired, cells either undergo a programmed cell death or override the checkpoint reentering the cell cycle in the presence of the lesion. This process, known as adaptation to the DNA damage checkpoint, represents an opportunity for cells to repair the damage in the following cell cycle. However, adaptation to the DNA damage checkpoint can be an unsafe event as daughter cells can accumulate genomic aberrations, therefore promoting genomic instability, and, indeed checkpoint adaptation has been described to occur also in cancer cells. Therefore, understanding the molecular mechanisms that drive checkpoint adaptation is a fundamental question to be addressed.

The molecular mechanism causing adaptation, as well as the players involved in this process, remains largely unknown. In budding yeast *S. cerevisiae*, the existence of a crosstalk between the cell cycle machinery and the DNA damage checkpoint have been suggested by two observations. First, the DNA damage checkpoint acts to halt cell cycle progression by directly inhibiting the pathways that control the exit from mitosis, namely the Cdc fourteen early anaphase release (FEAR) network and the mitotic exit network (MEN). Second, the activity of the FEAR network is required for checkpoint adaptation. Indeed, impairing the functions of single components of the FEAR network, namely Cdc5, Spo12 and Slk19, results in cells impaired in the adaptation process. While the molecular events for the DNA damage checkpoint activation have been intensely studied and

relatively well characterized, the molecular events that drive cell cycle resumption after checkpoint adaptation are less well understood. In the work presented in this thesis, we used and integrated different approaches, including genetics, single cell analyses, and fluorescence microscopy techniques to tackle this question.

Our findings indicate that the FEAR mutants (with the exception of Cdc5) are proficient in switching off the checkpoint but cannot exit mitosis, and suggest a more complex picture. As impairing the activity of single FEAR components does not affect exit from mitosis both in unperturbed conditions, and following checkpoint recovery, our studies unveil checkpoint adaptation as the rewiring of a cell cycle with peculiar features. From our investigations, we expect to elucidate the molecular circuitry underlying the rewiring of the cell cycle in persistent DNA damage conditions.

1. Introduction

1.1. An overview of the cell cycle

All organisms, from the simplest bacteria to the most complex animals, share a common feature: they are composed of structural units called cells. In order to ensure the proper physiology of the organisms and propagation of the species, cells have to correctly duplicate themselves in a process called 'the cell cycle'. The eukaryotic cell cycle is a complex process, characterized by a sequence of coordinated and interconnected events defined as cell cycle phases. Each phase of the cell cycle has to be completed before the cell can proceed to the following one. Four cell cycle phases can be distinguished: G_1 (Gap phase 1), S phase (DNA Synthesis phase), G_2 (Gap phase 2) and M phase (mitotic phase). The G_1, S and G_2 phases are collectively grouped in the interphase, namely the period between one mitosis and the following one (Figure 1.1).

In G_1 phase, also called the growth phase, the cell increases its supply of proteins and the number of organelles, and grows in size. If environmental conditions are favorable, the cell can proceed through the START (known as Restriction Point in mammals), which is a non-return point after which cells are irreversibly committed to enter the cell cycle and progress through it independently of signals from the environment. In S phase, the cell begins to replicate its DNA. The replication of the DNA is initiated at specific sites called replication origins that are situated in different parts of the genome. The genetic material of the cell must be completely and accurately replicated only once in order to guarantee a faithful transmission of the genetic information. Next, cells enter in the G_2 phase, in which cells grow until they reach an adequate size and prepare all the structures needed for the subsequent mitotic division that takes place in the M phase. During mitosis, the replicated DNA is segregated into the two daughter cells that are formed. Mitosis is composed of four different phases. In prophase, the DNA is condensed into chromosomes, composed of two sister chromatids bound together at the centromere, and the mitotic spindle is assembled.

In metaphase, chromosomes align at the equator of the spindle and sister chromatids are attached to kinetochore microtubules coming from opposite poles of the spindle. During anaphase, sister chromatids separate and segregate toward opposite poles in order that each daughter cell receives only one of the two sister chromatids. In most eukaryotic cells, the nuclear envelope breaks down in early stages of mitosis and re-forms around the segregated chromosomes in a process termed open mitosis. Conversely, budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) and other eukaryotes, including *Schizosaccharomyces pombe* (*S. pombe*) and other fungi, maintain the nuclear envelope intact throughout mitosis, a process termed closed mitosis. Finally, in telophase, chromosomes decondense and during cytokinesis the cytoplasm is physically divided and gives rise to two identical daughter cells.

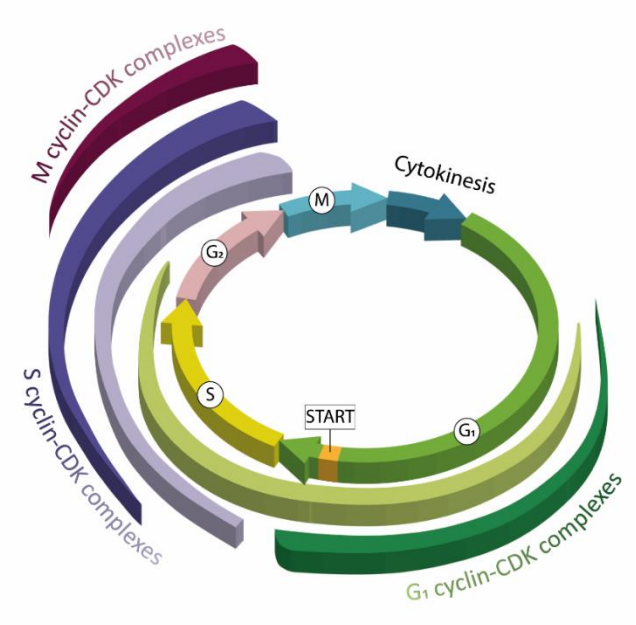

Figure 1.1 **The mitotic cell cycle.** The cell cycle comprises four discrete phases: G_1 phase, S phase (synthesis), G_2 phase and M phase (mitosis and cytokinesis). Activation of each phase is dependent on the proper progression and completion of the previous one. Cyclin-dependent kinases (CDKs) through association with different cyclin subunits control of the progression through the different cell cycle phases.

The central dogma of the eukaryotic cell cycle is the control of the progression through the different phases by the activity of Cyclin-dependent kinases (CDK), a specific class of serine/threonine kinases, whose activity and substrate specificity is dictated by their

association with cyclin subunits. Cyclins are proteins that show an oscillatory expression patter across the cell cycle. They can be subdivided in G_1 cyclins, S cyclins and mitotic cyclins, depending on the cell cycle stage in which they exert their functions. Therefore, different cyclin-CDK complexes exist, each of which is specific for a particular cell cycle stage (Figure 1.1). Passage through START requires the activity of G_1 cyclin-CDK complexes that set the conditions for the S phase by activating the expression of S cyclins. The activity of S cyclin-CDK complexes is initially kept inactive through binding with specific inhibitors. In late G_1, G_1 cyclin-CDK complexes induce the degradation of the inhibitors, which allows the S cyclin-CDK complexes to stimulate entry into the S phase. S cyclin-CDK complexes are required for initiation of DNA replication and they ensure that each chromosome is replicated only once, thus guaranteeing that the proper chromosome number is maintained in all daughter cells. Mitotic cyclins are expressed during S and G_2 phases but are kept inactive until DNA synthesis is completed. Mitotic cyclin-CDK complexes are responsible for chromosome condensation, breakdown of the nuclear envelope (in open mitoses), assembly of the mitotic spindle apparatus, and alignment of condensed chromosomes at the metaphase plate. Once all chromosomes are properly associated with spindle microtubules, the anaphase-promoting complex, also known as cyclosome (APC/C), is activated. This large multiprotein complex is responsible for the ubiquitination of several substrates, which are therefore targeted for proteasomal degradation. The activation of the APC/C complex results in the proteolysis of anaphase inhibitors, leading to inactivation of the protein complexes that hold together sister chromatids, thus allowing their segregation. The APC/C complex is also responsible for the targeting of mitotic cyclins for degradation by the proteasome. The decrease in the activity of mitotic cyclin-CDK complexes and the dephosphorylation of their substrates by phosphatases allow the decondensation of chromosomes, the reformation of the nuclear envelope, and the

division of the cytoplasm at cytokinesis. Finally, G_1 cyclin-CDK complexes phosphorylate the APC/C complex, thus inactivating it, allowing the accumulation of mitotic cyclins in the following cycle.

1.2. The DNA damage response

Genome integrity is constantly challenged by exogenous and endogenous damages, resulting from normal cellular metabolism, exposure to radiation, genotoxic compounds, and others. Keeping a stable and intact genome is a key task for all living organisms to ensure a faithful transmission of the genetic material across generations, thus, the survival of species. However, a completely stable genome is not as desirable as one might think. Indeed, a stable genome would not allow any changes required for the evolution of the species. It is also becoming more and more clear that fine tuning of genomic stability is not only a key aspect for the evolution of species, but also for the evolutionary potential of tumor cells, that is, for their ability to adapt to different external changes (Bohlander and Kakadia, 2015). Indeed, there are evidences that the development of tumors is governed by the same evolutionary laws as those of the selection of the fittest (Yates and Campbell, 2012). Therefore, genomic stability results from a balance between DNA damage and DNA repair, where the dominance of the former can lead to detrimental consequences, such as cancer, while the dominance of the latter results in that species do not evolve (Bohlander and Kakadia, 2015).

To ensure a reliable transmission of genetic information to the offspring, cells have evolved a variety of safeguard mechanisms to early detect, signal and repair DNA lesions. These interconnected cellular pathways are collectively known as the DNA damage response (DDR). Surveillance proteins that monitor DNA integrity in response to DNA damage can activate cell cycle checkpoints and DNA repair pathways to stop cells from further proceeding in the cell cycle and to, instead, allow for the repair of the lesion, respectively.

Following successful repair of the lesion, the DDR is turned off and cells are allowed to proceed in the cell cycle. However, if the damage is too extensive or irreparable, the DDR activates a programmed cell death or the entry in a senescent state.

In the following sections, I will cover the types of DNA damage, the pathways of DNA repair, the steps of DNA damage checkpoint activation, the targets for achievement of the cell cycle arrest and, finally, the outcomes of the termination of the checkpoint, with a special focus on pathways in *S. cerevisiae*. Before going into mechanistic details, a brief historical perspective on early stages of DDR research is presented.

1.2.1. A historical perspective on DDR research

Historically, the study of biological responses to DNA damage began with the fields of classical radiobiology and photobiology. Back in the 1877, Downes and Blunt reported the lethal effects of sunlight on bacteria (DOWNES and BLUNT, 1877) (Figure 1.2). However, it took another half a century before the understanding that the biological target of radiation was the genetic material inside cells. The work of the geneticist Hermann Muller highlighted that exposure to ionizing radiation dramatically increased the frequency of lethal mutations in the X chromosome in *Drosophila melanogaster* (*D. melanogaster*) (Muller, 1927). For many years, scientists exploited the inactivation of gene function by exposing cells to ionizing and ultraviolet (UV) radiation to study gene functions, a sort of primitive genetics.

In 1947, Albert Kelner serendipitously discovered enzymatic photoreactivation (Kelner, 1949), a highly efficient process for repairing pyrimidine dimers in DNA in a completely error-free manner. Following this finding, the research on DNA repair formally began. Indeed, in the 1960s, the discovery of photoreactivation was followed by that of excision repair of photoproducts from DNA, both in bacteria and in mammalian cells (BOYCE and

HOWARD-FLANDERS, 1964; SETLOW and CARRIER, 1964; Regan, Trosko and Carrier, 1968). Later on, the finding of multiple mechanisms by which lesions in DNA bases are repaired by excision, which we now recognize as base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR), dominated the study of biological responses to DNA damage. In the early 1970s, studies from Miroslav Radman, Evelyn Witkin and others on the SOS response (Radman, 1975; Witkin, 1976) led to the identification of another primary biological response to DNA damage, namely the DNA damage tolerance, in which the damage is physically tolerated in the genome through various replicative bypass mechanisms. Importantly, the elucidation of the SOS response led to the understanding of that DNA lesions can act as signals to activate complex regulatory response pathways. Besides, the study of DNA damage itself provided insights on biological processes in response to DNA lesions. In this sense, an important contribution came from the work from Tomas Lindahl, who adopted the general principle that the identification of novel forms of DNA damage that arise spontaneously in cells, or from environmental agents, can likely lead to the identification of a DNA repair mechanism(s) for such damage (Lindahl, 1993).

In the 1980s, the repertoire of biological processes in response to DNA damage expanded with the introduction of the important notion of checkpoint controls by Leland Hartwell and colleagues, who observed that cells arrest their progression through the G_2 phase of the cell cycle in the presence of a DNA damage (Weinert and Hartwell, 1988). In particular, they identified the first checkpoint gene, *RAD9*, showing that mutants *rad9* cells were impaired in damage-induced cell cycle arrest (Weinert and Hartwell, 1988). Following this finding, the research field on DNA checkpoints formally began and led to the discovery of multiple checkpoint controls that operate in other phases of the cell cycle. Thereafter, Hartwell and colleagues identified several additional mutations, including mutants in *MEC1* (mitotis entry checkpoint) and *RAD53* (originally identified as an X-ray sensitive mutation)

(Weinert, Kiser and Hartwell, 1994). However, the enzymatic activities of the corresponding proteins were unknown. Concomitantly, Stephen Elledge and colleagues identified a series of S phase arrest-defective (*sad*) mutants, among which an allele of *RAD53* and an allele of *MEC1* (Allen *et al.*, 1994). Moreover, they showed that Rad53 is a protein kinase, that Dun1 is a target of Rad53 and, finally, that Rad53 activity was required for the regulation of three distinct processes: a) a pause in G_1 after DNA damage, b) the induction of ribonucleotide reductase (*RNR)* genes in response to incomplete replication, and c) mitotic delay in response to DNA damage (Allen *et al.*, 1994). During the following years, Elledge and colleagues significantly contributed to the elucidation of the DDR activation pathway. In collaboration with Errol Friedberg's laboratory, they established a homology link between Mec1 and the mammalian ATM gene, as well as between Tel1 and the ATM-related ATR gene (Siede *et al.*, 1996). Moreover, they significantly contributed to draw the preliminary blueprint for DNA damage checkpoint activation pathway both in budding yeast and in mammalian cells. Indeed, they showed that the PI3K-like kinases Mec1 and Tel1 control a protein kinase cascade that culminates with the activation of downstream targets Rad53 and Chk1 (Matsuoka, Huang and Elledge, 1998) that, in turn, target important cell cycle proteins to prevent anaphase entry and mitotic exit after DNA damage (Sanchez *et al.*, 1999). Thereafter, they demonstrated that this regulation was conserved in mammals, with ATM and ATR controlling Chk1 and Rad53's mammalian homolog, Chk2, respectively (Matsuoka, Huang and Elledge, 1998; Sanchez *et al.*, 1999; Liu *et al.*, 2000). In the 1990s, many other scientists significantly contributed to the blossom of today's DDR vision. Among them, it is important to remember Yosef Shiloh's group, who first cloned and sequenced ATM (Savitsky *et al.*, 1995), Michael Kastan, who showed a key control of p53 by ATM (Kastan *et al.*, 1992), and Antony Carr and Karlene Cimprich, who characterized ATR (Bentley *et al.*, 1996; Cimprich *et al.*, 1996).

The already complex framework of DNA damage response took on another dimension when checkpoint adaptation was discovered. Checkpoint adaptation is a process that allows cells to resume their cell cycle and divide following a protracted checkpoint-mediated cell cycle arrest despite the presence of persistent DNA damage. Adaptation to the G_2/M DNA damage checkpoint was first described in *S. cerevisiae* by Sandell and Zakian in 1993, who demonstrated that following the induction of double strand break (DSB) in a repair-defective background, cells arrested in G_2/M for approximately 10 hours and then adapted and resumed division in the presence of the damaged DNA (Sandell and Zakian, 1993). Thereafter, in 1997, in a genetic screen for regulators of adaptation in budding yeast, the first two adaptation mutants were identified by Hartwell and collaborators (Toczyski, Galgoczy and Hartwell, 1997).

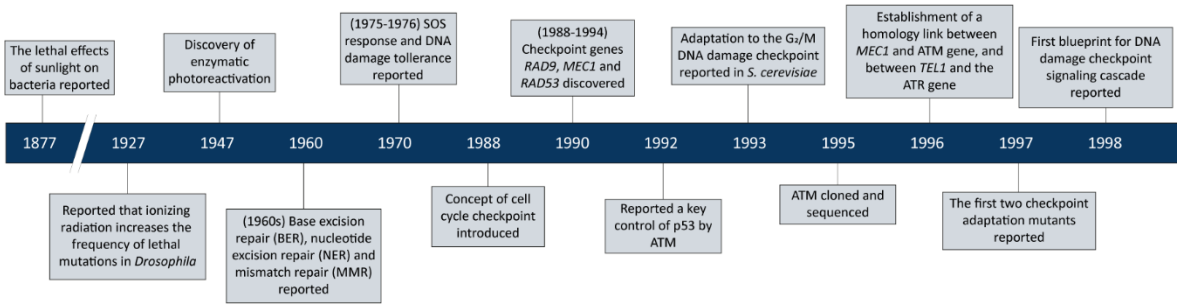

Figure 1.2 **Milestones in the DNA damage response research**.

1.3. Types and causes of DNA damage

The DNA in our cells is constantly challenged by a variety of damaging agents. It has been estimated that DNA lesions occur at a frequency of approximately 70,000 lesions per day in each cell in humans (LINDAHL and BARNES, 2000). Most of such lesions affects the regular helical structure of the DNA by introducing non-native chemical bonds or bulky adducts, and are caused by both normal metabolic processes and/or exogenous factors (Figure 1.3).

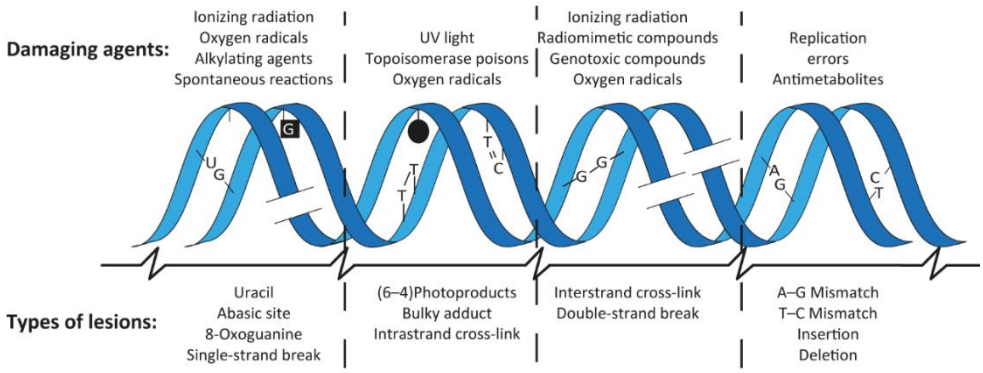

Figure 1.3 **DNA damaging agents and types of DNA lesions.** Common DNA damaging agents and examples of DNA lesions induced by these agents are shown.

1.3.1. Endogenous sources of DNA damage

The simple fact that DNA molecules in living cells are dissolved in water represents a potential source of damage. Indeed, the DNA molecule can be subjected to spontaneous hydrolysis reactions of different chemical groups (reviewed in (Gates, 2009)) (Figure 1.4). One example is the hydrolysis of the phosphodiester groups in the DNA backbone (although this does not occur to a significant extent, it can be accelerated by catalysts), that results in a single strand break (SSB). It has been estimated that spontaneous hydrolysis of the phosphodiester groups occurs at a frequency of approximately 55 000 lesions per day in each cell in humans (Tubbs and Nussenzweig, 2017). Moreover, depurination of DNA bases results from the hydrolysis of the bond between a purine and the deoxyribose sugar of the DNA, leading to abasic sites. If those sites are left unrepaired, they can cause mismatches during DNA replication It has been estimated that depurination of DNA occurs at a frequency of approximately 12 000 lesions per day in each cell in humans (Tubbs and Nussenzweig, 2017). Lastly, deamination of DNA bases results from the spontaneous removal of amine groups from adenine, guanine or cytosine. Deamination is a mutagenic event since the deamination of cytosine results in uracil residue (Gates, 2009), therefore, during DNA replication it can result in a guanine:cytosine to adenine:thymine transition. It has been estimated that cytosine deamination occurs at a frequency of approximately 192 lesions per day in each cell in humans (Tubbs and Nussenzweig, 2017).

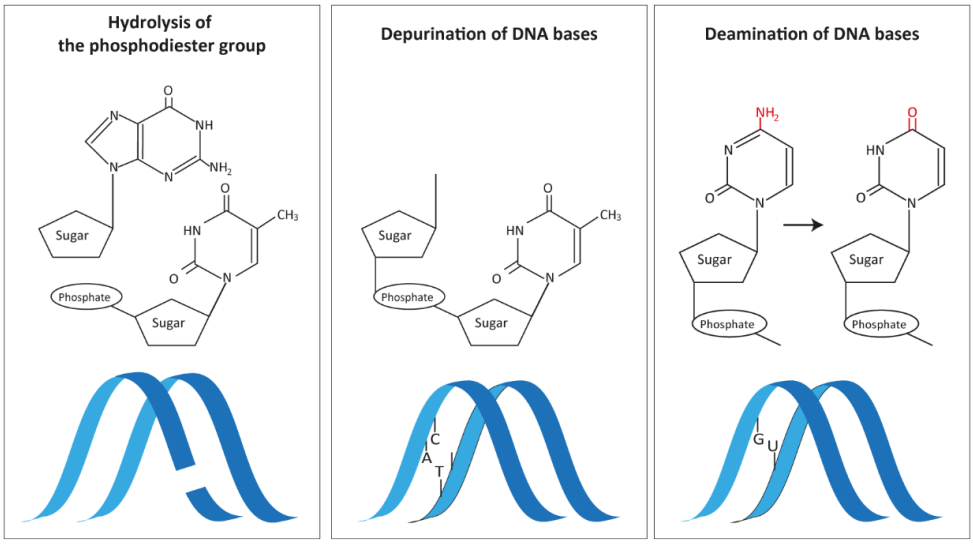

Figure 1.4 **Spontaneous hydrolysis reactions can occur on different residues of the DNA molecule**.

Normal cellular metabolism normally produces reactive oxygen species (ROS), which are highly reactive compounds, able to react with the DNA molecule and potentially give rise to multiple forms of oxidative damage (reviewed in (Jena, 2012)). One of the most common lesions caused by ROS is 8-oxoguanine (8-oxoG) (Figure 1.5). 8-oxoG can pair with adenine, and if it is not removed before DNA replication, 8-oxoG may generate guanine:cytosine to adenine:thymine transitions. It has been estimated that 8-oxoG occurs at a frequency of approximately 2 800 lesions per day in each cell in humans (Tubbs and Nussenzweig, 2017). Other lesions caused by ROS are modification of bases, intrastrand crosslinks (a covalent bond is formed between two adjacent bases on the same strand), interstrand crosslinks (a covalent bond is formed between two bases on different strands), covalent protein-DNA crosslinks, and DNA strand breaks (Jena, 2012). Given the number of different types of lesions that can be introduced in the DNA, ROS are considered especially harmful for DNA integrity. Indeed, DNA damage by ROS has been involved in several pathological conditions, including cancer, aging, and neurodegenerative diseases among others (Waris and Ahsan, 2006; Brieger *et al.*, 2012).

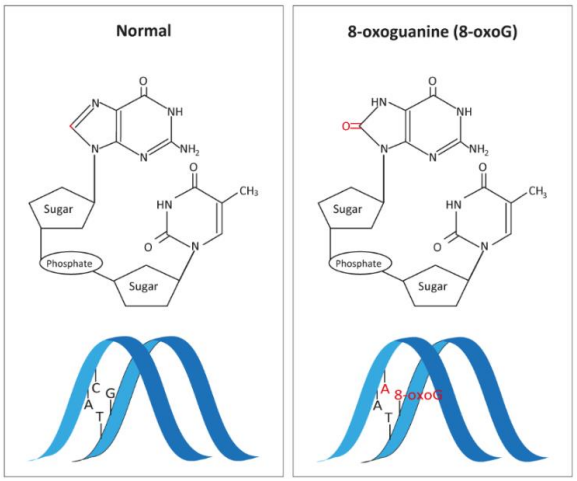

Figure 1.5 **8-oxoguanine**. Oxidation of the guanine by reactive oxygen species generates an 8-oxoguanine residue that pairs with adenine. If the residue is not removed before DNA replication, it causes a guanine:cytosine to adenine:thymine transitions.

In addition to ROS, also other endogenous molecules can act as genotoxic agents. The best characterized genotoxic molecule is S-adenosylmethionine (SAM), a methyl group donor used as a cofactor in most cellular transmethylation reactions. The reaction of SAM with the DNA molecule occurs mainly at the level of ring nitrogens of purine residues, leading to 7-methylguanine and 3-methyladenine. While the former is considered relatively harmless, as the modification does not alter the normal base pairing, the latter acts as a cytotoxic DNA agent by blocking DNA replication (Lindahl, 1993).

Normal replication of the DNA is also a source of errors and mismatches. Indeed, although normal cells replicate their DNA with extraordinary fidelity (about 10^{10} mutations per nucleotide per division (Drake *et al.*, 1998)), it was estimated that eukaryotic DNA polymerases introduce a wrong nucleotide approximately once every 10 000-100 000 nucleotides polymerized (McCulloch and Kunkel, 2008; Kunkel, 2009). Most of these events are base-base mismatches, base insertions or base deletions (McCulloch and Kunkel, 2008; Kunkel, 2009). Factors influencing the fidelity of DNA replication include the nucleotide selection step, the intrinsic proofreading activity of the DNA polymerase, and maintenance of normal nucleoside triphosphate (dNTP) pools. Regarding dNTPs, it was shown that large

excess of ribonucleoside triphosphate (rNTPs), compared to dNTPs, result in misincorporation of rNTPs in the genome. As a consequence, rNTP misincorporation represents one of the most common form of DNA damage (Ganai and Johansson, 2016).

1.3.2. Exogenous sources of DNA damage

Besides endogenous sources of DNA damage, cells have to face also DNA damage coming from environmental sources. Environmental sources that can cause DNA damage can be physical agents, such as UV light and ionizing radiations, or chemical agents, such as intercalating compounds, alkylating molecules, and base analogs. In addition, these agents can damage the DNA either directly or indirectly, or in both ways simultaneously (examples are presented in the following paragraphs).

1.3.2.1. Radiations and radiomimetic agents

There are two types of radiation, ionizing or non-ionizing radiations, depending on the energy of the radiated particles. This is an important distinction due to the large differences in radiation harmfulness to living organisms. Indeed, ionizing radiation carries enough energy to ionize atoms and molecules, and to break chemical bonds, while non-ionizing radiation only damages cells if the intensity is high enough to cause excessive heating of a tissue. Gamma rays, X-rays and the higher energy range of ultraviolet light constitute the ionizing part of the electromagnetic spectrum. The lower-energy, longer-wavelength part of the spectrum, including visible light, infrared light, microwaves and radio waves, constitute the non-ionizing part of the spectrum. Ultraviolet radiation has features of both ionizing and non-ionizing radiation, but the ultraviolet spectrum that penetrates the Earth's atmosphere is non-ionizing.

Ionizing radiations can damage DNA both directly, accounting for 30-40% of all lesions, or

by indirect mechanisms, which accounts for 60-70% (reviewed in (Ward, 1988)). Direct effects of ionizing radiations are due to ionization of the DNA molecule, which breaks the chemical bonds and generates SSB or DSB. Indirect effects of ionizing radiations are due to ionization of molecules that results in ROS production and that, in turn, damage the DNA by the mechanisms discussed above.

Similarly to ionizing radiation, also ultraviolet (UV) light harms the DNA molecule both directly, via photochemical reactions, and indirectly, via ROS production (reviewed in (Roy, 2017)). UV light induces the formation of covalent linkages in the DNA structure, commonly known as UV photoproducts (Figure 1.6). Two common UV photoproducts are cyclobutane pyrimidine dimers (CPDs, including thymine dimers) and 6,4 photoproducts. Pyrimidine dimers are molecular lesions formed from two thymine or cytosine bases located in proximity on the same DNA strand. These premutagenic lesions introduce local conformational changes in the DNA structure that consequently inhibit polymerases and arrest replication.

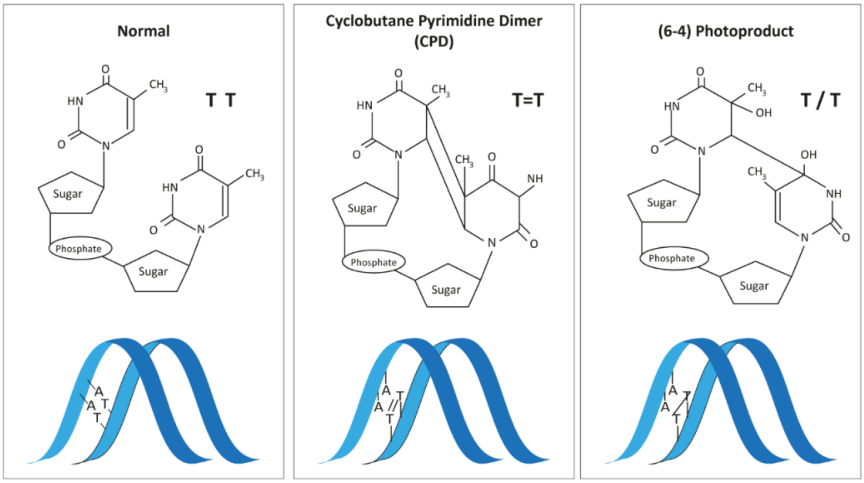

Figure 1.6 **UV-induced photoproducts: 6–4 photoproducts and cyclobutane pyrimidine dimers**. Two adjacent thymine residues (T T) are a potential substrate for UV-induced ionization. Ionization can result in either the formation of cyclobutane pyrimidine dimers (CPD, (T=T)) or 6-4 photoproducts (T/T).

Besides physical agents, also chemical compounds, known as radiomimetic drugs, affect the DNA in similar ways to radiation exposure (reviewed in (Povirk, 1996)). Radiomimetic

compounds induce DSB via highly specific reactions in which free-radical molecules attack the deoxyribose groups in both strands of the DNA. Therefore, the induced lesions are only a small subset of those induced by radiation, however, the final effects on cells are remarkably similar. Examples of such compounds are bleomycin, zeocin and enediyne.

1.3.2.2. Genotoxic compounds

The genetic material can be damaged by a variety of chemical compounds, classified based on the mechanism of action and types of DNA damage induced (reviewed in (Swift and Golsteyn, 2014)).

Alkylating agents are molecules with electrophilic properties that covalently transfer alkyl groups onto the DNA molecule, inducing the formation of DNA adducts, intra and interstrand crosslinks, and protein-DNA crosslinks. These structures, in turn, block the replication fork machinery, leading to DNA strand breaks. One example of alkylating agents is methyl methanesulfonate (MMS), a drug commonly used to study the replication checkpoint.

Antimetabolites are a class of molecules with a chemical structure that is very close to that of canonical nucleotides. Antimetabolites act either by inhibiting biosynthetic processes or by being incorporated into nucleic acids. The inhibition of nucleotide metabolism pathways results in depletion of dNTPs that, in turn, prevents DNA replication (Swift and Golsteyn, 2014). Conversely, incorporation of antimetabolites into DNA stalls or blocks DNA replication (Helleday *et al.*, 2008). Examples of both classes of antimetabolites are hydroxyurea (HU) and 5-Fluorouracil (5-FU). HU inhibits the ribonucleotide reductase (RNR) enzyme by reducing the reactive tyrosyl radical in the active center of the enzyme (Elleingand *et al.*, 1998). Inhibition of RNR causes a rapid depletion of dNTP pools and leads to replication fork stalling (Elleingand *et al.*, 1998). 5-FU, on the other hand, can be

incorporated into DNA or RNA in place of thymine or uracil, respectively (reviewed in (Longley, Harkin and Johnston, 2003)). The repair of 5-FU-containing DNA by the nucleotide excision repair results in further false-nucleotide incorporation. Continuous futile cycles of misincorporation, excision and repair eventually lead to DNA strand breaks (Longley, Harkin and Johnston, 2003).

Crosslinking agents are molecules that are able to form covalent bonds with the DNA molecule, giving rise to DNA adducts, intra and interstrand crosslinks, and protein-DNA crosslinks. These aberrant DNA structures, in turn, causes the replication arrest , leading to DNA strand breaks (Swift and Golsteyn, 2014). Among crosslinking agents, the best known compound is Cisplatin, a genotoxic agent widely used to treat a range of cancers (Swift and Golsteyn, 2016).

Finally, topoisomerase poisons, such as Camptothecin, are genotoxic agents with a mechanism of action aimed at interrupting DNA replication (reviewed in (Pommier, 2006)). Topoisomerases are enzymes responsible for relaxing DNA supercoiling during DNA replication and transcription, and they act by introducing transient nicks into the DNA molecule to relieve torsional stress. To avoid mistakes during this thorny process, the strand cleavage is coupled with the covalent attachment to the topoisomerase itself, forming enzyme-DNA complexes. Topoisomerase inhibitors act by transiently trapping the enzymes in these intermediate complexes, thus resulting in bulk structures on the DNA that, in turn, block the replication fork machinery, leading to DNA strand breaks. One example of topoisomerase inhibitors is Camptothecin.

1.3.3. DNA damage arising from dysfunctional telomeres

Telomeres, deriving from the Greek words “telos” (meaning end) and “mer” (meaning part), are the extremities of linear chromosomes. Telomeres are nucleoprotein complexes

that carry out two main functions: first, they disguise chromosome ends from being identified as DSBs; and second, they ensure self-preservation of the length of terminal DNA, which otherwise would be eroded by successive rounds of DNA replications.

The basic structure of chromosome ends is conserved among eukaryotes and consists of short tandem G-rich DNA repeats (Figure 1.7). The telomere length ranges from approximately 300 bp in budding yeast, to 10-15 kb in humans, and 20-60 kb in mice (Jain and Cooper, 2010). The G-rich strand typically forms a 3' single-stranded overhang at the terminus, which is approximately 12-15 b in budding yeast and approximately 50-150 b in humans and mice (Jain and Cooper, 2010).

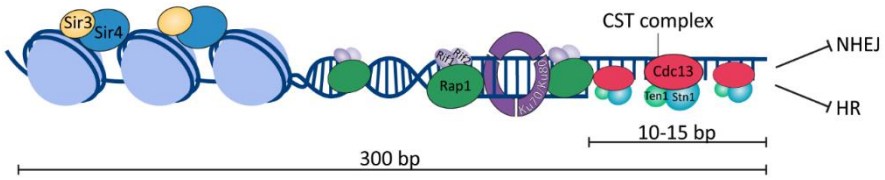

Figure 1.7 **Telomere structure and telomere-associated proteins in *S. cerevisiae*.**

Since the structure of telomeres highly resembles a DSBs, specialized proteins coat the telomeres to prevent them from being recognized by the checkpoint machinery. In budding yeast, the double stranded region contains specific sequences that are bound directly by Rap1, which in turn recruits the additional factors Rif1/Rif2 and Sir3/Sir4. These proteins act synergistically to help control telomere length and establish telomeric silencing. Indeed, loss of Rap1 causes telomere shortening and telomere fusions (Conrad *et al.*, 1990; Lustig, Kurtz and Shore, 1990; Marcand *et al.*, 2008). Furthermore, Rif2 prevents the association of the DSB recognition factors Tel1 and Mre11-Rad50-Xrs2 (MRX) complex (two players of the DSB recognition machinery), therefore disguising telomeres from being recognized as DSB and preventing checkpoint activation (Hirano, Fukunaga and Sugimoto, 2009; Bonetti *et al.*, 2010). Rap1 and the silencing proteins Sir3/Sir4 are responsible for the telomere

position effect (TPE), that is, the silencing of genes adjacent to telomere regions (Moretti *et al.*, 1994; Feeser and Wolberger, 2008).

In budding yeast, the single stranded region is bound by the CST complex, composed of Cdc13, Stn1 and Ten1 (Grandin, 2001). The main function of this complex is to avoid the G-strand recognition by the DNA damage checkpoint machinery. In particular, Cdc13 inhibits the binding of Mec1, the most important checkpoint kinase in yeast, and of RPA, the ssDNA-binding protein involved in DNA repair via HR (Hirano and Sugimoto, 2006; Gao *et al.*, 2007; Gelinas *et al.*, 2009). Consistent with this function, cells carrying a temperature-sensitive *cdc13-1* allele show telomere degradation and activate the DNA damage checkpoint when exposed to high temperatures (Weinert and Hartwell, 1993; Garvik, Carson and Hartwell, 1995). The activation of the DNA damage checkpoint is achieved through the recruitment of the 9-1-1 checkpoint clamp onto telomeres and is aided by the clamp loader, followed by the activation of Exo1, responsible for the degradation of the C-rich strand (Jia, 2004; Zubko, 2004). The newly created ssDNA functions as a docking platform for the recruitment of DNA damage sensors, such as Ddc1 (Melo, Cohen and Toczyski, 2001). Following the recruitment of Mec1, the checkpoint adaptor Rad9 is required to activate the downstream checkpoint effector Rad53 (Garvik, Carson and Hartwell, 1995), which is responsible for mediating the cellular responses to deal with the DNA lesion.

As the main function of telomere nucleoproteins is to protect chromosome ends from repair and checkpoint activation, it seems paradoxical that many checkpoint and DNA repair proteins contribute to telomeric functions. One example is represented by the Ku complex that is composed of two proteins, Ku70 and Ku80. The best known function of the Ku complex is in DNA repair through non-homologous end joining (NHEJ) (Milne *et al.*, 1996), thus its association with telomeres is counterintuitive. The Ku complex binds DNA ends and protects them from resection mediated by the homologous recombination (HR)

machinery (Mimitou and Symington, 2010; Shim *et al.*, 2010) and, consistently, it acts at telomere ends by protecting C-strand from excessive nuclease degradation and inappropriate recombination (Polotnianka, Li and Lustig, 1998).

Another important function on telomeres is the resection of the C-strand after DNA replication to regenerate the 3' single stranded overhang. Remarkably, this process is carried out by the same nucleases that resect the ends of DSBs, the first step for HR-mediated repair. However, excessive resection is prevented on telomeres by the concerted activity of the Ku complex, the CST complex and the Rif proteins (Gravel *et al.*, 1998; Polotnianka, Li and Lustig, 1998; Maringele and Lydall, 2002a; Bonetti *et al.*, 2010)

When the protection of chromosome ends fails, telomeres are recognized by the DNA repair and recombination machineries for repair (Figure 1.8). The possible outcomes range from the generation of circular or dicentric chromosomes to extrachromosomal telomeric circles (Larrivée and Wellinger, 2006). As mentioned before, the lack of the capping protein Cdc13 leads to the activation of the DNA damage checkpoint to prevent anaphase entry (Weinert and Hartwell, 1993; Garvik, Carson and Hartwell, 1995). In addition, telomere erosion by inhibition of telomere replication also elicits a DNA damage response (IJpma and Greider, 2003).

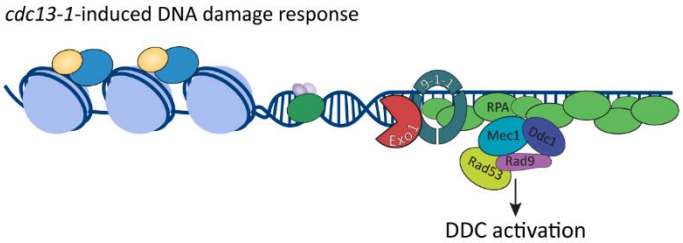

Figure 1.8 **DNA damage response elicited at dysfunctional telomeres**.

1.4. DNA damage repair pathways

As the genome can be damaged by a wide variety of DNA lesions, eukaryotic cells have evolved many highly conserved DNA repair mechanisms, specialized in recognizing and

repairing specific types of DNA lesions with different degrees of fidelity (Figure 1.9). Mismatch repair (MMR) recognizes and replaces mispaired DNA bases, base excision repair (BER) detects chemical alterations of DNA bases and repairs of the damaged base through excision. Nucleotide excision repair (NER) senses more complex lesions, such as pyrimidine dimers and bulky DNA adducts, and removes approximately 30 bp of DNA containing the lesion, which are then refilled by DNA polymerases. DSBs are perhaps the most dangerous form of DNA damage. Consistently, different pathways that repair DSBs have been evolved, and cells choose among them depending on the structure of the DNA ends and the phase of the cell cycle in which the DSB occurs (Figure 1.10). The DNA ends can be directly relegated by the NHEJ pathway, which is an error-prone process since it does not need a homology template. Alternatively, the DSB is repaired using an undamaged homologous sequence as template by the HR, hence in an error-free fashion. Recently, a third repair pathway was identified, namely the microhomology-mediated end joining (MMEJ) which uses the annealing of short homologous sequences to align DNA ends prior to ligation. The existence of multiple repair pathways guarantees backup mechanisms in the eventuality that the primary repair pathway fails (Moldave and Mitra, 2001). Indeed, synergistic interactions among genes involved in different repair pathways have been highlighted (Swanson *et al.*, 1999; Gellon *et al.*, 2001).

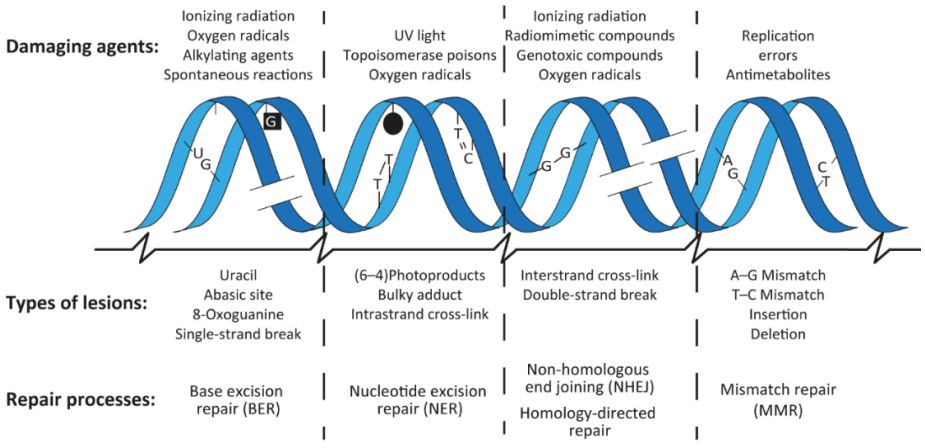

Figure 1.9 **Overview of the DNA repair mechanisms.** Common DNA damaging agents, examples of DNA lesions induced by these agents, together with the pathways involved in the repair of such lesions are shown.

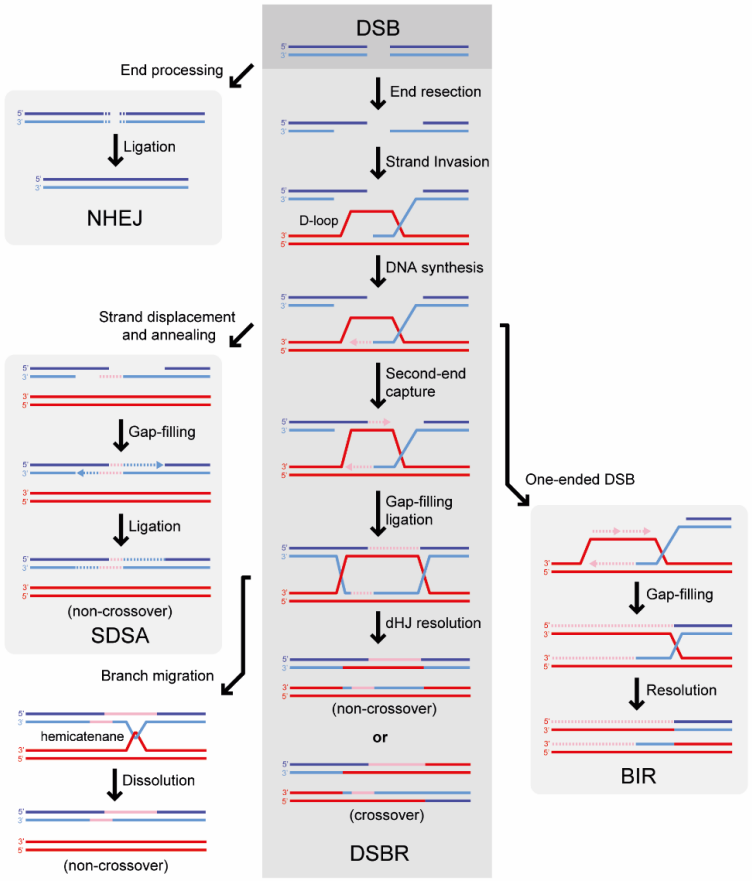

Figure 1.10 **Pathways of DNA double strand breaks (DSB) repair**. *Adapted from (Mathiasen and Lisby, 2014).*

1.4.1. Non-homologous end joining

DSBs repair through NHEJ is highly conserved across eukaryotes. The major players in yeast are the Ku complex, Dnl4-Lif1, Nej1, and the MRX complex, (composed of Mre11-Rad50-Xrs2), the mammalian counterparts of these are Ku70-Ku86, LIG4-XRCC4, Cernunnos (XLF), and the Mre11-Rad50-Nbs1 (MRN) complex, respectively. However, in vertebrates, the MRN complex is not involved in NHEJ, but other factors that carry out the same function exists. Indeed, DNA-PKcs, a catalytic subunit that binds DNA-bound Ku and forms the DNA-dependent protein kinase (DNA-PK), is absent in yeast (Ceccaldi, Rondinelli and D'Andrea, 2016).

The NHEJ mechanism consists of three phases: (i) end protection and tethering, (ii) strand annealing, complex assembly and end-processing, and (iii) ligation and complex disassembly (Figure 1.11). As soon as a DSB occurs, the Ku and MRX complexes reach the

lesion site in an independent manner (Wu, Topper and Wilson, 2008). In these circumstances, the Ku complex exerts the same function as in telomere maintenance, namely it protects DNA ends from degradation by exonucleases (Clerici *et al.*, 2008; Mimitou and Symington, 2010). The inhibitory effect of the Ku complex on resection is central for DSBs repair via NHEJ. Indeed, DSB end resection commits to repair the lesion through HR. The functions of the MRX complex in NHEJ are less understood, especially in light of its role in HR (Bressan, Baxter and Petrini, 1999). Structural studies of the human Mre11/Rad50 complex have shown that it consists of a globular head domain with a long coiled-coil domain of Rad50 that can dimerize, hence forming molecular bridges for keeping the DNA ends tethered to each other (de Jager *et al.*, 2001). Mre11 exhibits different nuclease activities, including 3' exonuclease, endonuclease and DNA helicase activities (Trujillo and Sung, 2001; Ghodke and Muniyappa, 2013). Rad50 possesses ATPase activity that drives the endonuclease and DNA unwinding functions of Mre11. Indeed, ATP-binding of Rad50 induces a closed configuration of the Mre11-Rad50 complex, thus inhibiting the Mre11 nuclease activity, while ATP hydrolysis causes a large conformational change that unmask the Mre11 nuclease active site (Hopfner *et al.*, 2000, 2001; Trujillo and Sung, 2001). Finally, Xrs2 is thought to assist in targeting the MRX complex to DSB ends by direct DNA binding (Trujillo *et al.*, 2003). The current model for MRX functions in NHEJ foresees that the complex acts as a flexible tether to keep DSB ends together and assists the ligation of DNA ends (Emerson and Bertuch, 2016; Gobbini *et al.*, 2016).

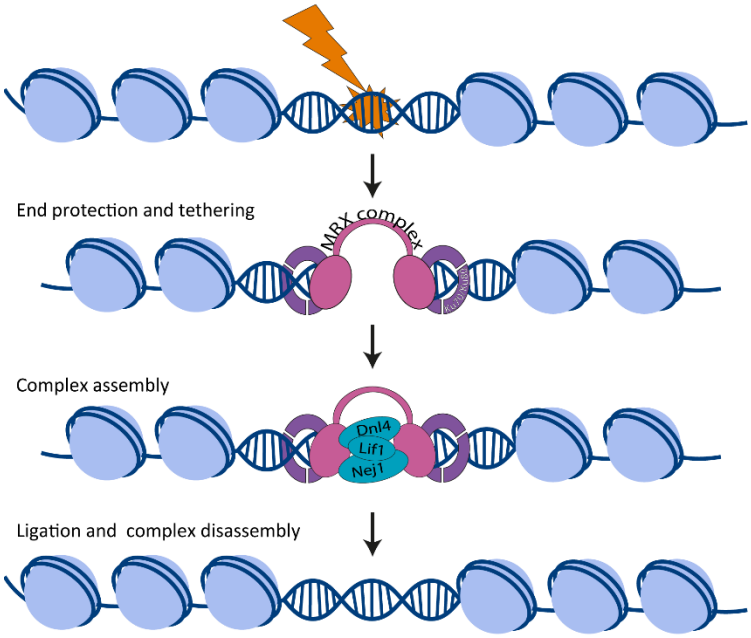

Figure 1.11 **Non-homologous end joining (NHEJ) pathway in *S. cerevisiae*.**

After the Ku and MRX complexes reach the DSB site, the Ku complex is responsible for the recruitment of Dnl4-Lif1 and Nej1, which in turn further stabilize the binding of the Ku complex (Zhang *et al.*, 2007; Chen and Tomkinson, 2011). Dnl4 is an ATP-dependent DNA ligase and, together with its accessory protein Lif1, is responsible for the ligation of two DNA ends (Wilson, Grawunder and Lieber, 1997; Herrmann, Lindahl and Schä, 1998). Nej1 is the most recently identified core NHEJ factor in yeast and, together with the Dnl4-Lif1 complex, is responsible for the recruitment of the end-processing factors Pol4 and Rad27 (Yang *et al.*, 2015). Since most DSB ends generated by ionizing radiation and/or oxygen free radicals have termini that cannot be directly ligated, end-processing factors are responsible for generating the complementary ends needed for successful ligation by Dnl4 (Wilson and Lieber, 1999). Once the two DSB ends have been processed, the Dnl4 can successfully ligate the nicks, thus completing the DSB repair.

1.4.2. Homology-directed repair

HR is a mechanistically conserved pathway from bacteria to human (Amunugama and Fishel, 2012). In germ cells, HR produces genetic diversity through DNA crossover events

between homologous chromosomes during meiosis. In somatic cells, HR ensures the maintenance of genomic stability by repairing DSBs or by restoring replication when a replication fork has encountered a DNA lesion.

As soon as a DSB occurs, the Ku and MRX complexes bind to free DNA ends to tether them (Wu, Topper and Wilson, 2008). The first step for HR-mediated DNA repair is the resection of the DNA ends, which is carried out by specific nucleases to form an extended region of ssDNA (Figure 1.12). This event commits the repair of the DSB via HR instead of via NHEJ, therefore it is subjected to a tight regulatory control (see section 1.4.3 for details). End resection consists of two phases, an initial resection step of 50–200 bp, catalyzed by the MRX complex and the endonuclease Sae2, followed by an extensive resection step of up to several kilobases executed via two parallel pathways, one that is catalyzed by the 5'-3' exonuclease Exo1, and the second by the Sgs1-Top3-Rmi1 (STR) complex together with the Dna2 nuclease. Sgs1 is a 3'-5' DNA helicase that physically interacts with the topoisomerase Top3, which in turn interacts with its accessory factor Rmi1. The STR complex is required for unwinding of dsDNA, which is then degraded by the endonuclease Dna2. The ssDNA ends generated both by initial and extensive resections are bound by the ssDNA-binding protein RPA to protect the ends from degradation and formation of secondary structures.

Following extensive resection, ssDNA-bound RPA is displaced by Rad51 in a process known as Rad51 nucleation. To facilitate the formation of Rad51 filaments, Rad52 mediates the displacement of RPA and Rad55-Rad57 stabilizes the Rad51 filament. Subsequently, the binding of Rad51 to the ssDNA forms a nucleoprotein filament. The Shu complex, an heterotetramer consisting of Csm2, Psy3, Shu1 and Shu2, protects the Rad51 nucleoprotein filament from disassembly by the helicase and anti-recombinase Srs2 (Bernstein *et al.*, 2011; Godin *et al.*, 2013). Rad51 performs two central functions for a successful HR: homology search and DNA-strand invasion. The structure composed by the damaged DNA

and its homologous template is known as synapsis. When synapsis is formed, Rad51 catalyzes the invasion of the 3' single stranded tail, generating a displacement loop (D-loop). The invading 3' end serves to prime DNA synthesis using the homologous sequence as a template.

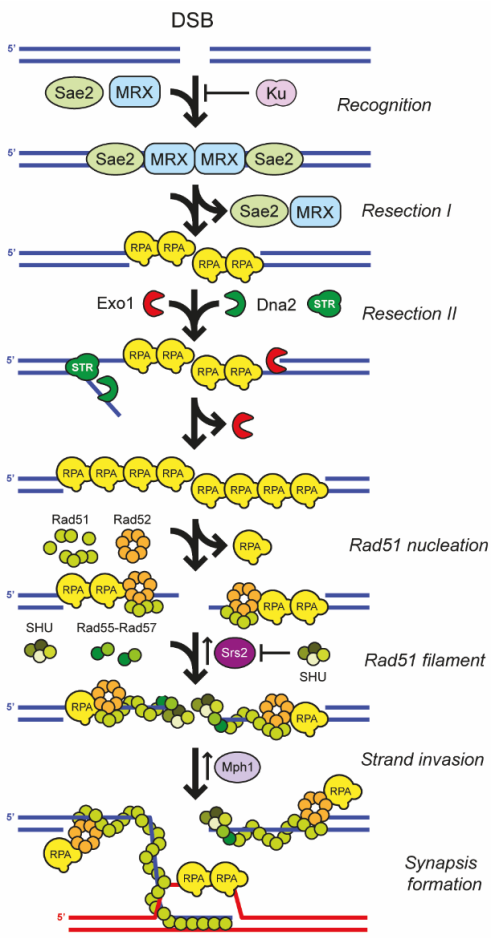

Figure 1.12 **Homology-directed repair (HDR) pathways**. Initial common steps in DSB processing via HDR pathways are shown. *Adapted from (Mathiasen and Lisby, 2014).*

After the formation of the D-loop, multiple subpathways for the resolution of synapsis exist: break-induced replication (BIR), synthesis-dependent strand annealing (SDSA) and double Holliday junction (dHJ) (Figure 1.13). BIR occurs in case of a one-ended DSB, perhaps arising after a replication fork collapse. In this case, the DNA synthesis within the D-loop may continue to the end of the chromosome. SDSA is the predominant pathway in somatic cells and occurs in the presence of a second DSB end. In this case, the extended invading strand is displaced and can then anneal back to the complementary strand of the other DSB

end. The repair is completed by gap filling and ligation, resulting exclusively in non-crossover products. Finally, dHJ occurs when the second DSB end is captured by the displaced strand of the D-loop. In this case, the second DSB end also serves as a template for DNA synthesis. The resolution of dHJ can produce either crossover or non-crossover recombination products. It has been shown that, while in meiotic recombination the favored subpathway is dHJ producing crossover products, in somatic cells SDSA is the favored subpathway, as second-end capture is suppressed.

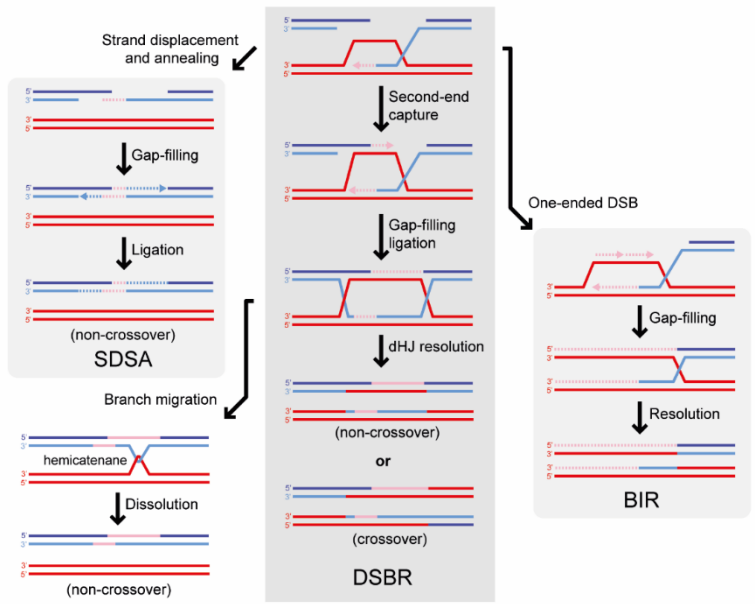

Figure 1.13 **Homology-directed repair pathways differs in the mechanism of synapsis resolution.** Particular from Figure 1.10. *Adapted from (Mathiasen and Lisby, 2014).*

1.4.3. Cell cycle regulation of DSB repair pathway

In haploid yeast cells, DSBs that occur during the G_1 phase are mainly repaired by NHEJ, while DSBs that occur during the S and G_2 phases are repaired by HR. A key determinant in the choice of DSBs repair pathway is whether the DSB is resected to generate the 3' ssDNA tail, essential for repair through HR. Once resected, NHEJ is blocked and the repair of the DSB is committed to HR. Therefore, cells regulate the initiation of resection to prevent inappropriate attempts of HR in the absence of sister chromatids. Indeed, DNA end resection is the critical node for the regulation of DSB repair by the cell cycle.

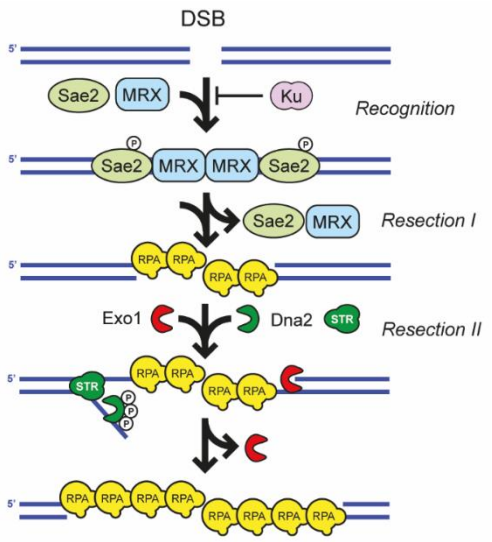

Figure 1.14 **CDK-mediated phosphorylation of double strand break repair components commits the repair via homology-directed mechanisms**. Particular from Figure 1.12. *Adapted from (Mathiasen and Lisby, 2014).*

Initiation of resection is regulated by cyclin-CDK complex activity at the transcription level and at the protein level through posttranslational modification, degradation and subcellular localization. The expression of Exo1, Sgs1 and Sae2 is cell cycle-regulated (Figure 1.14). The transcripts of both Exo1 and Sae2 peak in late G_1, while the transcript of Sgs1 peaks in early S phase. Additionally, both Sae2 and Dna2 are targeted by CDK-dependent phosphorylation in S/G_2 (Huertas *et al.*, 2008; Kosugi *et al.*, 2009; Chen *et al.*, 2011). Phosphorylation of Sae2 causes its transition from an oligomeric inactive configuration to a monomeric/dimeric active one (Fu *et al.*, 2014), while the phosphorylation of Dna2 facilitates its relocalization from the cytoplasm to the nucleus (Kosugi *et al.*, 2009; Chen *et al.*, 2011). The activity of the Ku complex also seems to be regulated by CDK complexes. Both components of the Ku complex carry putative CDK phosphorylation sites, however, their removal does not affect DSB repair, thus suggesting an indirect regulation of the Ku complex (Y. Zhang *et al.*, 2009).

1.4.4. Other pathways involved in DNA damage repair

1.4.4.1. Base excision repair (BER)

Oxidized bases and abasic sites result from endogenous sources such as oxidative stress, hydrolysis or deamination. This type of lesions should be repaired before DNA replication, otherwise they can act as obstacles for the replication fork, therefore causing fork stalling and, potentially, fork collapse. The BER pathway plays an essential role in restoring oxidized bases and abasic sites. The BER pathway involves the sequential action of several enzymes to carry out specific DNA-modifying activities (Figure 1.15). Briefly, repair through the BER pathway begins with the activity of DNA N-glycosylase enzymes, responsible for the cleavage of the N-glycosylic bond between the damaged base and its deoxyribose. As a result, the cleaved base is released and an apyrimidinic/apurinic (AP) site is formed. AP endonucleases catalyze the cleavage of the phosphodiester backbone, therefore producing a nick with a residual deoxyribose phosphate residue, which is removed by 3'-or 5'-phosphodiesterases. The small gap generated is subsequently filled by a DNA polymerase and, finally, the nick is sealed by a DNA ligase (Hoeijmakers, 2001).

In yeast, five DNA N-glycosylases are present, and they can be subdivided in two groups according to their enzymatic activity. The first class comprises monofunctional enzymes that only catalyze cleavage of the N-glycosylic bond (Ung1 and Mag1). The second class comprises bifunctional DNA N-glycosylases/AP lyases that catalyze both cleavage of the N-glycosylic bond and nicking of the phosphodiester backbone at AP sites (Ntg1, Ntg2 and Ogg1). In addition, yeast possesses other two AP endonucleases (Apn1 and Apn2), which are required for nick introduction. DNA polymerase ε (Pol ε) is responsible for gap filling and the DNA ligase Cdc9 seals the nick (Boiteux and Jinks-Robertson, 2013).

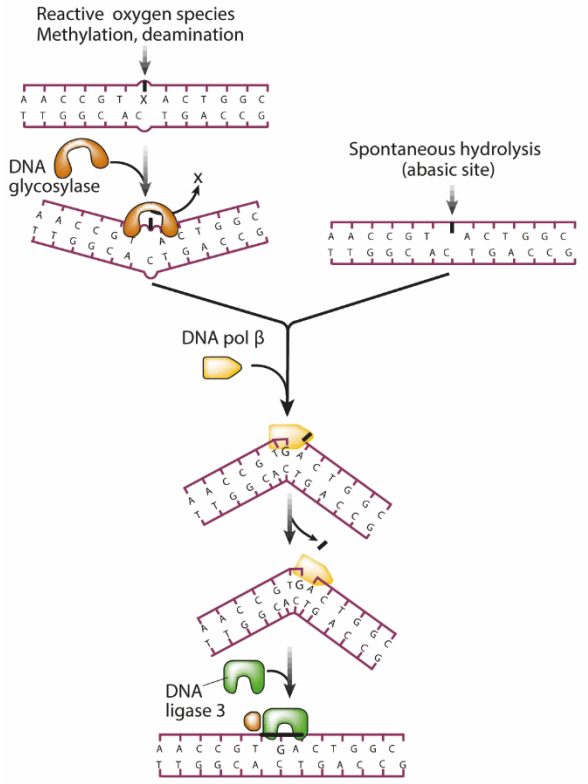

Figure 1.15 **Base excision repair (BER) pathway.** *Adapted from (Hoeijmakers, 2001).*

1.4.4.2. Nucleotide excision repair (NER)

The NER repair pathway senses and processes a plethora of structurally unrelated, helix-distorting lesions that are able to interfere with base pairing and impair replication and transcription, such as pyrimidine dimers and intra-strand crosslinks. NER also represents a backup mechanism to repair oxidized bases and abasic site (Swanson *et al.*, 1999; Torres-Ramos *et al.*, 2000). Based on the modality of the recognition of the lesion, the NER pathway is subdivided in two subpathways: global-genome NER (GG-NER) and transcription-coupled NER (TC-NER) (Figure 1.16). In GG-NER, the initiating lesion is recognized by a specific set of proteins that globally "scan" the genome, while in TC-NER, the lesion is recognized after a stall of the transcribing RNA polymerase. Once a lesion has been recognized, the two subpathways converge in a mechanism that foresee the incision of the 25–30 b ssDNA stretch containing the lesion. Following the release of the stretch, the gap is filled by DNA polymerase and the remaining nick is sealed by the DNA ligase.

The GG-NER sensing machinery is represented by a trimeric Rad4-Rad23-Rad33 complex that senses distortion of the DNA helix and opens the helix. The resulting DNA structure convenes the TFIIH transcription factor, which is composed of seven subunits and comprises two ATP-dependent DNA helicases, namely Rad3 and Rad25. Therefore, the TFIIH transcription factor further opens the helix and allows for the recruitment of Rad14 and RPA to stabilize the pre-incision complex and perform a "verification step". In the absence of the verification of the lesion, the NER reaction aborts before the incision of the damage strand.

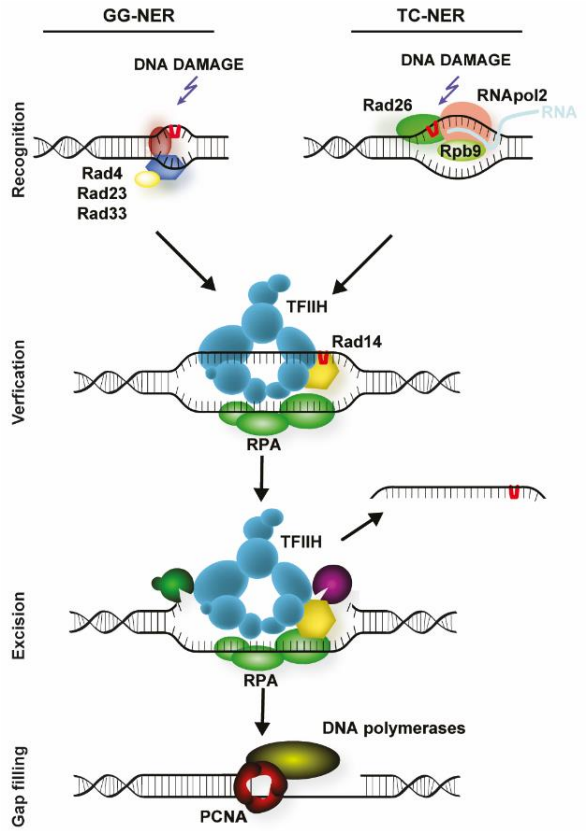

Figure 1.16 **Nucleotide excision repair (NER) pathways.** *Adapted from (Lans, Marteijn and Vermeulen, 2012).*

The TC-NER pathway is triggered when RNA Pol II stalls, and it comprises two subpathways: one dependent on the Rad26 protein and a second one dependent on the Rpb9 subunit of RNA Pol II. These subpathways show different efficiencies in different genes and regions of a gene. Indeed, it was shown that the Rad26-dependent mechanism is predominant in slowly and moderately transcribed genes (Tijsterman *et al.*, 1997; Li, 2002), while the Rpb9-

dependent mechanism is predominant in highly transcribed genes (Li, 2002; Li and Smerdon, 2004). Moreover, the Rad26-dependent mechanism can be active in certain regions upstream of the transcription start site (Tijsterman *et al.*, 1997; Li, 2002), while the Rpb9-dependent mechanism acts more effectively in the region downstream of the transcription start site (Li, 2002; Li and Smerdon, 2004).

Recent studies suggest that Rad26 may be already associated with RNA Pol II during transcriptional elongation, therefore, its recruitment to the lesion site would be dependent on the stalling of RNA Pol II (Malik *et al.*, 2009). Depending on whether the lesion stalls the transcription machinery transiently or permanently, the stalled complex has two alternative outcomes. Rad26 presumably promotes the bypass of the lesion at moderately blocking lesions, such as pyrimidine dimers, allowing for the transcription to continue. On the other hand, at strongly blocking lesions, such as intra-strand crosslinks, the bypass of the damage is impaired and the TC-NER pathway is triggered, resulting in that Rad26 recruits additional NER factors that allow the backtracking of RNA Pol II without dissociation from the template. In this way, the lesion is exposed to the NER machinery for its repair. Similarly, in the Rpb9-dependent TC-NER, it has been proposed that Rpb9 exerts functions comparable to the ones described above for Rad26 (Boiteux and Jinks-Robertson, 2013).

1.4.4.3. Mismatch repair (MMR)

The mismatch repair (MMR) pathway is a specialized mechanism that detects helical distortions that arise when errors are made during DNA synthesis or when non-identical duplexes exchange strands during recombination (Hsieh and Yamane, 2008).

Much of the knowledge of eukaryotic MMR derives from studies in *E. coli*, in which the process is best understood. *E. coli* possesses three specialized mutator or “Mut” proteins,

namely MutS, MutL and MutH (Modrich and Lahue, 1996). The MutS homodimer detects and binds to mismatches and the MutL homodimer bridges mismatch identification with the recruitment of MutH, which cuts specifically the newly synthetized strand but not the template strand. The discrimination of the newly synthetized strand is based on the transient, hemi-methylation of DNA following replication. Finally, the combined activity of a helicase (UvrC) and a single-strand exonuclease results in the degradation of the nicked strand, followed by filling of the gap by the DNA polymerase and sealing of the remaining nick by the DNA ligase.

In eukaryotes, several MutS and MutL homologs have been identified, and specialized, highly conserved functions have been attributed to each one of them. However, a MutH-like protein is absent in eukaryotes, and discrimination of newly synthetized strands is not based on hemi-methylation of DNA.

In budding yeast, six MutS homologs can be found: Msh1 is specifically involved in MMR in the mitochondria (Mookerjee, Lyon and Sia, 2005; Sia and Kirkpatrick, 2005); Msh2, Msh3 and Msh6 are involved in MMR of the genomic DNA and form two functionally redundant heterodimeric complexes, MutSα and MutSβ, composed of Msh2/Msh6 or Msh2/Msh3, respectively (Marsischky *et al.*, 1996; Robert E. Johnson *et al.*, 1996); finally, Msh4 and Msh5 associate to form a heterodimeric MutS-like complex, known as MutSγ, specifically involved in MMR during meiosis (Pochart, Woltering and Hollingsworth, 1997).

In budding yeast, four MutL homologs are present: Mlh1, Mlh2, Mlh3 and Pms1, and they form three different MutL-like complexes: MutLα, MutLβ and MutLγ, composed of Mlh1-Pms1, Mlh1-Mlh2 and Mlh1-Mlh3, respectively (Wang, Kleckner and Hunter, 1999). After the activation of MMR, MutLα can interact with both MutSα and MutSβ, while during meiotic crossover, MutLα interacts with MutSγ.

The proposed mechanism for repair mediated by MMR is depicted in Figure 1.17. Since a MutH-like protein is absent in eukaryotes, other proteins mediate the discrimination of newly synthetized strands, as well as the generation of the nick and the degradation of the mismatch-containing stretch. In budding yeast, such proteins are the proliferating cell nuclear antigen (PCNA) sliding clamp, the MutLα complex and the Exo1 exonuclease. The PCNA sliding clamp is a ring-shaped homotrimer of the Pol30 protein that encircles DNA and functions as a sliding clamp and processivity factor for replicative DNA polymerases (Paunesku *et al.*, 2001). Since PCNA can physically interact with MutSα (Clark *et al.*, 2000; Flores-Rozas, Clark and Kolodner, 2000), MutSβ (R E Johnson *et al.*, 1996) and Mlh1 (Umar *et al.*, 1996; Lee and Alani, 2006), it has been suggested that PCNA may act as a scaffold to targets MMR proteins to sites of new DNA synthesis and to contribute, at least partially, to strand discrimination (Umar *et al.*, 1996; Lee and Alani, 2006). The yeast MutLα is a strand-directed endonuclease that incises DNA in a MutSα- and PCNA-dependent manner (Erdeniz *et al.*, 2007; Kadyrov *et al.*, 2007).

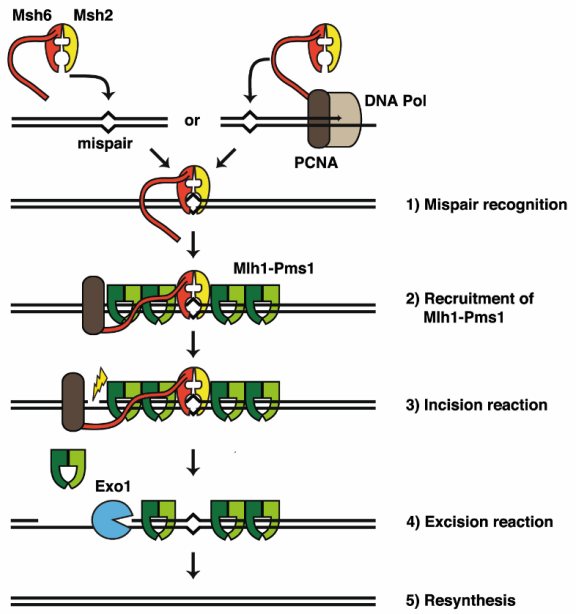

Figure 1.17 **Mismatch repair (MMR) pathway.** *Adapted from (Lens, Voest and Medema, 2010).*

The newly created nick is then used as an access point for the Exo1 endonuclease. The budding yeast Exo1 has been shown to physically interact with Msh2 (Tishkoff *et al.*, 1997)

and Mlh1 (Tran, Simon and Liskay, 2001). Moreover, it has been suggested that Exo1 has a structural and an enzymatic role during MMR (Sokolsky and Alani, 2000; Amin *et al.*, 2001; Tran, Simon and Liskay, 2001), where it catalyzes the degradation of mismatch-containing DNA.

The DNA mismatch repair is completed when the resected strand is filled by the DNA polymerase δ and the remaining nick is sealed by the DNA ligase Cdc9.

1.5. Responses to genome-wide DNA damage

The function of the DNA damage response is to early detect DNA lesions and to coordinate cellular responses to promptly restore the genome integrity (Lowndes and Murguia, 2000; Zhou and Elledge, 2000; Bartek and Lukas, 2007). Such cellular responses include cell cycle arrest, activation of transcriptional programs, initiation of DNA repair or, if the damage is too severe, cellular senescence or programmed cell death (Harper and Elledge, 2007). Therefore, the DNA damage response can be defined as a network of interacting pathways operating in concert, where the checkpoint proteins act as a hub between detection and cellular responses.

Checkpoint proteins can be formally divided into sensors, transducers and effectors. The role of sensor proteins is to recognize DNA damages, directly or indirectly, and to recruit transducer proteins to the damage site. Transducer proteins, in turn, relay the signal to effector proteins, which are responsible for the activation of downstream cellular responses. Although it is simpler to think of the DNA damage checkpoint in this linear way, the situation is far more complex. Indeed, there is an extensive communication, and in some cases overlap, between the DNA damage checkpoint proteins and proteins involved in DNA replication and repair. For instance, the MRX complex (composed of Mre11-Rad50-Xrs2) is one of the first complexes recruited at the site of DSB, where it takes part in the

first steps of the repair but, most importantly, it is also essential for the initiation of the checkpoint signal (Yamamoto, 1996; Ubersax *et al.*, 2003).

1.5.1. DNA damage checkpoints in *S. cerevisiae*

In budding yeast, the DNA damage checkpoints act to control three distinct cell cycle transitions, the START, the G_1/S and the G_2/M transitions. The G_1 checkpoint arrests cells prior to START, hence before cells are irreversibly committed to the next cell cycle. DNA lesions that arise during the G_1 phase must be promptly repaired before replication onset. However, certain DNA lesions do not activate the G_1 checkpoint and persist into S phase, where they can hinder the replication of DNA of the replication fork and cause its stalling at the lesion sites. During S phase, two checkpoints detect the presence of a DNA damage (intra-S) or replication stress (replication checkpoint) (Segurado and Tercero, 2009). Although the intra-S and replication checkpoints are two genetically separable pathways, these checkpoints show a significant redundancy in functions and components due to the occurrence of similar or common DNA structures (Myung and Kolodner, 2002; Zegerman and Diffley, 2009). Differently from other organisms, like *S. pombe* and vertebrates, in *S. cerevisiae* the G_2/M DNA damage checkpoint regulates the transition from metaphase to anaphase rather than the transition from G_2 to M, thus preventing exit from mitosis instead of mitotic entry (Weinert and Hartwell, 1988).

1.5.2. DNA damage checkpoint players in *S. cerevisiae*

The DNA damage checkpoint mechanism is evolutionarily conserved among eukaryotes and the main actors are members of a family of phosphoinositide 3-kinase-related kinases (PIKKs), which include Tel1 and Mec1 in *S. cerevisiae*, Tel1 and Rad3 in *S. pombe*, and ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), DNA-PKcs (DNA-dependent protein kinase catalytic subunit) in mammals (Zhou and Elledge, 2000; Lovejoy and Cortez, 2009). A homologue of DNA-PKcs has not been identified in *S. cerevisiae*. Both Tel1 and

Mec1 play important roles in DSB signaling: Tel1 is recruited at DSB sites (Usui, Ogawa and Petrini, 2001), while Mec1 is recruited at RPA-coated ssDNA sites (Zou, 2003; Dart *et al.*, 2004). ssDNA sites are an intermediate of DNA repair that can arise from different processes, such as nucleotide and base excision repair, at stalled replication forks, or following DSB resection (Carr, 2002; Sogo, Lopes and Foiani, 2002). The two apical PIKKs, in turn, promote the activation of downstream effector kinases Chk1 and Rad53 in *S. cerevisiae*, Chk1 and Cds1 in *S. pombe*, and CHK1 and CHK2 in mammals, that are essential for amplification of the DDR signal, as well as for the activation of downstream components (Stracker, Usui and Petrini, 2009). Differently from vertebrates, where ATM activates primarily CHK2 and ATR activates CHK1 (Stracker, Usui and Petrini, 2009), in *S. cerevisiae*, Mec1 is responsible for the activation of both Rad53 and Chk1 (Sanchez *et al.*, 1999). Mediator proteins, Rad9 in *S. cerevisiae*, Crb2 in *S. pombe* and 53BP1 in mammals, act as molecular scaffolds to recruit downstream effector kinases to sites of damage and trigger the PIKK-dependent phosphorylation (Harper and Elledge, 2007). Downstream effector kinases ultimately activate downstream targets, which are responsible for checkpoint-mediated cellular responses, such as regulation of transcription level of repair genes and regulation of cell cycle transitions by influencing the stability and/or localization of proteins involved in cell cycle progression or checkpoint maintenance (Branzei and Foiani, 2008).

1.5.3. DNA damage checkpoint activation mechanism in response to DSBs

DSBs are perhaps the most dangerous form of DNA damage due to their ability to induce chromosomal aberrations (Natarajan *et al.*, 1980), including deletions, inversions, duplications and translocations.

The first step for a successful DNA damage checkpoint activation is sensing of the lesion, which is not a proper role of checkpoint proteins, but of the DSB repair proteins (Figure 1.18). Indeed, as soon as a DSB occurs, it is specifically and independently recognized by

both the Ku and MRX complexes (Martin *et al.*, 1999; Lisby *et al.*, 2004; Zhang *et al.*, 2007; Clerici *et al.*, 2008; Wu, Topper and Wilson, 2008; Shim *et al.*, 2010), two components that initiate the DSB repair through NHEJ and HR, respectively. The MRX complex is considered the principal sensor of DSB-induced checkpoint activation in *S. cerevisiae* (Rupnik, Lowndes and Grenon, 2010), and its binding to DNA ends elicits the recruitment of Tel1. The physical interaction between Tel1 and Xrs2 leads to Tel1-dependent checkpoint activation prior to DNA end processing (D'Amours, 2001; Grenon, Gilbert and Lowndes, 2001; Usui, Ogawa and Petrini, 2001; Nakada, 2003). Tel1 phosphorylates serine 129 on the histone variant H2AX (γ-H2AX), giving rise to a region that is approximately 50 kb large and surrounds the break, further amplifying the signal from the lesion site (Downs, Lowndes and Jackson, 2000; Shroff *et al.*, 2004). Indeed, in both *S. cerevisiae* and vertebrates, γ-H2AX acts as a chromatin modulator by recruiting cohesin, histone modifiers and chromatin remodeling complexes (van Attikum and Gasser, 2009; Rossetto *et al.*, 2010). Moreover, Tel1 also promotes a robust checkpoint response by mediating the accumulation and stable retention of checkpoint and repair proteins at the site of the damage (Huertas, Sendra and Muñoz, 2009).

A key step in the switch from Tel1 to Mec1 signaling is represented by resection of the DSB ends, which is also the first step for DBS repair through HR (Mantiero *et al.*, 2007; Shiotani and Zou, 2009). Resection occurs by a two-step mechanism (Mimitou and Symington, 2008; Zhu *et al.*, 2008), an initial, "short", processing mediated by the MRX complex and Sae2, followed by an extensive processing mediated by the exonuclease Exo1 (see section 1.4.2 for details). Once an extensive resection of DSB ends has taken place, Sae2, MRX and Tel1 dissociate from the DSB (Lisby and Rothstein, 2009), and RPA binds concomitantly to the 3' ssDNA tails (Lisby *et al.*, 2004). RPA-coated ssDNA is responsible for the recruitment of Mec1 to the lesion site (Lydall and Weinert, 1995; Zou, 2003). Moreover, RPA-coated ssDNA

recruits the 9-1-1 checkpoint clamp (Ddc1-Rad17-Mec3 in *S. cerevisiae*; Rad9-Rad1-Hus1 in *S. pombe*, and RAD9-RAD1-HUS1 in mammals), which structurally resembles the replication clamp PCNA (Doré *et al.*, 2009; Sohn and Cho, 2009), and clamp loader (Rad24-Rfc2-5 in *S. cerevisiae*; RAD17-RFC2-5 in mammals), which resembles the PCNA loader (Green *et al.*, 2000). The main function of the 9-1-1 complex is to promote Mec1 kinase activity in both G_1 and G_2 phases (Kondo, 2001; Melo, Cohen and Toczyski, 2001; Wu, Shell and Zou, 2005; Majka, Niedziela-Majka and Burgers, 2006), which is achieved through a direct interaction between Ddc1 and Mec1 (Navadgi-Patil and Burgers, 2011), as well as through Ddc1-mediated recruitment of the replication initiation protein Dpb11 that stimulates Mec1 kinase activity in the G_2/M phase (Mordes, Nam and Cortez, 2008; Navadgi-Patil and Burgers, 2008, 2009; Pfander and Diffley, 2011).

Following Mec1 binding at resected DNA ends, the checkpoint adaptor protein Rad9 is recruited. In unperturbed conditions, Rad9 is in an hypophosphorylated form and it is associated with Ssa1 and/or Ssa2 chaperone proteins (Gilbert, Green and Lowndes, 2001; Gilbert *et al.*, 2003) that presumably facilitate Mec1-dependent remodeling of the Rad9 complex (Gilbert *et al.*, 2003). A small portion of hypophosphorylated Rad9 is already associated with chromatin also in unperturbed conditions, likely to ensure a prompt and efficient Rad9-dependent DNA damage checkpoint response (Hammet *et al.*, 2007; Granata *et al.*, 2010). In DNA damage conditions, Rad9 is recruited to the lesion sites by two different mechanisms, one involves histone modifications (γ-H2AX and H3K79 methylation) (Huyen *et al.*, 2004; Grenon *et al.*, 2007; Hammet *et al.*, 2007), and the other involves Ddc1/Dpb11 (Puddu *et al.*, 2008). Briefly, the constitutive methylation of histone H3K79

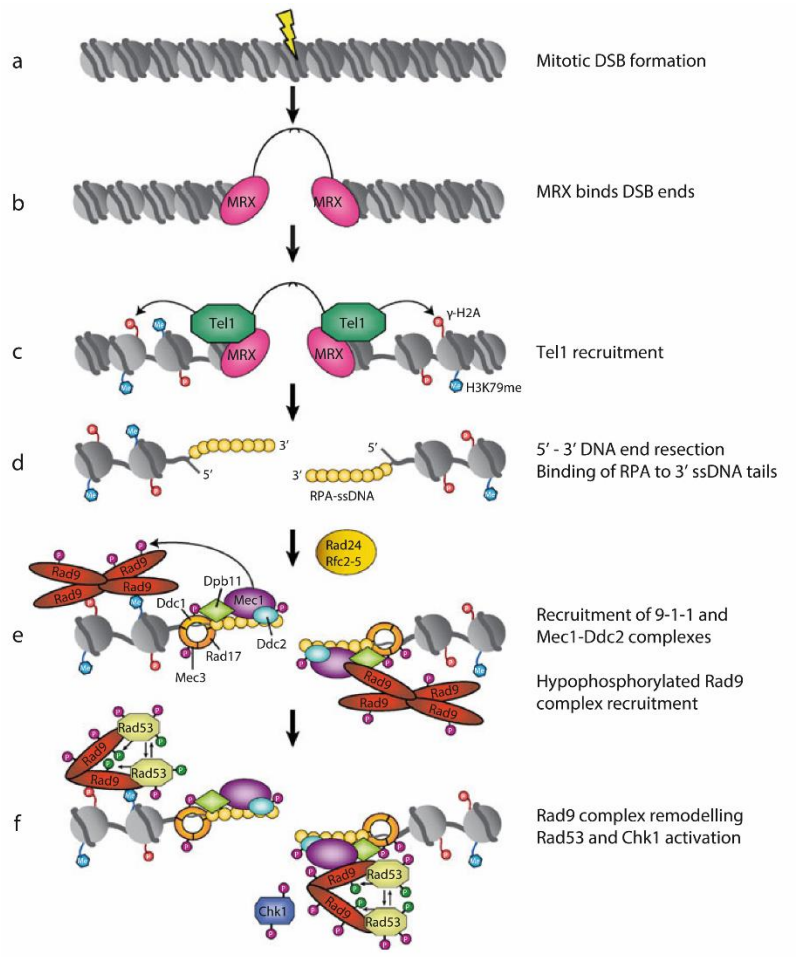

Figure 1.18 **DNA damage checkpoint activation in response to double strand breaks in *S. cerevisiae***. *Adapted from (Finn, Lowndes and Grenon, 2012).*

(H3K79me) is normally hidden in the nucleosome core and it is inaccessible until DNA damage-induced chromatin remodelers expose it (Luger *et al.*, 1997; van Leeuwen, Gafken and Gottschling, 2002). It has been suggested that both H3K79me and γ-H2AX act as a docking site for Rad9 recruitment at the site of the damage (Giannattasio *et al.*, 2005; Wysocki *et al.*, 2005; Toh *et al.*, 2006; Hammet *et al.*, 2007; Huertas, Sendra and Muñoz, 2009). At the lesion sites, Rad9 amplifies the DNA damage signal and facilitates the activation of Rad53 by acting as a molecular scaffold to bring in close proximity Mec1 and Rad53 molecules. Mec1-dependent phosphorylation of Rad9 creates a docking site for Rad53 recruitment (Gilbert, Green and Lowndes, 2001; Schwartz *et al.*, 2002; Sweeney *et al.*, 2005) and this results in Mec1-dependent phosphorylation of Rad53 (Sweeney *et al.*, 2005), as well as in-trans autophosphorylation of Rad53, which is required for the full

activation of Rad53 and its release from the hyperphosphorylated Rad9 complex (Gilbert, Green and Lowndes, 2001).

Besides its role in Rad53 activation, hyperphosphorylated Rad9 also mediates the activation of the parallel downstream kinase Chk1 with a similar mechanism. Indeed, Chk1 is recruited at the lesion site in a Rad9-dependent manner (Abreu *et al.*, 2013), where it is activated through a Mec1-dependent phosphorylation (Sanchez *et al.*, 1999; Blankley, 2004). Following their activation, Rad53 and Chk1 kinases phosphorylate several downstream targets that are involved in cell cycle control and transcriptional regulation.

1.5.4. G_1 checkpoint targets

The G_1 checkpoint surveils the integrity of the genome before passing through START, or the restriction point, when cells become irreversibly committed to the next cell cycle. The irreversible commitment is achieved through a self-sustaining wave of transcription of genes involved in the transition from G_1 to S phase. This mechanism of G_1/S transcriptional activation is conserved from yeast to humans. Briefly, in *S. cerevisiae*, the transcriptional factor SCB-binding factor (SBF) activates the expression of genes involved in the transition from G_1 to S phase transition. SBF is kept inactive in early G_1 phase by the transcriptional inhibitor Whi5, which is a target of G_1 cyclin–CDK (G_1 cyclins in *S. cerevisiae* are Cln1, Cln2, and Cln3). Phosphorylation of Whi5 by Cln3–CDK results in its inactivation and activation of Cln1 and Cln2 transcription. In turn, Cln1 and Cln2 in complex with CDK further inactivate Whi5, thus providing a positive feedback that leads to cell cycle commitment. Activation of G_1/S transcription results in the accumulation of S phase cyclins, Clb5 and Clb6 in budding yeast, responsible for the regulation of DNA replication initiation. The Clb5-CDK and Clb6-CDK complexes are kept inactive in the G_1 phase by the Clb–CDK-specific inhibitor Sic1. This inhibition is relieved a two-step process where Cln–CDK phosphorylation, followed by Clb-CDK phosphorylation, primes Sic1 for degradation (Kõivomägi *et al.*, 2011).

When DNA lesions occur in the G_1 phase, following checkpoint activation, Rad53 directly phosphorylates one subunit of SBF transcription factor. This inhibitory phosphorylation prevents transcription of G_1 and S cyclins (Sidorova and Breeden, 1997, 2003) and the consequent degradation of Sic1 (Schwob, 1994; Verma, 1997), therefore inhibiting cell cycle entry.

1.5.5. S phase checkpoint targets

Faithful genome replication is of primary importance for cells to maintain the genome integrity, therefore it is not surprising that two checkpoints act in the S phase to surveil the presence of a DNA damage (intra-S DNA damage checkpoint) or replication stress (replication checkpoint) (Segurado and Tercero, 2009).

In *S. cerevisiae*, intra-S checkpoint activation depends on Mec1 and Rad53 kinases (Paulovich and Hartwell, 1995) through the mechanism discussed above. In response to replication stress, Mec1 can be activated by two independent pathways, depending on whether the signal comes from the leading or the lagging strand (Puddu *et al.*, 2011). On the leading strand, the activation of Mec1 requires the leading strand factors Dpb4, Dpb11 and Sld2, while, on the lagging strand, it depends on the recruitment of the 9-1-1 complex, which is loaded following stabilization of the 5' ends of primer-template junctions that result from polymerase stalling. Following the activation of Mec1, Rad53 is activated and mediates all the molecular events required for the stabilization of DNA replication forks (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Tercero, Longhese and Diffley, 2003) and inhibits the firing of late replication origins (Santocanale and Diffley, 1998; Shirahige *et al.*, 1998). Stabilization of the replication fork is achieved through Rad53-dependent phosphorylation of Exo1 and results in the inhibition of its exonuclease activity, thus preventing the accumulation of ssDNA and consequent recombination events (Morin *et al.*, 2008; Segurado and Diffley, 2008). Inhibition of late origin firing is achieved through Rad53-

mediated phosphorylation, and consequent inhibition of several targets, including the DNA polymerase α-primase (Pol α-primase) (Longhese *et al.*, 1996; Marini *et al.*, 1997) and two replication initiation factors, namely Sld3 and Dbf4 (Lopez-Mosqueda *et al.*, 2010; Zegerman and Diffley, 2010).

Moreover, the activity of Rad53 is required for the activation of DNA damage-induced transcriptional programs. Dun1 is a protein kinase downstream of Rad53 and it is required for the transcriptional activation of several DNA repair genes and genes encoding RNR subunits (Allen *et al.*, 1994; Gasch *et al.*, 2001; de Bruin and Wittenberg, 2009). RNR is involved in the modulation of dNTP pools, which are important for the completion of replication (Zhou and Elledge, 1993; de la Torre Ruiz and Lowndes, 2000; Chen, Smolka and Zhou, 2007).

Following S phase checkpoint activation, the cell cycle is arrested or delayed until the DNA lesions have been repaired and DNA replication is completed. In vertebrates and in *S. pombe*, this is achieved through the inhibition of CDK activity, therefore preventing the G_2/M transition. However, in *S. cerevisiae*, cyclin-CDK complexes are not targeted in response to replication stress (Sorger and Murray, 1992). Instead, mitotic exit is inhibited at multiple levels to ensure that chromosome segregation does not occur in the presence of damaged DNA. The regulation of mitotic exit by checkpoint proteins will be discussed in section 1.6.2.

1.6. The G_2/M checkpoint

In most eukaryotes, the most prominent checkpoint response is the DNA damage-induced G_2 arrest, which prevents entry into mitosis by inhibiting CDK activity. In *S. pombe* and vertebrates, phosphorylation of two conserved residues in CDK1, tyrosine 15 and threonine 14, results in the inhibition of CDK activity (Gould and Nurse, 1989; Krek and Nigg, 1991;

Norbury, Blow and Nurse, 1991; Parker, Atherton-Fessler and Piwnica-Worms, 1992; Lee, Enoch and Piwnica-Worms, 1994; Den Haese *et al.*, 1995). The phosphorylation status of these residues is regulated by the Wee1 family of kinases (Swe1 in *S. cerevisiae*; Wee1 and Mik1 in *S. pombe*, and Wee1 and Myt1 in human), and the Cdc25 family of phosphatases (Mih1 in *S. cerevisiae*; Cdc25 in *S. pombe*, and Cdc25A, Cdc25B, Cdc25C in human) (Sancar *et al.*, 2004). In *S. pombe* and mammalian cells, Chk1 indirectly inhibits the activity of CDK complexes by acting on both Wee1 and Cdc25. Phosphorylation of Wee1 results in its stabilization, therefore ensuring that the phosphorylation of CDK complex on tyrosine 15 is maintained (O'Connell, 1997; Rhind, Furnari and Russell, 1997; Raleigh and O'Connell, 2000), while phosphorylation of Cdc25 causes its sequestration in the cytoplasm, therefore preventing the dephosphorylation and activation of CDK complexes (Zeng *et al.*, 1998; Lopez-Girona *et al.*, 1999; Zeng and Piwnica-Worms, 1999; Bartek and Lukas, 2007). As anticipated above, in *S. cerevisiae*, the DNA damage checkpoint does not target the CDK complex activity to prevent entry into mitosis (Amon *et al.*, 1992; Sorger and Murray, 1992). Rather, the G_2/M checkpoint induces a mitotic arrest by inhibiting the transition from metaphase to anaphase and mitotic exit (Cohen-Fix and Koshland, 1997; Gardner, 1999; Sanchez *et al.*, 1999; Liang and Wang, 2007). Before describing the molecular details of such regulation, I will introduce the molecular events and pathways that regulate exit from mitosis in *S. cerevisiae*.

1.6.1. Regulation of the mitotic exit in *S. cerevisiae*

"Mitotic exit" is the part of the cell cycle that begins with segregation of sister chromatids in anaphase and ends after the separation of the two daughter cells. As cells exit from mitosis, the mitotic cyclin-CDK complexes are inactivated and regulatory pathways that promote late cell cycle events are activated. Suppression of mitotic CDK inactivation causes cells to arrest in late anaphase/telophase with segregated chromosomes and an elongated

mitotic spindle (Irniger, 2002; Wäsch and Cross, 2002). In *S. cerevisiae*, inactivation of mitotic CDK complexes is carried out mainly by the ubiquitin-mediated degradation of mitotic cyclins, which is initiated at the transition from metaphase to anaphase by the APC/C in complex with Cdc20 (Bäumer, Braus and Irniger, 2000; Yeong *et al.*, 2000). However, a significant amount of mitotic CDK activity persists until telophase (Surana *et al.*, 1993; Shirayama, Matsui and Toh-E, 1994; Jaspersen *et al.*, 1998). In contrast, in most other eukaryotes, the bulk of mitotic cyclin degradation occurs at the transition from metaphase to anaphase (Surana *et al.*, 1993; Shirayama, Matsui and Toh-E, 1994; Jaspersen *et al.*, 1998). The residual pool of mitotic CDK complexes is targeted for degradation by the APC/C in complex with its other regulatory subunit Cdh1 (Schwab, Lutum and Seufert, 1997; Visintin, Prinz and Amon, 1997; Yeong *et al.*, 2000; Wäsch and Cross, 2002). Moreover, mitotic exit requires the reversal of all phosphorylation events mediated by mitotic CDK complexes. In *S. cerevisiae*, this task is mainly performed by the proline-directed phosphatase Cdc14 (Visintin *et al.*, 1998; Stegmeier and Amon, 2004).

In budding yeast, the control of exit from mitosis can be divided into three interlaced regulatory pathways that are consecutively activated as cells pass from metaphase into G_1. First, the APC/C^{Cdc20} complex is activated, which is responsible for the first wave of degradation of the mitotic cyclins and chromosome segregation. The second and third activated pathways are the Cdc fourteen early anaphase release (FEAR) pathway and the mitotic exit network (MEN), respectively. While the former is dispensable for mitotic exit, the latter is strictly required. Indeed, FEAR mutants delay but do not fail to exit from mitosis (Pereira *et al.*, 2002; Stegmeier, Visintin and Amon, 2002; Yoshida, Asakawa and Toh-e, 2002), whereas MEN mutants arrest in late anaphase with high mitotic CDK activity (Surana *et al.*, 1993; Shirayama, Matsui and Toh-E, 1994; Jaspersen *et al.*, 1998). Both pathways

control the activity of Cdc14, a key cell cycle protein that plays a plethora of tasks essential for the mitotic exit.

1.6.1.1. The APC/C

The onset of anaphase is characterized by the separation and segregation of sister chromatids, which are moved apart in the two daughter cells by the mitotic spindle. Therefore, sister chromatids have to be correctly attached by microtubules emanating from opposite poles. This process is carefully supervised by the spindle assembly checkpoint (SAC), a checkpoint signal generated by kinetochores that inhibit the activity of APC/C as long as incorrectly attached chromosomes persist. Once the SAC has been satisfied, the inhibition of the APC/C is relieved and it associates with the Cdc20 subunit. Activated APC/C^{Cdc20} ubiquitinates mitotic cyclins as well as the securin (Pds1 in budding yeast), an inhibitor of the caspase-like cysteine protease known as separase (Esp1 in budding yeast) for degradation by the proteasome (Figure 1.19 A). Separase is responsible for the cleavage of cohesin, the protein complex that keeps together replicated sister chromatids at metaphase. The cleavage of cohesin results in the separation of sister chromatids, which are subsequently segregated by the mitotic spindle. In addition to its role in cohesin cleavage, separase also promotes the release of the phosphatase Cdc14 from its inhibitory sequestration (Yeong *et al.*, 2000; Sullivan, Lehane and Uhlmann, 2001; Surana, Yeong and Lim, 2002; Buonomo *et al.*, 2003a; Sullivan, Hornig, *et al.*, 2004) (see section 1.6.1.2.1 for details).

The APC/C^{Cdc20} begins the destruction of mitotic cyclins, however, their complete degradation requires that the APC/C associates with the Cdh1 subunit (APC/C^{Cdh1} complex) (Figure 1.19 B). The APC/C^{Cdh1} complex promotes the destruction of other key mitotic proteins that are not targeted by the APC/C^{Cdc20}, including the Cdc20 itself (Prinz *et al.*, 1998). The transition from APC/C^{Cdc20} to APC/C^{Cdh1} is a consequence of the lowering in

mitotic CDK activity. Indeed, while the activity of the APC/C^{Cdc20} is optimally maintained when the cyclin-CDK activity is high, the activity of the APC/C^{Cdh1} is inhibited by mitotic CDK (Zachariae *et al.*, 1998; Yeong *et al.*, 2000; Tóth *et al.*, 2007; Holt, Krutchinsky and Morgan, 2008; Pines, 2011). Once mitosis is completed, APC/C^{Cdh1} helps to reset the conditions for the G_1 state: it targets the polo-family protein kinase Cdc5 for destruction in early G_1 phase, and targets mitotic cyclins for degradation throughout the G_1 phase (Wäsch and Cross, 2002; Visintin *et al.*, 2008).

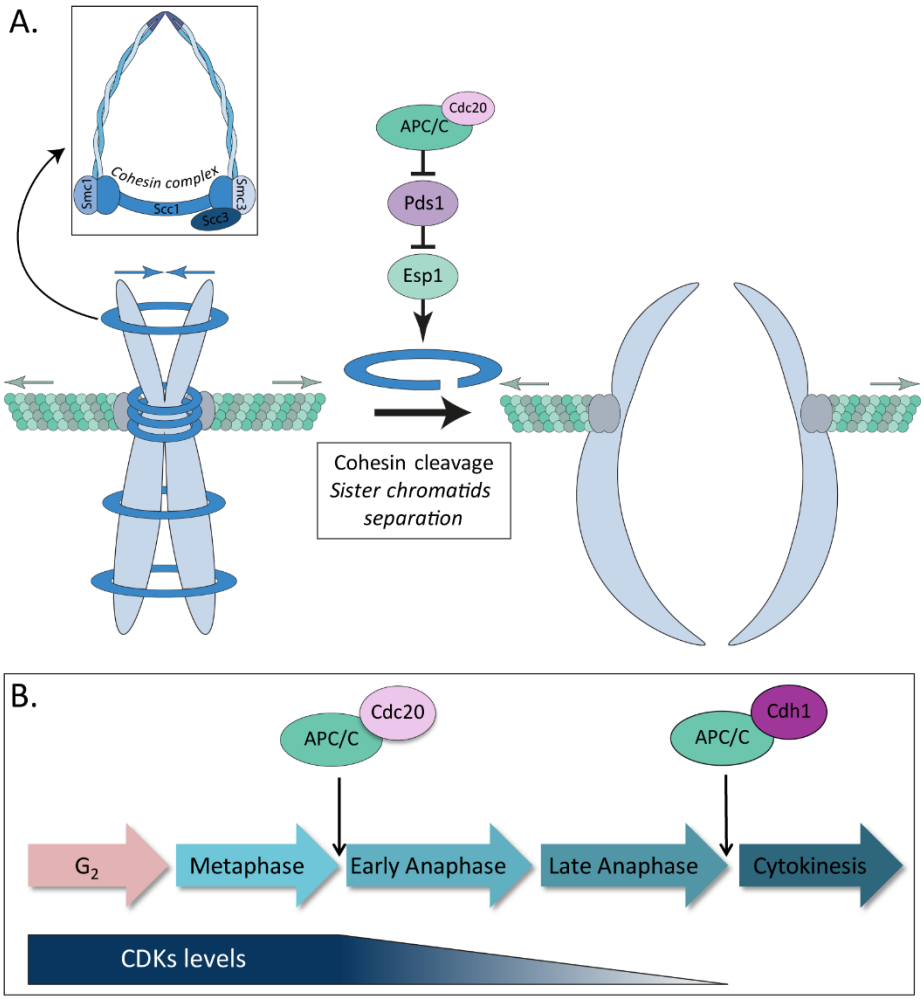

Figure 1.19 **Functions of the APC/C complex in exit from mitosis**. A. The APC/C^{Cdc20} is required to promote Clb2 degradation and cohesin cleavage at metaphase to anaphase transition. B. The APC/C^{Cdc20} and APC/C^{Cdh1} are required for lowering the CDK activity throughout anaphase and mitotic exit.

1.6.1.2. Cdc14

In *S. cerevisiae*, phosphorylation of CDK substrates is reversed by the phosphatase Cdc14 (Visintin *et al.*, 1998; Wenying Shou *et al.*, 1999; Jaspersen and Morgan, 2000; Shou *et al.*,

2001). Cdc14 is a dual-specificity phosphatase, namely it can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within its substrates. As Cdc14 strongly favors residues immediately followed by proline, a motif that corresponds to a minimal CDK phosphorylation site, Cdc14 can dephosphorylate mitotic CDK substrates. Moreover, Cdc14 contributes to anaphase progression and completion by regulating a variety of other cellular events, including segregation of rDNA and telomeres (D'Amours, Stegmeier and Amon, 2004; Sullivan, Higuchi, *et al.*, 2004; Wang, Yong-Gonzalez and Strunnikov, 2004; Geil, Schwab and Seufert, 2008; Clemente-Blanco *et al.*, 2009, 2011), mitotic spindle dynamics (Higuchi and Uhlmann, 2005; Khmelinskii *et al.*, 2007, 2009; Roccuzzo *et al.*, 2015), and cytokinesis (Sanchez-Diaz *et al.*, 2012).

Cdc14 is kept inactive inside the nucleolus during most of the cell cycle through its binding with the nucleolar protein Cfi1, also known as Net1 (Visintin, Hwang and Amon, 1999) (Figure 1.20). However, other nucleolar proteins participate in the sequestration of Cdc14. One such proteins is Fob1, which is required for the blocking of the replication fork at specific sites in the rDNA repeats (Kobayashi and Horiuchi, 1996; Mohanty and Bastia, 2004). Fob1 keeps the Cdc14 sequestration machinery in the nucleolus by anchoring the Cdc14–Cfi1 complex in association with the silencing factor Sir2 to the rDNA repeats (Toyn and Johnston, 1993; Stegmeier, Visintin and Amon, 2002; Buonomo *et al.*, 2003a; Tomson *et al.*, 2009; Bairwa *et al.*, 2010). Moreover, Fob1 binds and keeps Spo12 inhibited (Stegmeier, Visintin and Amon, 2002; Tomson *et al.*, 2009). Spo12 is a protein of unknown biochemical activity involved in promoting the release of Cdc14 from the nucleolus in early anaphase (Stegmeier, Visintin and Amon, 2002; Tomson *et al.*, 2009). The complex formed by Sir2, Cfi1 and Fob1 is known as REgulator of Nucleolar silencing and Telophase (RENT) and, besides its role in Cdc14 localization, it also suppresses transcription and

recombination in rDNA repeats (Straight *et al.*, 1999; Visintin, Hwang and Amon, 1999; W Shou *et al.*, 1999; Huang and Moazed, 2003; Kobayashi *et al.*, 2004; Huang *et al.*, 2006).

At the onset of anaphase, sequestration of Cdc14 is weakened due to phosphorylation of key components of the anchoring system. Two distinct waves of Cdc14 release occur as cells proceed through the exit from mitosis. The first wave is triggered by the FEAR network, which causes Cdc14 to localize mainly in the nucleoplasm and only a very small amount in the cytoplasm. A second wave of Cdc14 release is triggered by MEN and causes Cdc14 to localize in the cytoplasm.

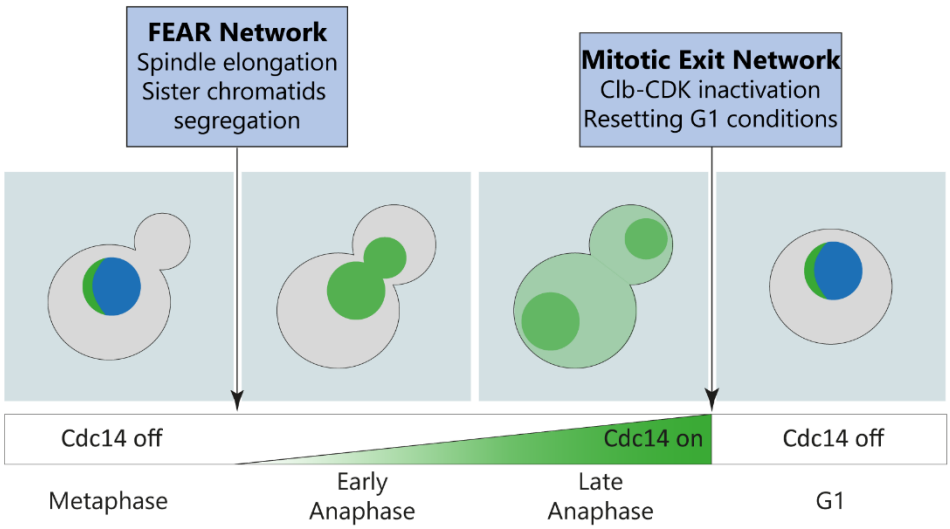

Figure 1.20 **Overview of the regulation of localization, hence activity, of Cdc14 during exit from mitosis**.

1.6.1.2.1. The FEAR network

As cells pass from metaphase to anaphase, the sequestration of Cdc14 in the nucleolus is relieved by the activation of the FEAR network that causes Cdc14 to localize mainly in the nucleus. The FEAR network was originally identified in MEN mutants, where a transient release of Cdc14 was observed during early anaphase, followed by its relocalization in the nucleolus when cells entered the late anaphase arrest (Stegmeier, Visintin and Amon, 2002). Further studies identified a number of proteins that function in a network to regulate the early release of Cdc14. These proteins include the separase Esp1, the separase-binding protein Slk19, the polo-like kinase Cdc5, the nucleolar protein Spo12, the

replication fork block protein Fob1, the "Zillion Different Screens" proteins Zds1 and Zds2, CDK associated with the cyclins Clb1 and Clb2, and, finally, the phosphatase PP2A, which is a multiprotein complex that associates with different regulatory subunits to mediate substrate specific dephosphorylation. Recently, new studies helped to define the interconnections of FEAR components inside the network, which foresee a three-branched organization with Esp1, Spo12 and Cdc5, which are representative members of each branch (Roccuzzo *et al.*, 2015) (Figure 1.21).

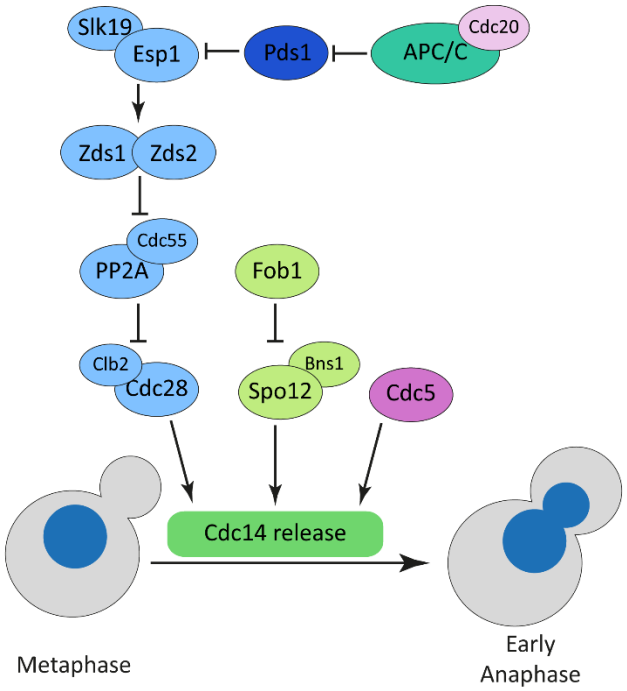

Figure 1.21 **Current model of the genetic organization of the FEAR network**.

The first wave of Cdc14 release is promoted by mitotic CDK-mediated and Cdc5-mediated phosphorylation of Cfi1 and Cdc14 (Shou *et al.*, 2002; Yoshida and Toh-e, 2002; Visintin, Stegmeier and Amon, 2003; Azzam *et al.*, 2004; Rodriguez-Rodriguez *et al.*, 2016), which weakens the binding between Cfi1 and Cdc14. Moreover, mitotic CDK-mediated phosphorylation of Spo12 further incentives this disengagement (Tomson *et al.*, 2009). A dynamic balance of kinase and phosphatase activities regulates the release of Cdc14 from the nucleolus. In metaphase, activating phosphorylation events are counteracted by the protein phosphatase PP2A bound to its regulatory subunit Cdc55 (Wang and Burke, 1997;

Queralt *et al.*, 2006; Wang and Ng, 2006; Yellman and Burke, 2006). At the onset of anaphase, the activity of the FEAR network promotes the inhibition of $PP2A^{Cdc55}$, therefore tipping the balance in favor of CDK-mediated phosphorylation (Queralt and Uhlmann, 2008a; Rossio and Yoshida, 2011). A critical role in the inhibition of $PP2A^{Cdc55}$ is played by Esp1, a function that is independent from the role of the separase in the cleavage of cohesin (Sullivan and Uhlmann, 2003). Once the Esp1 inhibitor Pds1 is targeted for degradation by APC/C^{Cdc20}, Esp1 associates with Slk19 (Stegmeier, Visintin and Amon, 2002) and, through a poorly understood mechanism, the Esp1-Slk19 complex promotes the mitotic CDK-mediated phosphorylation of Cfi1 that triggers the release of Cdc14 (Sullivan, Lehane and Uhlmann, 2001; Sullivan and Uhlmann, 2003; Azzam *et al.*, 2004). Moreover, the Zds1 and Zds2 proteins also participate in this process by counteracting $PP2A^{Cdc55}$ (Queralt and Uhlmann, 2008b; Yasutis *et al.*, 2010; Wicky *et al.*, 2011), which is probably achieved by binding of $PP2A^{Cdc55}$ that, in turn, causes its sequestration in the cytoplasm, therefore lowering its nuclear concentration (Rossio and Yoshida, 2011).

The activation of Cdc14 by FEAR is dispensable for sustaining cell growth, although it affects cell viability due to occurrence of aberrant chromosome segregation in anaphase. Indeed, double mutants for both FEAR and MEN, or the abrogation of Cdc14 activity using a thermosensitive allele, arrest in anaphase and is characterized by i) incomplete positioning of the spindle pole bodies (SPBs, the centrosome counterpart in budding yeast) in the mother-to-daughter axis, ii) blended and broken spindles, iii) lagging DNA masses at the bud neck, iv) nucleolus segregation failure, and v) unresolved regions near the telomeres of several chromosomes (D'Amours, Stegmeier and Amon, 2004; Ross and Cohen-Fix, 2004; Sullivan, Higuchi, *et al.*, 2004; Torres-Rosell *et al.*, 2004; Machín *et al.*, 2005; Jin *et al.*, 2008; Clemente-Blanco *et al.*, 2009). However, such defects are not detectable in conditional mutants for MEN, where the first wave of Cdc14 activation still takes place, therefore,

implicating these phenotypes to deficient FEAR-mediated Cdc14 functions. However, the molecular bases for all these defects remain elusive. What is known so far is that Cdc14 activates important players in the dynamics and stability of the anaphase spindle, including the spindle-stabilizing protein Fin1 (Woodbury and Morgan, 2007b, 2007a), the kinetochore protein Ask1 (Higuchi and Uhlmann, 2005), the microtubule-associated protein Ase1 (Khmelinskii *et al.*, 2007, 2009; Khmelinskii and Schiebel, 2008), the chromosome passenger protein Sli15 (Pereira and Schiebel, 2003), and the kinesin-5 motor protein Cin8 (Khmelinskii *et al.*, 2009; Rozelle, Hansen and Kaplan, 2011). Moreover, the activity of Cdc14 is important for the resolution of late-segregating chromosomal regions, namely rDNA and telomers (D'Amours, Stegmeier and Amon, 2004; Machín *et al.*, 2006; Clemente-Blanco *et al.*, 2009, 2011), which is achieved through direct inhibition of RNA polymerase I and RNA polymerase II transcription of these regions, a process required for the loading of the condensin complex onto rDNA and telomeres in anaphase (Clemente-Blanco *et al.*, 2009, 2011). Condensin is a fundamental player for the separation and segregation of sister chromatids as it mediates chromosome arm compaction and promotes decatenation activity of topoisomerase II (Top2) (Renshaw *et al.*, 2010; Charbin, Bouchoux and Uhlmann, 2014). In addition, FEAR-mediated activation of Cdc14 targets the Holliday junction resolvase Yen1 to the nucleus, therefore providing a backup mechanism for complete sister chromatid resolution in early anaphase (Blanco, Matos and West, 2014; Eissler *et al.*, 2014; García-Luis *et al.*, 2014). Recently, the activity of Cdc14 was implicated in DNA repair. More specifically, it was shown that Cdc14 is transiently released in response to genotoxic stresses to promote recombinational repair (Villoria *et al.*, 2017). Last but not least, Cdc14 released by FEAR directly promotes the activation of MEN, thus self-sustaining its own release in late anaphase through a positive feedback loop (Jaspersen and Morgan,

2000; Pereira *et al.*, 2002; Stegmeier, Visintin and Amon, 2002; Bardin, Boselli and Amon, 2003)

1.6.1.2.2. The mitotic exit network

The FEAR-mediated release of Cdc14 is dispensable for mitotic exit. Consistently, the FEAR-mediated release of Cdc14 alone is not sufficient to drive full exit from mitosis, and a complete mitotic exit requires the MEN activation that allows for a full cytoplasmic release of Cdc14 and subsequent full dephosphorylation of cytoplasmic CDK substrates that mediate numerous downstream events (Charles *et al.*, 1998; Bosl and Li, 2005; Tóth *et al.*, 2007).

The MEN pathway is a Ras-like GTPase signaling cascade, involving the action of the GTPase Tem1, the putative guanine-nucleotide exchange factor (GEF) Lte1, the two-component GTPase activating protein (GAP) Bub2-Bfa1, the protein kinases Cdc5, Cdc15, Dbf2, the Dbf2-activating protein Mob1, and, finally, a scaffold protein Nud1 (Visintin *et al.*, 1998; Tinker-Kulbetg and Morgan, 1999; W Shou *et al.*, 1999; Bardin, Visintin and Amon, 2000; Jaspersen and Morgan, 2000; Pereira and Schiebel, 2001; Visintin and Amon, 2001) (Figure 1.22).

Proper subcellular localization of the MEN components is critical for its activation in response to a proper spindle position. Indeed, some MEN components localize at the SPB that enters the cytoplasm of the daughter cell, while the MEN activating protein Lte1 localizes at the bud cortex (Cenamor *et al.*, 1999; Menssen, Neutzner and Seufert, 2001; Visintin and Amon, 2001; Yoshida, Asakawa and Toh-e, 2002). Therefore, the activation of MEN is prevented until the mitotic spindle has been properly oriented, namely when one SPB enters the cytoplasm of a daughter cell. The peculiar localization of MEN components at the SPB that enters the daughter cell is largely determined by the scaffold protein Nud1,

which associates with the cytoplasmic face of the SPB (Jaspersen *et al.*, 1998; Cenamor *et al.*, 1999; W Shou *et al.*, 1999; Luca *et al.*, 2001; Visintin and Amon, 2001; Yoshida, Asakawa and Toh-e, 2002; Molk *et al.*, 2004; Rock and Amon, 2011; Valerio-Santiago and Monje-Casas, 2011). Nud1-mediated recruitment of Tem1, Cdc15 and Dbf2-Mob1 at the SPB promotes the activation of the network. The MEN core pathway activation is subjected to several layers of regulation. The RAS-related GTPase protein Tem1 is in an active form only when loaded with GTP. It has been proposed that the Bub2-Bfa1 complex, which also localizes at the SPB, keeps Tem1 inactive by acting as a GAP that converts Tem1 to an inactive GDP-bound form (Bardin, Visintin and Amon, 2000; Krishnan *et al.*, 2000; Pereira *et al.*, 2000; Wang, Hu and Elledge, 2000; Sarah E Lee *et al.*, 2001). At the onset of anaphase, Cdc5 phosphorylates and inactivates the Bub2-Bfa1 complex (Hu *et al.*, 2001; Geymonat *et al.*, 2003). This phosphorylation is counteracted by the phosphatase $PP2A^{Cdc55}$ (Baro *et al.*, 2013), therefore, the inhibition of $PP2A^{Cdc55}$ activity is important for both the FEAR-dependent Cdc14 release as well as for the activation of MEN. A second layer of regulation for the activation of MEN is represented by the CDK-mediated phosphorylation of Cdc15 that results in Cdc15 inhibition. However, this phosphorylation event seems to influence the localization of Cdc15 rather than its kinase activity (Jaspersen and Morgan, 2000; Stegmeier, Visintin and Amon, 2002; König, Maekawa and Schiebel, 2010). At the onset of anaphase, FEAR-released Cdc14 relieves the inhibition on Cdc15, therefore promoting the activation of MEN (Cenamor *et al.*, 1999; Jaspersen and Morgan, 2000; Xu *et al.*, 2000; Gruneberg *et al.*, 2001; Menssen, Neutzner and Seufert, 2001). Lastly, a third layer of regulation for the activation of MEN is represented by the CDK-mediated phosphorylation of Mob1. In metaphase, Mob1 is in a hyperphosphorylated state that results in the inhibition of the Dbf2-Mob1 complex (König, Maekawa and Schiebel, 2010). During mitotic

exit, rapid dephosphorylation of Mob1, presumably mediated by FEAR-released Cdc14, allows for activation of the Dbf2-Mob1 complex (König, Maekawa and Schiebel, 2010).

The activation of MEN is closely bound to the proper orientation of the mitotic spindle. When the daughter SPB is in the mother cytoplasm, the activation of MEN is inhibited through the mechanisms discussed above. Conversely, the movement of the daughter SPB into the daughter cytoplasm results in the activation of MEN, that is achieved through the activity of Lte1, which does not promote GTP loading on Tem1, rather, it counteracts the inhibition provided by the spindle orientation checkpoint through a poorly understood mechanism (Geymonat *et al.*, 2009; Chan and Amon, 2010; Bertazzi, Kurtulmus and Pereira, 2011). Thereafter, Tem1 promotes the recruitment of Cdc15 on Nud1 (Valerio-Santiago and Monje-Casas, 2011), which in turn determines the recruitment of Dbf2-Mob1. Cdc15 directly phosphorylates Dbf2, causing its activation (Mah, Jang and Deshaies, 2001). Finally, active Dbf2 presumably phosphorylates Cfi1 in order to relieve the inhibition on Cdc14 (Mah *et al.*, 2005), however, this phosphorylation still needs to be demonstrated *in vivo*. Remarkably, Dbf2 can also phosphorylate Cdc14 in a residue near its nuclear localization signal (NLS), which results in the inactivation of NLS and the exclusion of Cdc14 form the nucleus (Mohl *et al.*, 2009).

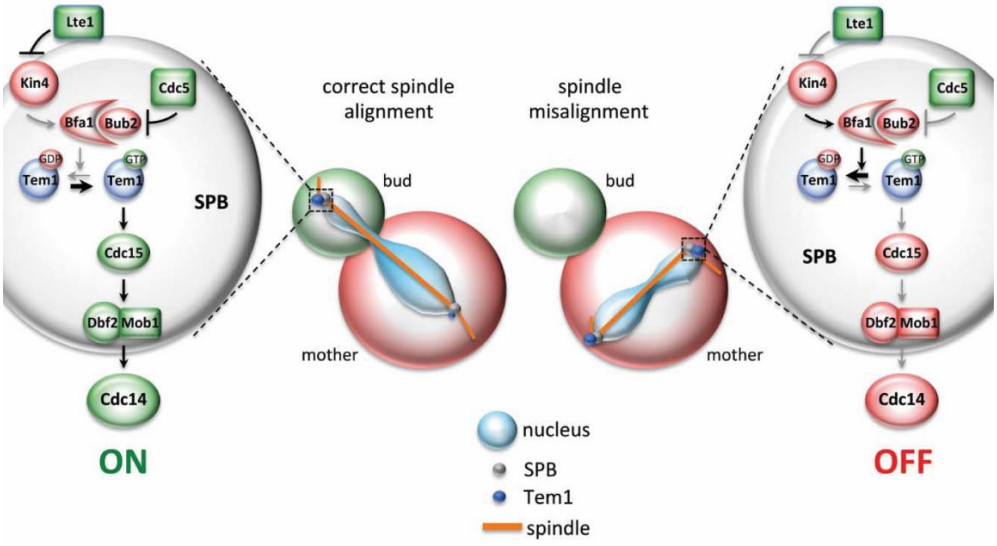

Figure 1.22 **Model for activation of the MEN pathway**. *Adapted from (Scarfone and Piatti, 2015).*

A fully released cytoplasmic Cdc14 is responsible for resetting the conditions for the subsequent G_1 phase through three important activities: i) Cdc14 dephosphorylates CDK substrates in the cytoplasm; ii) it promotes the formation of the APC/C^{Cdh1} complex in late anaphase, which is responsible for the last wave of mitotic cyclin degradation; and, finally, iii) it causes the accumulation of the Cdk1 inhibitor Sic1, which further contributes to the complete inactivation of Cdk1. Thereafter, the cells finally undergo cytokinesis and MEN is inactivated during the entry into the next G_1 phase. Inactivation of MEN is promoted by Cdc14 itself through a negative feedback loop. Indeed, the APC/C^{Cdh1} complex induces the degradation of Cdc5, whose activity is essential to sustain MEN activity (Shirayama *et al.*, 1998; Visintin *et al.*, 2008). Moreover, Cdc14 also dephosphorylates the Bub2-Bfa1 complex, therefore restoring its GAP activity (Pereira *et al.*, 2002). Finally, Cdc14 dephosphorylates Lte1, resulting in the delocalization of Lte1 from the bud cortex to the cytoplasm in late anaphase. The delocalization of Lte1 contributes to its inactivation, and therefore to the termination of MEN signaling (Jensen *et al.*, 2002; Seshan, Bardin and Amon, 2002; Seshan and Amon, 2005).

1.6.1.3. Cdc5

The Polo-like kinases (PLKs) are evolutionarily conserved kinases and have an essential role in the regulation of the cell cycle., comprising Cdc5 in *S. cerevisiae*, Plo1 in *S. pombe*, and Plk1 in human. The Polo kinase was first reported in *Drosophila* neuroblasts, where mutations in the *polo* gene resulted in circular metaphase chromosome arrangements (Sunkel and Glover, 1988). All Plk1 homologs share the same domain organization, with an N-terminal serine/threonine catalytic kinase domain separated by a linker segment, known as the T-loop, important for regulation of the kinase activity, and a C-terminal non-catalytic polo-box domain (PBD), which consists of two separate polo-box motifs. The polo boxes

fold together to form a pincer-like structure that binds to phosphoserine/threonine on Polo substrates (Elia *et al.*, 2003).

The quantities of the Cdc5 protein in the cell are strongly linked with cell cycle progression: the protein accumulates in S phase, reaches its maximum levels in anaphase, and is rapidly degraded in the G_1 phase (Charles *et al.*, 1998; Cheng, Hunke and Hardy, 1998; Shirayama *et al.*, 1998). The activity of the Cdc5 kinase is regulated by a CDK-mediated phosphorylation (Mortensen *et al.*, 2005; Bloom and Cross, 2007; Benanti, 2016). Cdc5 contains five CDK consensus sites, one strict (S/T-P-X-K/R) and four minimal sites (S/T-P). Of these, the minimal site threonine 242 seems to be the most important in Cdc5 regulation, since its mutation results in complete abrogation of the kinase activity (Mortensen *et al.*, 2005). Moreover, CDK-mediated phosphorylation of threonine 70 is required for the activation of MEN (Rodriguez-Rodriguez *et al.*, 2016), while phosphorylation of threonine 238 is not essential for cell viability (Rawal *et al.*, 2016). However, *cdc5-T238A* has a reduced kinase activity and DNA damage checkpoint adaptation defects (Rawal *et al.*, 2016; Rodriguez-Rodriguez *et al.*, 2016).

Cdc5 performs a plethora of functions in mitosis. Biochemical studies have identified hundreds of Cdc5 substrates, many of which remain to be fully characterized (Snead *et al.*, 2007). Besides the roles in pathways that control the exit from mitosis described above, Cdc5 promotes timely mitotic entry at different levels (Sakchaisri *et al.*, 2004; Nakashima *et al.*, 2008), acting as a positive regulator of both cyclin Clb2 expression (Darieva *et al.*, 2006) and Clb2-CDK interaction (Asano *et al.*, 2005). Furthermore, Cdc5 promotes the efficient cleavage of the cohesin ring subunit Scc1 by the separase Esp1 through phosphorylation of ten residues on Scc1 (Ciosk *et al.*, 1998; Alexandru *et al.*, 2001; Hornig and Uhlmann, 2004). These phosphorylation events are counteracted by the phosphatase $PP2A^{Cdc55}$ (Yaakov, Thorn and Morgan, 2012). At the transition from metaphase to

anaphase, the activity of Cdc5 is required for the removal of cohesin from chromatin (Mishra *et al.*, 2016), while, in anaphase, the activity of Cdc5 activity is important for chromosome condensation, as it targets the condensin components Brn1, Ycg1 and Ycs4 for hyperactivation of condensin activity (St-Pierre *et al.*, 2009), and for mitotic spindle elongation (Park *et al.*, 2008; Roccuzzo *et al.*, 2015).

One remarkable feature of Cdc5 is that the kinase "knows" where to target each of its substrates in a timely manner to promote mitotic progression, which is achieved through two important mechanisms, namely via priming-phosphorylation of substrates and inducing changes in the localization of Cdc5 itself during the cell cycle. As mentioned above, the PBD domain is able to bind to phosphoserine/threonine on substrates, therefore, previous phosphorylation by other kinases serves as a recruitment signal (priming) for Cdc5. Furthermore, the localization of Cdc5 is regulated throughout the cell cycle. Cdc5 is first localized in the nucleus until early anaphase (Charles *et al.*, 1998; Cheng, Hunke and Hardy, 1998; Shirayama *et al.*, 1998; Song *et al.*, 2000; Nakashima *et al.*, 2008; Botchkarev, Rossio and Yoshida, 2014). In late anaphase, Cdc5 is released into the cytoplasm in a Cdc14-dependent manner through a poorly understood mechanism (Botchkarev, Rossio and Yoshida, 2014). In addition, Cdc5 also changes its localization at the SPBs during the cell cycle. In S phase, prior to SPB duplication, Cdc5 levels begin to increase and the protein localizes at the SPB (Lee *et al.*, 2005; Botchkarev, Rossio and Yoshida, 2014). After the duplication of SPB, Cdc5 localizes at the nuclear surface of both SPBs (Botchkarev *et al.*, 2017) through binding to the nuclear SPB component Spc110 and the kinetochore proteins Slk19, Cse4 and Tid3 (Snead *et al.*, 2007; Rahal and Amon, 2008). Following the activation of the FEAR network, Cdc5 is released from the inner plaque of both SPBs and it is translocated in the cytosol in a Cdc14-dependent manner (Botchkarev, Rossio and Yoshida, 2014; Botchkarev *et al.*, 2017). In the cytosol, Cdc5 associates with the outer plaque of the

SPB destined to the daughter cell in a Bfa1-dependent manner (Botchkarev *et al.*, 2017) and stays there until cell cycle completion (Cheng, Hunke and Hardy, 1998; Shirayama *et al.*, 1998; Song *et al.*, 2000; Maekawa *et al.*, 2007; Botchkarev, Rossio and Yoshida, 2014). The recruitment of Cdc5 to the SPBs probably depends on priming kinase-mediated phosphorylation of SPB components. Indeed, mutations affecting the ability of the PBD to bind phospho-primed substrates results in a defective localization of Cdc5 to the SPBs in mitosis (Park *et al.*, 2004; Ratsima *et al.*, 2011; Botchkarev, Rossio and Yoshida, 2014). Finally, Cdc5 localizes at the bud neck in metaphase and anaphase (Song *et al.*, 2000; Sakchaisri *et al.*, 2004; Meitinger *et al.*, 2011; Botchkarev, Rossio and Yoshida, 2014), where it likely contributes to cytokinesis (Meitinger *et al.*, 2011; Lepore *et al.*, 2016). The Cdc5 kinase remains active until mitosis and cytokinesis have been completed. Thereafter, the APC/C^{Cdh1} complex targets Cdc5 for proteasome-mediated degradation (Charles *et al.*, 1998; Cheng, Hunke and Hardy, 1998; Shirayama *et al.*, 1998).

1.6.2. The G_2/M checkpoint targets

Separation of sister chromatids at the onset of anaphase represents a point of no return for mitosis, therefore, it is not surprising that multiple regulatory processes control the separation of sister chromatids, and, in particular, the timing at which sister chromatid cohesion is dissolved. Besides SAC, which supervises the correct attachment of sister chromatids to microtubules emanating from opposite poles of the mitotic spindle, the DNA damage checkpoint also acts at this cell cycle transition in order to prevent chromosome segregation in case of unrepaired DNA lesions (Cohen-Fix and Koshland, 1997; Gardner, 1999; Liang and Wang, 2007) (Figure 1.23). Several studies provided insights on how the DNA damage checkpoint effector kinases co-operate to ensure efficient checkpoint-mediated metaphase arrest. Two independent mechanisms act to stabilize Pds1: i) Chk1-dependent phosphorylation of Pds1 prevents its degradation by the APC/C^{Cdc20} complex

(Sanchez *et al.*, 1999; Wang, 2001; Agarwal *et al.*, 2003); and ii) Rad53 is responsible for the inhibition of the interaction between Pds1 and Cdc20 (Agarwal *et al.*, 2003). The stabilization of Pds1 ensures both inhibition of cohesin cleavage and FEAR network activation. Recently, other processes not strictly related to checkpoint signaling have been found to influence the activity of Pds1. For instance, autophagy, a process involved in dealing with cellular stresses of different nature, is involved also in DDR by inducing the nuclear exclusion of Pds1 associated with the separase Esp1, thus preventing activation of the separase, namely anaphase onset and chromosome segregation (Dotiwala *et al.*, 2013; Eapen and Haber, 2013).

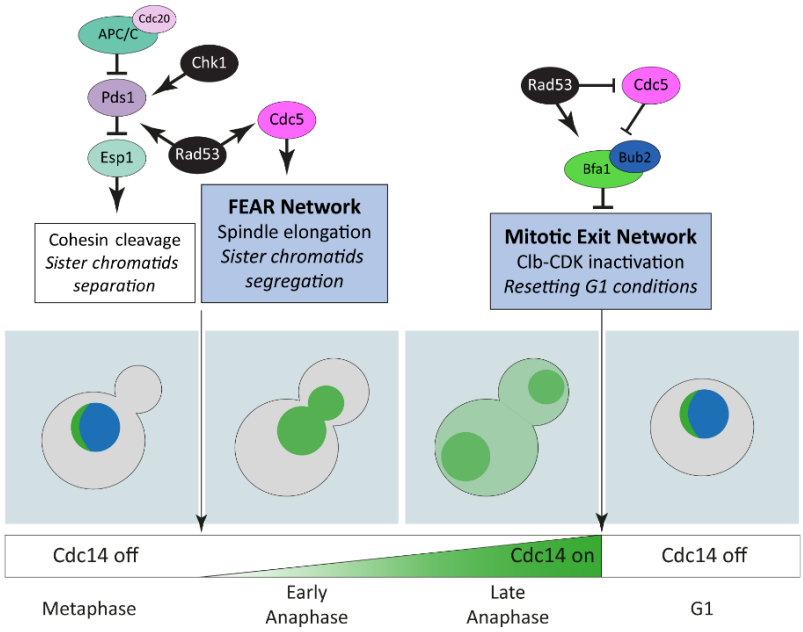

Figure 1.23 **Targets of the DNA damage checkpoint for inhibition of exit from mitosis in *S. cerevisiae***. The crosstalk between the DNA damage checkpoint and the cell cycle machinery is shown.

Moreover, the DNA damage checkpoint targets the Cdc5 kinase in a Rad53- and Rad9-dependent manner (Cheng, Hunke and Hardy, 1998), leading to the inactivation of Cdc5, preventing thus mitotic exit (Sanchez *et al.*, 1999). Consistently, following DDR activation, Cdc5 activity has been found to be downregulated after DDR activation (T. Zhang *et al.*, 2009), and Cdc5 is kept sequestered in the nucleus to prevent the activation of the MEN network (Valerio-Santiago, de Los Santos-Velázquez and Monje-Casas, 2013; Botchkarev *et*

al., 2017). Consistent with these observations is the finding that Rad53 suppresses the activation of MEN by phosphorylating the MEN inhibitor Bfa1, likely preventing the dissociation of Bfa1 from Tem1 (Liang and Wang, 2007).

1.7. Termination of the checkpoint: recovery and adaptation

Following the detection of a DNA lesion, a cell cycle arrest is elicited in order to allow the required time for repair. To accomplish this task, a number of repair pathways have evolved to cope with different kinds of damages introduced by endogenous or exogenous agents. If the lesion is successfully repaired, the DDR has to be switched off to resume the cell cycle. This process is known as checkpoint recovery (Figure 1.24). Conversely, if the damage is too extensive or irreparable, sustained activation of the DDR can initiate a programmed cell death pathway. However, a different possibility also exists, where, despite the continuous presence of a damage, the DDR signaling is turned off and cells resume the cell cycle. This process is known as checkpoint adaptation. While the molecular events for DDR activation are relatively well characterized, the molecular events that drive checkpoint recovery and adaptation are less understood. In the following sections, I will outline factors involved in the termination of the DDR following successful or failed repair of a lesion.

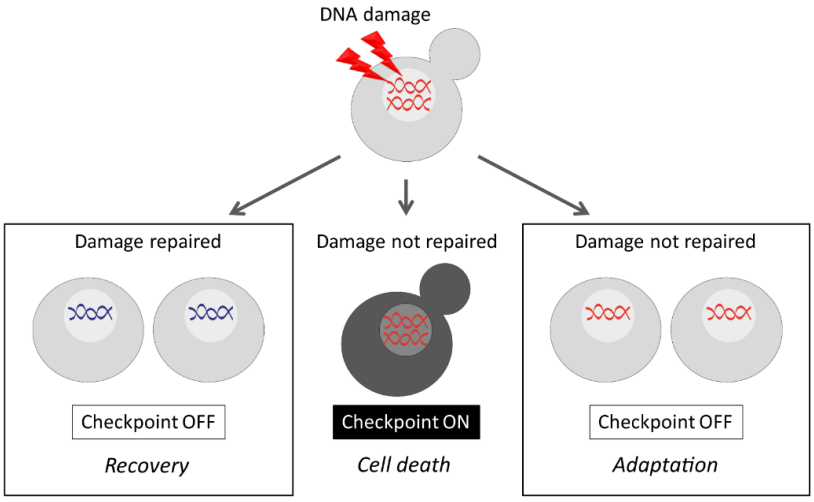

Figure 1.24 **Possible outcomes for termination of the checkpoint**. Following DNA damage, several cellular outcomes are possible: if the damage is repaired, cells undergo checkpoint recovery, namely cell cycle progression following repair of the lesion. If the damage is not repaired, cells either die or undergo checkpoint adaptation, namely cell cycle progression in the presence of the lesion.

1.7.1. Checkpoint recovery

Several studies have showed that factors implicated in checkpoint recovery are involved in different phases of the DNA damage checkpoint, therefore depicting checkpoint recovery as a complex multistep process rather than a single inactivation mechanism. Several, not mutually exclusive mechanisms, have been proposed, including i) disassembly of DNA repair and checkpoint proteins on the site of a damage (Vaze *et al.*, 2002); ii) removal of DNA lesions (Yeung and Durocher, 2011); iii) recruitment of protein phosphatases to revert checkpoint-mediated phosphorylation events (Keogh *et al.*, 2006; O'Neill *et al.*, 2007); and, finally, iv) feedback control via transcriptional changes (Woolstencroft *et al.*, 2006).

A proper termination of the checkpoint signaling requires the reversal of the same mechanisms that are essential for its activation. For example, following repair of a DNA lesion, checkpoint sensors and activators of the checkpoint, including Mec1, Tel1, the 9-1-1 and MRX complexes, are disengaged, thus preventing further signaling to Rad53. Several studies have shown that the DNA repair proteins Sae2 and Srs2 play important roles in checkpoint inactivation. Sae2 works together with the MRX complex to promote the initial DNA resection of DSB ends (Clerici *et al.*, 2005). However, *SAE2* deletion or mutation of its Mec1/Tel1 phosphorylation sites result in a prominent checkpoint activation and cell cycle arrest that cannot be only associated to a defective repair of a lesion (Baroni *et al.*, 2004; Clerici *et al.*, 2006). These observations suggest that Mec1/Tel1-dependent phosphorylation of Sae2 may act as a mediator between DNA repair and the DNA damage checkpoint. Srs2 acts as an anti-recombinase factor by counteracting Rad51 nucleoprotein filament formation. Defects in the recovery of *srs2* are relieved by the inhibition of Mec1 and Tel1 activities, suggesting that the function of Srs2 is required for removal of the checkpoint from the site of a lesion and/or for restoring chromatin structure (Vaze *et al.*, 2002).

As the DDR signaling is mainly accomplished through the activation of phosphorylation events, it is not surprising that a series of phosphatases have been implicated in checkpoint termination. For instance, full activation of Rad53 is mediated by a Mec1-dependent phosphorylation (Sweeney *et al.*, 2005), as well as in-trans autophosphorylation (Gilbert, Green and Lowndes, 2001). In order to terminate the checkpoint signaling, Rad53 has to be dephosphorylated. The phosphatases Ptc2 and Ptc3 have been implicated in the dephosphorylation of Rad53, and share redundant roles in the inactivation of Rad53 (Leroy *et al.*, 2003). Following a DSB, the overexpression of *PTC2* results in the dephosphorylation of Rad53 and, consequently, in a faster resumption of cell cycle progression (Leroy *et al.*, 2003). Moreover, Ptc2 directly interacts with Rad53, suggesting that the phosphatase is the main inactivator of Rad53 (Leroy *et al.*, 2003; Smolka *et al.*, 2006; Guillemain *et al.*, 2007). However, the molecular details regarding the regulation of Ptc2 and the activation of Ptc3 are not fully understood. Besides Ptc2 and Ptc3, a third phosphatase, Pph3, has also been implicated in the inactivation of Rad53 (Keogh *et al.*, 2006; O'Neill *et al.*, 2007). However, since impairing the activity of all these phosphatases fails to impair the dephosphorylation of Rad53, at least another phosphatase (Travesa, Duch and Quintana, 2008; Bazzi *et al.*, 2010; Kim *et al.*, 2011) or additional pathways responsible for a complete checkpoint inactivation must be envisioned.

Checkpoint termination could also be achieved through negative feedback mechanisms activated by downstream targets of effector kinases. For instance, impairing the function of the downstream kinase Dun1 causes a prolonged Rad53 activation, which is further worsened by the deletion of *CCR4* (Woolstencroft *et al.*, 2006). Ccr4 is the catalytic subunit of the mRNA deadenylase complex and regulates mRNA turnover (Tucker *et al.*, 2002). It has been shown that Ccr4 can affect transcriptional targets of Rad53 and Dun1 (Traven *et al.*, 2005). Moreover, Ccr4 influences the abundance of Crt1, a transcriptional repressor of

DNA damage-induced genes (Woolstencroft *et al.*, 2006), therefore Ccr4 can indirectly affect many other DNA damage-induced transcriptional targets. Given these considerations, it is likely that the termination of the checkpoint signaling can be promoted also by transcriptional inhibition of specific DNA damage-induced genes.

1.7.2. Checkpoint adaptation

Related to, but conceptually distinct from, checkpoint recovery is checkpoint adaptation, a process that allows cells to resume their cell cycle and divide following a protracted checkpoint-mediated cell cycle arrest despite the presence of a persistent DNA damage.

As mentioned above, adaptation to the G_2/M DNA damage checkpoint was first described in *S. cerevisiae* by Sandell and Zakian in 1993 who demonstrated that following DSB induction in a repair-defective background, cells arrested in G_2/M for approximately 10 hours and then adapted and resumed division in the presence of damaged DNA (Sandell and Zakian, 1993). Thereafter, in 1997, in a genetic screen for regulators of adaptation in budding yeast, the first two adaptation mutants (affecting the *CDC5* gene and the casein kinase II subunit *CKB2* gene) were identified by Hartwell and colleagues (Toczyski, Galgoczy and Hartwell, 1997). At that time, checkpoint adaptation was considered to occur only in unicellular organisms, where an escape from long-term arrest would be beneficial because it provides a second chance to repair the damage in the following cell cycle. Conversely, as checkpoint adaptation could promote genomic instability, checkpoint adaptation was considered unlikely to occur in multicellular organisms (Lupardus and Cimprich, 2004; Yoo *et al.*, 2004). Unexpectedly, in 2004, Yoo and colleagues described checkpoint adaptation in *Xenopus* egg extracts that entered mitosis despite a protracted DNA replication stress. Interestingly, they found that this process is promoted by the Cdc5 homolog Plx1, suggesting a conserved mechanism among budding yeast and vertebrates (Yoo *et al.*, 2004). Following this observation, the question of whether checkpoint adaptation occurred

in mammalian cells arose, and in 2006, Syljuåsen and colleagues published the first report of checkpoint adaptation in human cells (Syljuåsen *et al.*, 2006). Yet, they found that this process was delayed because of the depletion of the Cdc5 homolog Plk1, further supporting the existence of an evolutionary conserved mechanism for adaptation (Syljuåsen *et al.*, 2006).

The cell decision to adapt to the DNA damage checkpoint seems to be tightly regulated. In particular, the load of damage has been proposed to be a critical determinant, as a single DSB leads to adaptation after about 10 hours of checkpoint arrest, while two DSBs lead to a permanent cell cycle arrest (Sandell and Zakian, 1993; Lee *et al.*, 1998). Remarkably, *cdc13-1* mutant cells grown at the non-permissive temperature eventually adapt to the DNA damage checkpoint (Toczyski, Galgoczy and Hartwell, 1997), suggesting that the DDR elicited by telomere dysfunction, although representing a more "global" damage condition (as all telomeres are affected simultaneously), is sensed or evaluated in a slightly different manner compared to proper DSBs.

Although it has been 25 years since the first observation of the adaptation phenomenon in 1993, and several studies have been published using both yeast and human cell models, the molecular mechanisms mediating checkpoint adaptation remain largely unknown. In the following sections, I will describe the molecular players identified so far and their mechanism of action, if known.

1.7.2.1. Cdc5

In the first screen for adaptation components back in 1997, Hartwell and colleagues identified two mutants that were unable to adapt to the G_2/M DNA damage checkpoint arrest (Toczyski, Galgoczy and Hartwell, 1997). One of these mutants was affected at the *CDC5* gene. Differently from other loss-of-function alleles of *CDC5*, this allele has been

found to be specifically defective in checkpoint adaptation, it was therefore called *cdc5-adaptation defective* (*cdc5-ad*). The *cdc5-ad* allele carries a leucine to tryptophan substitution at position 251 within the kinase domain. However, this allele has been found to be kinase-proficient (Charles *et al.*, 1998; Serrano and D'Amours, 2016). Moreover, *CDC5* and *cdc5-ad* gene products show comparable kinetics of expression and degradation along the cell cycle (Serrano and D'Amours, 2016). Finally, the Cdc5-ad protein variant has been found to prematurely localize at SPB in response to persistent DNA damage compared to its wild type counterpart (Serrano and D'Amours, 2016), however, how this mislocalization impacts on the checkpoint termination is unclear.

New Cdc5 adaptation-defective mutants that carry specific mutations in the T-loop region were recently identified. As mentioned above, all Plk1 homologs share the same domain organization, with an N-terminal serine/threonine catalytic kinase domain separated by a linker segment, known as the T-loop, important for regulation of kinase activity, and a C-terminal non-catalytic PBD, which consists of two separate polo-box motifs. The polo boxes fold together to form a pincer-like structure that binds to phosphoserine/threonine on Polo substrates (Elia *et al.*, 2003). It was shown that phosphorylation of threonine 238 in the Cdc5 T-loop is crucial to maintain full kinase activity in mitosis, although it is not essential for cell viability (Rawal *et al.*, 2016). Moreover, impairing the phosphorylation of threonine 238 (*cdc5-T238A*) completely prevents checkpoint adaptation, and alters the regulation of the Mus81-Mms4 resolvase (Rawal *et al.*, 2016), consistent with the recent finding that, in unperturbed conditions, *cdc5-T238A* mutant cells are defective in the activation of Cdc14 in early anaphase, suggesting that Cdc5 promotes checkpoint adaptation, at least in part, by stimulating events required for exit from mitosis (Valerio-Santiago, de Los Santos-Velázquez and Monje-Casas, 2013).

Besides the T-loop, the PBD was also found to be important for the functions of Cdc5 during the adaptation process. Indeed, it was found that impairment of the PBD of Cdc5 results in an impaired checkpoint adaptation (Ratsima *et al.*, 2016). In particular, the PBD has been found to be important for the localization of Cdc5 at SPBs to promote adaptation, thereby suggesting that SPB components might act as a platform or a target for Cdc5 in this process (Ratsima *et al.*, 2016).

The important role played by Cdc5 in the adaptation process is further stressed by the observation that high levels of Cdc5 lead to the complete loss of Rad53 phosphorylation despite a persistent damage (Donnianni *et al.*, 2010; Vidanes *et al.*, 2010). Indeed, the overexpression of Cdc5 affects the ability of Rad9 to promote the autophosphorylation of Rad53, therefore interfering with checkpoint maintenance (Vidanes *et al.*, 2010). Furthermore, the overexpression of Cdc5 delays resection following DSB induction (Donnianni *et al.*, 2010).

1.7.2.2. Other factors involved in adaptation

The second adaptation-defective mutant identified by Hartwell and colleagues was affected at the *CKB2* gene (Toczyski, Galgoczy and Hartwell, 1997), that encodes for the non-essential regulatory subunit of casein kinase 2 (CK2) (Reed, Bidwai and Glover, 1994). CK2 is a multiprotein complex that possesses protein serine/threonine kinase activity, and contains two catalytic alpha subunits, Cka1 and Cka2, and two regulatory beta subunits, Ckb1 and Ckb2 (reviewed in (Glover, Bidwai and Reed, 1994)). CK2 plays important functions in many cell physiology processes, including cell morphology and size (reviewed in (Canton and Litchfield, 2006)), and in cell cycle progression. Regarding the latter, CK2 has been found to participate in G_1/S and G_2/M transitions (Hanna, Rethinaswamy and Glover, 1995), however, the mechanisms for the regulation of the transition from G_2 to M is currently unknown. In G_1 phase, in case of unfavorable environment or until a critical cell

size has been reached, CK2 restrains G_1 cyclin-CDK complex activity through two mechanisms: first, CK2-mediated phosphorylation of CDK has been proposed to attenuate the interaction between CDK and G_1 cyclins (Russo *et al.*, 2000; Russo, van den Bos and Marshak, 2001); second, CK2-mediated phosphorylation of the CDK inhibitor Sic1 prevents Clb-CDK complex activation (Coccetti *et al.*, 2004, 2006).

Following the initial discovery of the involvement of CK2 in adaptation, subsequent studies provided evidences for its function in the adaptation process. More specifically, CK2 has been found to phosphorylate Ptc2, a phosphatase involved in checkpoint termination, and this phosphorylation event facilitates its interaction with Rad53, therefore promoting the inactivation of Rad53 (Guillemain *et al.*, 2007). However, *ckb1* and *ckb2* mutants exhibit a more severe adaptation defect compared to *ptc2* mutants, suggesting that the CK2 protein kinase likely has other functions in checkpoint adaptation (Guillemain *et al.*, 2007). To date, these additional functions remain unknown.

DSB end resection, the first step of DSB repair through HR, influences checkpoint adaptation. Indeed, the nuclease Sae2 and the anti-recombinase factor Srs2 shows both recovery and adaptation defects (Vaze *et al.*, 2002; Clerici *et al.*, 2006). Sae2, together with the MRX complex, promotes the initial DNA resection of DSB ends (Clerici *et al.*, 2005). However, it was shown that deletion of *SAE2* or mutation of its Mec1/Tel1 phosphorylation sites result in a prominent checkpoint activation and cell cycle arrest that cannot be only associated with a defective repair of the lesion (Baroni *et al.*, 2004; Clerici *et al.*, 2006). Sae2 may negatively regulate checkpoint signaling by modulating MRX association with the DNA ends at a damaged site. It was proposed that the Mec1- and Tel1-dependent phosphorylation of Sae2 is necessary for the regulation of checkpoint termination through a Sae2-mediated inhibition of MRX signaling (Clerici *et al.*, 2006). A similar function has been proposed for Srs2, the anti-recombinase factor that counteracts Rad51 nucleoprotein

filament formation. It was suggested that Srs2 may favor to initiate adaptation by promoting the removal of checkpoint proteins from the site of the lesion and/or restoring chromatin structure (Vaze *et al.*, 2002).

Consistent with the role of DSB end resection in checkpoint adaptation, it was shown that loss of the Ku70/80 complex, a negative regulator of resection, leads to adaptation defects, probably due to a significant increased resection rate, which was found to be similar to the resection rate observed for two DSBs resected at a normal rate (Lee *et al.*, 1998). The adaptation defect observed in *ku70Δ* cells is relieved by deletion of Mre11, a factor that promotes the initiation of resection, indicating that the extent of the resection influences the inactivation of the checkpoint activation, thus adaptation (Lee *et al.*, 1998). Moreover, the adaptation defect in *yku70Δ* cells is suppressed by mutations affecting the ssDNA binding protein RPA (Lee *et al.*, 1998; Ghospurkar *et al.*, 2015), suggesting that RPA counteracts adaptation by sustaining the checkpoint until the damage is repaired, promoting thus proper checkpoint recovery.

A process intimately linked with DNA end resection is that of nucleosome remodeling around the DSB site, which facilitates the exonucleases activity on nucleosome-bound DNA adjacent to the DSB. Therefore, it is not surprising that the chromatin remodeling factor Tid1 (also known as Rdh54) and its homolog Fun30 are required for checkpoint adaptation (Sang Eun Lee *et al.*, 2001; Eapen *et al.*, 2012; Ferrari *et al.*, 2013). Consistently, the chromatin remodeler Ino80 is also important for checkpoint adaptation. It was found that the adaptation defects associated with the absence of Ino80 are likely due to the inability of the chromatin to maintain high levels of histone H2AX phosphorylation at the site surrounding the DSB, and therefore the inability of chromatin to sustain a proper checkpoint signaling at the DSB site (Papamichos-Chronakis, Krebs and Peterson, 2006).

Since checkpoint adaptation correlates with the loss of Rad53 phosphorylation, it is not surprising that the phosphatases Ptc2 and Ptc3, involved in the dephosphorylation of Rad53 during checkpoint recovery, have been found defective in checkpoint adaptation (Leroy *et al.*, 2003).

In summary, checkpoint adaptation is affected by factors involved in the early steps of damage processing and the assessment of ssDNA levels, as well as proteins influencing the chromatin environment, all of which seem to share a common function in the modulation of checkpoint signaling. Recently, however, other processes not strictly related to checkpoint signaling have been found to influence checkpoint adaptation. For instance, autophagy, a process involved in dealing with cellular stresses of different nature, is also involved in DDR, where it participates in counteracting cell cycle progression. It was found that DNA damage triggers a specific subpathway of autophagy, namely genotoxin-induced targeted autophagy (GTA), in a Mec1- and Rad53-dependent manner and requires proteins involved in selective autophagy (Eapen *et al.*, 2017). Moreover, the activation of autophagy correlates with the nuclear exclusion of Pds1 associated with the separase Esp1, therefore preventing activation of the separase, namely anaphase onset and chromosome segregation (Dotiwala *et al.*, 2013; Eapen and Haber, 2013). Consistently, hyperactivation of autophagy results in a permanent cell cycle arrest and adaptation defects, and abrogation of autophagy, or artificially targeting Esp1 to the nucleus, is sufficient to re-establish adaptation (Dotiwala *et al.*, 2013; Eapen and Haber, 2013; Eapen *et al.*, 2017).

As checkpoint adaptation represses DDR activation and allows cells to progress into anaphase, it is not surprising that cell cycle components governing the onset of anaphase have been implicated in checkpoint adaptation. Among these cell cycle components, we have discussed the fundamental role played by Cdc5, a component of both the FEAR and the MEN networks that control mitotic events occurring at the transition from metaphase

to anaphase and in late anaphase, respectively. Moreover, autophagy counteracts adaptation by preventing the activation of the separase Esp1, a protein responsible for both cohesin cleavage and FEAR network activation, and that determines chromosome segregation and early nuclear release of Cdc14, respectively. Interestingly, other components of the FEAR network have been found to participate in the adaptation response (see section 1.6.1.2.1 for details about the FEAR network). Indeed, it was found that mutation of single components of the FEAR network, such as Slk19, Spo12 and, of course, Cdc5 causes defects in checkpoint adaptation (Jin and Wang, 2006). However, the molecular details of how the network activity participates in checkpoint inactivation remain unknown.

In conclusion, checkpoint adaptation involves the downregulation of the DDR at different levels, including modulation of the strength of the DDR signaling and promotion of cell cycle resumption. Although many components involved in this process have been identified, the molecular mechanism underlying checkpoint adaptation is still a mystery.

2. Materials and methods

2.1. Plasmids, primers and strains

2.1.1. Plasmids and primers

All plasmids and primers that were used in this study are listed in Table 2.1 **PCR-mediated gene deletions performed.** For each ORF deleted, DNA template, primers used for PCR-based amplifications, and primers used for control of deletion are listed.

ORF	Purpose	Template plasmid	Forward primer	Reverse primer
BNS1	Deletion	pFA6a-KanMX6	BNS1_1_F	BNS1_1_R
BNS1	Control of deletion		BNS1_2_F	BNS1_2_R
MAD1	Deletion	pFA6a-hphMX6	MAD1_F1	MAD1_R1
MAD1	Control of deletion		MAD1_F2	MAD1_R2
SGS1	Deletion	pFA6a-KanMX6	SGS1_1_F	SGS1_1_R
SGS1	Control of deletion		SGS1_2_F	SGS1_2_R
SLK19	Deletion	pFA6a-KanMX6	SLK19_1_F	SLK19_1_R
SLK19	Control of deletion		SLK19_2_F	SLK19_2_R
SPO12	Deletion	pFA6a-hphMX6	SPO12_1_F	SPO12_1_R
SPO12	Control of deletion		SPO12_2_F	SPO12_2_R
SRS2	Deletion	pFA6a-KanMX6	SRS2_1_FW	SRS2_1_REV
SRS2	Control of deletion		SRS2_2_FW	SRS2_2_REV
ZDS1	Deletion	pFA6a-KanMX6	ZDS1-F1	ZDS1-R1
ZDS1	Control of deletion		ZDS1-FC	ZDS1-RC
ZDS2	Deletion	pFA6a-hphMX6	ZDS2-F1	ZDS2-R1
ZDS2	Control of deletion		ZDS2-FC	ZDS2-RC

and Table 2.3, respectively.

2.1.2. Bacterial strains

The genotypes of the *Escherichia coli* bacterial (*E. coli*) strains that were used as hosts for plasmid amplification are listed in Table 2.4. Chemically competent cells were used for the transformations.

2.1.3. Yeast strains

All *Saccharomyces cerevisiae* (*S. cervisiae*) strains that were used in this study derive from the W303 background carrying a wild type copy of the *RAD5* gene (*ade2-1, can1-100, trp1-*

1 leu2-3,112, his3-11,15, ura3, RAD5), or from the JKM179 background (*delta-ho, hml::ADE1, hmr::ADE1, ade1-100, leu2-3,112, lys5, trp1::hisG, ura3-52, ade3::GAL10:HO*). The majority of the W303 strains were generated by dissecting sporulated heterozygous diploid strains obtained by crossing haploid strains of opposite mating type (see section 2.5.1 for procedure). The majority of the JKM179 strains were obtained by transformation using PCR-based cassettes. The relevant genotypes of all yeast strains used in this study are listed in Table 2.5.

2.2. Growth media and growth conditions

2.2.1. Growth media for Escherichia coli

Bacterial cells were grown in Lysogeny broth (LB) medium with the following composition.

LB: 1% bactotryptone (DIFCO)
0.5% yeast extract (DIFCO)
1% NaCl
pH 7.25

The LB medium was supplemented with 50 µg/ml ampicillin (LB + amp). For solid media, 2% agar (DIFCO) was added to the medium. All strains were grown at 37°C.

2.2.2. Growth media for Saccharomyces cerevisiae

Yeast cells were grown in rich medium yeast extract peptone (YEP).

YEP: 1% yeast extract
2% bactopeptone
0.015% L-tryptophan
pH 5.4

The YEP medium was supplemented with 300 µM adenine and either 2% glucose yeast extract peptone dextrose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 2% galactose (YEPRG) as carbon sources. For solid media, 2% agar (DIFCO) was added to the medium.

SC: 0.15% yeast nitrogen base (YNB, DIFCO) without amino acids and ammonium sulfate.

0.5% ammonium sulfate

200 nM inositol

All W303 strains were grown at 23°C and all JKM179 strains were grown at 28°C, unless otherwise stated. Growth conditions for individual experiments are described in the corresponding figure legend.

2.3. DNA-based procedures

2.3.1. *Escherichia coli* transformation

Fresh, chemically competent Top10 cells (50 µl) were thawed on ice for approximately 10 minutes prior to the addition of plasmid DNA or the ligation mixture. Cells were incubated with DNA on ice for 30 minutes and then subjected to a heat shock for 30-45 seconds at 37°C. After the heat shock, cells were returned to ice for 2 minutes. Finally, 950 µl LB medium was added to the reaction tube. The cell suspension was incubated on a shaker at 37°C for 45 minutes before plating onto LB and ampicillin plates. The plates were incubated overnight (O/N) at 37°C.

2.3.2. Plasmid DNA isolation from *Escherichia coli* (mini prep)

Clones were picked from individual colonies and used to inoculate 2 ml LB and ampicillin and grown O/N at 37°C. Bacterial cells were transferred to micro-centrifuge tubes and pelleted for 5 seconds at 8000 revolutions per minute (rpm). Minipreps were performed

with QIAprep Spin Miniprep Kit (Quiagen) following the manufacturer's instructions. Plasmids were eluted in 30 µl of sterile double-distilled water (ddH_2O).

2.3.3. High-efficiency LiAc-based yeast transformation

Yeast cells were grown O/N in 50 ml YEPD, or the appropriate medium, allowing them to reach the stationary phase. On the following morning, the cell culture was diluted to OD_{600} = 0.2 and allowed to grow several cycles until it reached an OD_{600} of 0.4-0.7. the cells were then harvested at 3000 rpm for 3 minutes and washed with 50 ml ddH_2O. The pellet was then transferred to an eppendorf tube with 1 ml ddH_2O and washed with 1 ml 1X Tris-EDTA/lithium acetate (TE/LiAc) solution. The cells were then resuspended in 250 µl 1X TE/LiAc solution. Competent cells were used for each transformation reaction, 50 µl aliquots with 300 µl 1X polyethylene glycol (PEG)/TE/LiAc solution, 5 µl 10 mg/ml single-stranded salmon sperm denatured DNA and "x" µl (up to 10 µl) DNA. After gentle mixing, the transformation reaction was incubated on a rotating wheel for 30 minutes at room temperature (RT). The cells were then heat-shocked at 42°C for 15 minutes, followed by centrifugation for 3 minutes at 3000 rpm. The pellet was resuspended in 200 µl 1X TE and the cell suspension was plated on appropriate auxotroph selective medium. In case of selection for resistance to the antibiotic geneticin (G418), the pellet was resuspended in 200 µl 1X TE and the cell suspension was plated on YEPD to allow the cells to recover after the heat-shock before exposure to antibiotics. After two days, the resulting colonies were replica plated on a YEPD plate containing 220 µm/ml G418.

10X TE:	0.1 mM Tris, brought to pH 8.0 with HCl
	10 mM EDTA, pH 8
10X LiAc:	1 M LiAc, brought to pH 7.0 with acetic acid
1X TE/LiAc:	1X TE

1X LiAc

1X PEG/TE/LiAc: 1X TE

1X LiAc

40% PEG 4000

2.3.4. Smash and Grab yeast genomic DNA isolation

Cells were picked from individual yeast colonies and inoculated in 200 µl Lysis buffer. 200 µl phenol/chloroform/isoamyl alcohol 25:24:1 (SIGMA) and 1 volume of glass beads were added to the cell suspensions and the tubes were shaken for 10 minutes on Vxr Ika-Vibrax shaker. The tubes were then centrifuged twice for 4 minutes at 13 000 rpm and the upper aqueous layer was transferred to new tubes. 1 ml ice-cold 100% ethanol was added to precipitate the DNA. After gently mixing the solution, the tubes were centrifuged for 4 minutes at 13 000 rpm. The supernatants were then removed, the pellets were air-dried, and the DNA resuspended in 50 µl 1X TE.

Lysis buffer: 2% Triton X-100

1% sodium dodecyl sulfate (SDS)

100 mM NaCl

10 mM Tris, brought to pH 8.0 with HCl

1 mM EDTA, pH 8.0

2.3.5. Teeny yeast genomic DNA extraction

Yeast cells of the desired strain were grown in 10 ml YEP containing the appropriate sugar till to reach the stationary phase. The cells were collected through centrifugation and then washed with 1 ml of solution I. The pellet was then transferred into 0.4 ml solution I, together with 14 mM β-mercaptoethanol. After mixing, 0.1 ml of a 2 mg/ml Zymolyase 100T solution were added and the tube was incubated at 37°C until spheroplast were

formed (20-30 minutes), which was verified by optical microscopy. After 30 seconds of centrifugation, the pellet was carefully resuspended in 0.4 ml 1X TE. After the addition of 90 µl of solution II, the tube was mixed and incubated for 30 minutes at 65°C. 80 µl potassium acetate (KOAc), 5 M, were then added and the tube was incubated on ice for at least 1 hour. The tube was then centrifuged for 15 minutes, the supernatant was transferred in a new tube, and the DNA was precipitated and washed with 100% ethanol. The dried pellet was carefully resuspended in 0.5 ml 1X TE. 25 µl of 1 mg/ml RNase was added and the solution was incubated for 20 minutes at 37°C. The DNA was then precipitated by the addition of 0.5 ml isopropanol, followed by centrifugation. The pellet was washed with cold 70% ethanol, air-dried and finally resuspended in 50 µl 1X TE.

Solution I: 0.9 M sorbitol

0.1 M EDTA, pH 7.5

Solution II: 1.5 ml EDTA, pH 8.5

0.6 ml Tris base

0.6 ml 10% SDS

2.3.6. Enzymatic restriction of DNA

For diagnostic DNA restriction, 0.5-2 µg plasmid DNA was digested for 2 hours at 37°C with 1-10 units of the appropriate restriction enzyme (New England Biolabs, NEB). The volume was adjusted depending on the DNA volume and concentration to 20-50 µl with the appropriate buffer and ddH_2O.

For preparative DNA restriction, 5-10 µg plasmid DNA were incubated for 2 hours at 37°C with 1-10 units of restriction enzyme. The enzymes sensitive to heat inactivation were inactivated at 65°C for 20 minutes. The enzymes not sensitive to heat inactivation for yeast transformation were inactivated at 65°C for 5 minutes with 6 mM EDTA pH 8. The DNA was

then precipitated by the addition of 1/10 volume 3 M sodium acetate (NaOAc) and 3 volumes 100% isopropanol, followed by centrifugation at 13,000 rpm for 15 minutes. The pellet was washed with 200 µl 70% ethanol and finally resuspended in 10 µl ddH_2O, in the case of integrative plasmids, for transformation into yeast.

2.3.7. Constructs for *CDC14^{TAB6-1}* and *mCherry-TUB1* strain construction

To obtain strains in the JKM179 background carrying the construct for *CDC14^{TAB6-1}* allele, the plasmid Rp82 (YIPlac204 – pCDC14-CDC14^{TAB6-1}) was linearized with the Bsu36I restriction enzyme before being transformed into yeast cells. Clones were controlled for single integration events via PCR on the entire genome of the transformed strain (see section 2.3.5 for details) using TRP1_F1 and TRP1_R1 primers for one integration event, and TRP1_F2 and TRP1_R1 primers for multiple integration events.

To obtain strains in the JKM179 background carrying the construct for *mCherry-TUB1*, the plasmid pAK011 (pRS306-mCherry-TUB1, gift from Dr. E. Schiebel) was linearized with the ApaI restriction enzyme before being transformed into yeast cells. Clones were controlled for single integration events via PCR on the entire genome of the transformed strain using URA3_F1 and URA3_R1 primers for one integration event, and URA3_F2 and URA3_R1 primers for multiple integration events.

2.3.8. DNA amplification

The DNA was amplified using polymerase chain reaction (PCR). PCR was performed using genomic yeast DNA or plasmid DNA as template. Amplification of a DNA fragment requires two oligonucleotides flanking the interesting region, working as primers for the DNA polymerase. Phusion DNA polymerase (Finnzymes) and ExTaq (TaKaRa) DNA polymerase were used.

Reaction mix: template DNA 1 µl

reaction buffer 1X

dNTPs 0.2 mM

forward primer 1 µM

reverse primer 1 µM

DNA polymerase 1-2 units

ddH_2O up to 20 µl

DNA amplification was performed with a Biometra T3000 Thermocycler with the following general procedure:

1. heat shock step 5 minutes at 95°C
2. denaturation step 1 minute at 95°C
3. annealing step 1 minute at 50-58°C
4. extension step 1 minute /kb at 72°C
5. steps from 2 to 4 repeated for 20-25 times
6. extension step 10 minutes at 72°C
7. end hold at 4°C

2.3.8.1. PCR-mediated gene deletion

Gene deletion was performed as described by Longtine et al. (Longtine *et al.*, 1998). Gene deletions performed in the W303 and/or JKM179 background are listed in Table 2.1. For each ORF deleted, DNA template and primers used for amplification are listed. Deletions were assessed via PCR on the entire genome of the transformed strain (see section 2.3.5 for details) using primers used for control of deletion, listed in Table 2.1.

2.3.8.2. PCR-mediated gene tagging

The auxin-inducible degron (AID) system allows for rapid degradation of target proteins in response to plant hormones of the auxin family through the SCF degradation pathway. In

order to use this system, (i) cells must ectopically express the F-box transport inhibitor response 1 (Tir1) protein; and (ii) the target protein has to be fused with the AID degron. To induce the degradation of the protein of interest, indole-3-acetic acid (IAA; a natural auxin) or 1-Naphthaleneacetic acid (NAA; synthetic hormone of the auxin family) are added to the medium. IAA (as well as NAA) is required to mediate the binding between Tir1 and the AID degron. This interaction allows for the activation of the SCF degradation pathway, resulting in the proteasomal degradation of the AID-fused protein.

Gene tagging with the auxin-inducible degron (AID) tag (Nishimura *et al.*, 2009) was performed as described by Morawska and Ulrich (Morawska and Ulrich, 2013). To tag the *CFI1* gene in the W303 and JKM179 backgrounds, wild type W303 (Ry2281) and wild type JKM179 (Ry2118) cells were transformed with the PCR fragment obtained using pMK43 plasmid as template and CFI1_AID_FW and CFI1_AID_REV as primers. *CFI1* tagging was assessed by PCR on the entire genome of the transformed strain using Cfi1-internal-F and Cfi1-300bpds-R as primers.

Gene tagging with the pGAL-3HA construct was performed as described by Longtine et al. (Longtine *et al.*, 1998). To tag the *FOB1* gene in the W303 background, wild type W303 (Ry2281) cells were transformed with the PCR fragment obtained using pFA6a-KanMX6-pGal1-3HA plasmid as template and FOB1-F4 and FOB1-R3 as primers. *FOB1* tagging was assessed by PCR on the entire genome of the transformed strain using FOB1-FC and FOB1-RC1 as primers.

2.3.8.3. PCR-mediated gene integration

Gene integration was as described by Longtine et al. (Longtine *et al.*, 1998). To integrate the pADH1-OsTIR1-9MYC construct in the URA3 locus in the JKM179 background, *CFI1-AID* (Ry7000) cells were transformed with the PCR fragment obtained using genomic DNA from

Ry4850 (*MATalpha, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, ura3::pADH1-OsTIR1-9MYC::URA3, cdc20-AID::KanMX*) as template and OsTir1_FW1 and OsTir1_REV2 as primers. The pADH1-OsTIR1-9MYC integration was assessed by PCR on the entire genome of the transformed strain using OsTir1_FW1 and OsTir1_REV2 as primers.

2.3.9. Agarose gel electrophoresis

Following the addition of 1/5 volume of bromophenol blue (BPB) solution, DNA samples were loaded on 0.8% - 1% agarose gels along with DNA markers. The gels were made in 1X Tris-Acetate-EDTA (TAE) buffer containing 10 µg/ml ethidium bromide and run at 80-120 volts (V) until the desired separation was achieved. The DNA bands were visualized under a UV lamp (radiation wavelength 260 nm).

BPB solution:	0.2% BFB in 50% glycerol
10X TAE buffer:	0.4 M Tris acetate
	0.01 M EDTA

2.3.10. Purification of DNA from agarose gel

Cut DNA was first loaded into an agarose gel to separate the DNA fragments by electrophoresis. The DNA fragment of interest was then excised from the agarose gel with a sharp scalpel. DNA extraction was performed with QIAquick Gel Extraction Kit (Quiagen) following the manufacturer's instructions. The DNA fragments were eluted in 30-50 µl ddH_2O.

2.4. Protein-based procedures

2.4.1. Yeast protein extraction

10 ml of a cell culture at OD_{600} = 0.2-1 were collected and centrifuged for 2 minutes at maximum speed. The resulting pellet was washed with 1 ml cold 10 mM Tris-HCl pH 7.5, transferred to 2 ml Sarstedt tubes and frozen in liquid nitrogen in order to better preserve

the protein integrity. The pellet was then resuspended in 100 µl lysis buffer supplemented with complete protease inhibitor cocktail (Roche) and phosphatases inhibitors (60 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 5 mM sodium fluoride (NaF), and 15 mM p-Nitrophenylphosphate). An equal volume of acid-washed glass beads (Sigma) was added (leaving a layer of supernatant over the beads) and the tubes were subjected to 3-5 rounds of Fast Prep (speed 6.5 for 45 seconds) at 4°C in order to break the cells. Cell breakage was verified using an optical microscope. Lysed cells were transferred to a fresh tube. In order to quantify the protein content, 10 µl of the lysate were diluted 1:3 with cold 50 mM Tris-HCl pH 7,5 / 0,3 M NaCl and 3 µl were used in the Biorad protein quantification assay. The absorbance was read at λ = 595 nm. 50 µl 3X SDS blue loading buffer were then added to each sample. The samples were boiled at 95°C for 5 minutes, centrifuged at 13,000 rpm for 3 minutes and the supernatant, containing the final protein extract, was collected in a new microcentrifuge tube. Extracts were stored at -20°C.

Lysis buffer:	50 mM Tris-HCl, pH 7,5
	1 mM EDTA, pH 8
	50 mM DTT
3X SDS blue loading buffer:	9% SDS
	30% glycerol
	0.05% Bromophenol blue
	6% β-mercaptoethanol
	0.1875 M Tris-HCl, pH 6.8

2.4.2. Yeast protein extraction from TCA treated yeast cells

10 ml of a cell culture at OD_{600} = 0.2-1 were collected and centrifuged for 2 minutes at maximum speed. The resulting pellet was resuspended in an equal volume of ice-cold 5%

trichloroacetic acid (TCA) and incubated for 10 minutes on ice. After centrifugation for 2 minutes at maximum speed at 4°C, the pellet was transferred with 1 ml 5% TCA to a 2 ml Sarstedt tube. The tube was centrifuged at 4°C and the supernatant discarded. The pellet was frozen in liquid nitrogen in order to better preserve the protein integrity. The pellet was then washed with 1 ml absolute acetone and air-dried. The pellet was then resuspended in 100 µl lysis buffer (see section 2.4.1) supplemented with complete protease inhibitor cocktail (Roche) and phosphatases inhibitors (60 mM β-glycerol phosphate, 0.1 mM Na orthovanadate, 5 mM NaF, and 15 mM p-Nitrophenylphosphate). An equal volume of acid-washed glass beads (Sigma) was added (leaving a layer of supernatant over the beads) and the tubes were subjected to 3-5 rounds of Fast Prep (speed 6.5 for 45 seconds) at 4°C in order to break the cells. Cell breakage was verified using an optical microscope. 50 µl 3X SDS blue loading buffer (see section 2.4.1) were then added to each sample. The samples were thereafter boiled at 95°C for 5 minutes, centrifuged at 13,000 rpm for 3 minutes and the supernatant, containing the final protein extract, was collected in a new microcentrifuge tube. Extracts were stored at -20°C.

2.4.3. SDS polyacrylamide gel electrophoresis

Appropriate amounts of proteins (50-100 µg of total extracts) were separated based on their molecular weight on 8% or 10% polyacrylamide gels. The gels were prepared from a 30% 30:0.8 acrylamide:bisacrylamide mixture (Sigma), 4X Separating buffer, 2X Stacking buffer and an appropriate amount of ddH_2O. As polymerization catalysts, ammonium persulfate (APS) and TEMED (BDH) were used. 1.5 mm thick polyacrylamide gels were run in 1X running buffer at 100-150 V for 2-3.5 hours.

4X Resolving buffer: 1.5 M Tris base, brought to pH 8.8 with glacial acetic acid

0.4% SDS

2X Stacking buffer: 0.25 M Tris base, brought to pH 6.8 with glacial acetic acid

0.2% SDS

10X Running buffer: 2 M glycine

0.25 M Tris-HCl

0.02 M SDS

pH 8.3

2.4.4. Western blot hybridization

Proteins were transferred in Western transfer tanks to nitrocellulose (Protran, Whatman) in 1X transfer buffer at 30 V O/N or 100 V for 1.5-2 hours. Ponceau S staining was used to roughly reveal the amount of proteins transferred onto the filters.

For Western blots, membranes were blocked with 1X phosphate-buffered saline (PBS) supplemented with 1% Tween (PBS-T) and 3% milk for 1 hour at RT.

After blocking, the membranes were incubated with the primary antibody as follows:

- 1:5000 mouse anti-Pgk1 (Invitrogen) diluted in 1% milk / 1% BSA / 1X PBS-T for 2 hours at RT or O/N at 4°C.
- 4 µg/mL mouse anti-Rad53 antibody (EL7.E1, gift from Dr. M. Foiani) diluted in 4% milk / 1% BSA / 1X PBS-T for 2 hours at RT or O/N at 4°C.
- 1 µg/mL mouse anti-AID antibody (Invitrogen) diluted in 4% milk / 1% BSA / 1X PBS-T for 2 hours at RT or O/N at 4°C.

The membranes were then washed 3 times for 15 minutes in 1X PBS-T. Afterwards, the membranes were incubated with the horseradish-peroxidase (HRP)-conjugated secondary antibody: 1:10 000 anti-mouse or 1:10 000 anti-rabbit in 1% milk / 1% BSA / 1X PBS-T for 1 hour.

After incubation with the secondary antibody, the membranes were washed 3 times for 15 minutes in 1X PBS-T and the bound secondary antibody was revealed using ECL (Enhanced Chemiluminescence, Amersham).

1X Transfer buffer:	0.2 M glycine
	0.025 M Tris base
	20% methanol
10X PBS buffer:	1.37 M NaCl
	27 mM KCl
	14.7 mM KH2PO4
	80 mM Na2HPO4
1X PBS-T buffer:	0.1% Tween
	1X PBS

2.5. Cell biology procedures

2.5.1. Yeast tetrad dissection and analysis

MATa and *MATα* strains were mixed on solid medium, appropriate for the growth of both haploids, and incubated O/N at permissive conditions. Next, cells from the cross mixture were streaked to single colonies on selective medium and incubated at the appropriate temperature, allowing for selection of diploid cells. Single colonies grown under selective conditions were then amplified on rich media for 1 day. This step greatly increases the efficiency of sporulation. Next, diploids were patched onto sporulation plates to induce meiosis and sporulation by starvation. After 3-5 days, the diploids had efficiently sporulated and tetrads were dissected. In order to separate individual spores, the wall of the ascus

containing the tetrad was removed by enzymatic digestion. A toothpick full of tetrads was resuspended into the digestion mixture. The digestion mixture was then incubated at 37°C for 3 minutes in order to enzymatically digest the ascus wall. Then, 1 ml ddH_2O was added to dilute the mixture and 20 µl of mixture were dripped in a line onto the appropriate agar plate. Individual tetrads were dissected using the Nikon dissection microscope. The spores were left growing at 23°C for 3-5 days. The colonies were replica plated onto selective media to define their genotype.

Digestion mixture:	198 µl ddH_2O
	2 µl 10 mg/ml Zymolyase 100T (Seikagaka, Biobusiness)
Sporulation plates:	30 g K-Acetate
	60 g Agar (DIFCO)
	all amino acids at 1/4 of the normal concentration
	diluted in 3 L ddH_2O

2.5.2. BAR-test/α-factor sensitivity

The *BAR1* gene (BARrier to the α-factor response) encodes a protease that is secreted into the periplasmic space of *MATa* cells (Sprague and Herskowitz, 1981). This protease cleaves and inactivates the α-mating factor pheromone, therefore allowing cells to recover from α-factor-induced cell cycle arrest. Transcription of *BAR1* in *MATα* haploids and *MATa/MATα* diploids is repressed. *BAR1* is a *MATa*-specific gene whose transcription is stimulated by the presence of α-factor (Manney, 1983; Kronstad, Holly and MacKay, 1987). *MATa* cells that lack the Bar1 protein are supersensitive to of α-factor-induced G_1 arrest (Sprague and Herskowitz, 1981). When assessing the genotype of yeast strains, the presence or absence of the Bar1 protein was assessed with what we call the "BAR-test". *MATa* cells (one streak

through a patch) were resuspended in 50 µl of an appropriate medium. A volume of the appropriate medium containing 0.8% agarose was boiled. When cooled down, 3 ml of the agarose/medium were mixed with the cell suspension mixture and poured into a small Petri dish. The Petri dish was put on ice until the mixture was solid. A small dot of *MATα* tester strain was put in the center of the dish and the dish was incubated at RT for 1-2 days. *bar1* mutant strains, being highly sensitive to the presence of α-factor, do not grow in proximity of the *MATα* cells but produce a halo around the tester strain.

2.5.3. Activation/inactivation of conditional mutants

2.5.3.1. Regulation of gene expression

To regulate the expression of specific proteins, yeast strains in which the encoding genes were cloned under the control of inducible promoters were used. The $pGAL_{1\text{-}10}$ promoter induces the expression of a downstream gene after the addition of galactose to cells growing in media containing a poor carbon source such as raffinose. The $pGAL_{1\text{-}10}$ promoter can be turned off by the addition of glucose to a galactose-containing medium, thus inhibiting the expression of the downstream gene. This system has been used to overproduce specific proteins, as well as to achieve the full repression of specific essential genes in a time-regulated manner.

2.5.3.2. Protein degradation

Degron systems can be used to induce the degradation of a specific protein in a time-regulated manner. The AID system allows for rapid degradation of target proteins in response to auxin hormones through the SCF degradation pathway (Nishimura *et al.*, 2009). To induce the degradation of a protein, indole-3-acetic acid (IAA; a natural auxin) or 1-Naphthaleneacetic acid (NAA; synthetic hormone in the auxin family) were added to the medium (see Figure legends for concentration of the specific compound used).

2.5.3.3. Regulation of conditionally mutant genes

Temperature sensitive alleles were inactivated by incubating cells at the restrictive temperature (32°C for *cdc13-1*).

2.5.3.4. In situ indirect immunofluorescence

Samples of 1ml of cell culture at OD_{600} = 0.2 - 0.4 were collected by centrifugation for 1 minute at 13 000 rpm at room temperature and incubated 10 minutes at room temperature in 1ml of fixative solution. Cells were then pelleted and washed 3 times with 1ml 0.1M KPi pH 6.4 and once with 1ml of sorbitol-citrate solution. Cells were then resuspended in 200µl of digestion solution and incubated at 35°C for 10 minutes to enzymatically digest the cell wall, creating spheroplasts. Digestion was checked by looking for burst spheroplasts when mixed with an equal volume of 1% SDS under an optical microscope. Spheroplasts were pelleted at 2 000 rpm for 2 minutes and washed with 1ml of sorbitol-citrate solution. Pellets were then resuspended in an appropriate volume of sorbitol-citrate solution (from 20 to 200µl, depending on pellet size).

The resuspended spheroplasts (5 µl) were then loaded on a 30-well slide (ThermoScientific) previously treated for 10 minutes with 5 µl 0.1% polylysine (Sigma). To fix cells onto the slide, the slide was incubated in ice cold methanol for 3 minutes and in ice cold acetone for 10 seconds. Cells were then incubated for 2 hours in a humid dark incubation chamber with the primary antibodies diluted in PBS-ovalbumin, washed 5 times with PBS-ovalbumin and incubated with the secondary antibodies diluted in PBS-ovalbumin for further 60 minutes. Cells were then washed 5 times with PBS-ovalbumin and covered with a DAPI mount solution. The slide was covered with a coverslip and sealed with nail polish.

<u>0.1 M KPi buffer pH 6.4</u>: 27.8ml 1 M K_2HPO_4

72.2ml 1 M KH_2PO_4

	diluted in 900 mL ddH_2O
Fixative solution:	3.7% formaldehyde
	0.1 M KPi pH 6.4
1.2 M Sorbitol-citrate:	17.4 g anhydrous KH_2PO_4
	7 g citric acid
	218.64 g sorbitol
	diluted in 1 L ddH_2O
Digestion solution:	1.2 M sorbitol-citrate
	10% glusulase (Perkin-Elmer)
	0.1 mg/mL Zymolyase 100T
PBS-ovalbumin:	1% ovalbumin (Sigma)
	0.04 M K_2HPO_4
	0.01 M KH_2PO_4
	0.15 M NaCl
	0.1% NaN_3
DAPI mount solution:	0.04 M K_2HPO_4
	0.01 M KH_2PO_4
	0.1 5M NaCl
	0.1% NaN_3
	0.05 µg/ml DAPI

0.1% p-phenylenediamine

90% glycerol

Primary antibodies: 1:100 rat anti-tubulin (Oxford-Biotechonology)

1:300 goat anti-Cdc14 (Santa Cruz)

Secondary antibodies:1:100 FITC-conjugated anti-rat

1:500 CY3-conjugated anti-mouse

2.5.3.5. Scoring of indirect immunofluorescence samples

Cell cycle progression was scored by analysis of nuclear and spindle morphologies and dividing cells into three categories:

a. Interphase cells (which includes cells in the G1, S, and G2 phases of the cell cycle): these cells are typically unbudded cells (or cells with a small bud) with one nucleus, one single SPB (or two side-by-side SPBs) and 3-5 short cytoplasmic microtubules emanating from each SPB.
b. Metaphase cells: these cells are typically medium or large budded cells with an undivided nucleus closed to the bud-neck, two separated SPBs and a short and thick bipolar spindle.
c. Anaphase cells (including both anaphase and telophase cells): these cells are typically large budded cells with two nuclear masses (one in the mother cell and the other in the daughter cell), one SPB associated to each nucleus and an elongated spindle.

2.5.4. Synchronization experiments

2.5.4.1. G_1 phase arrest and release

Cells were grown O/N in the appropriate medium at 23°C in a water shaking bath. The cells were then diluted to OD_{600} = 0.2 in fresh medium and left to grow for 2 hours. The cells were then diluted again to OD_{600} = 0.2 and 5 µg/ml α-mating factor synthetic peptide dissolved in ddH_2O (Primm) was added to the medium. After 90 minutes incubation, 2.5 µg/ml α-factor was re-added to the culture. The G_1 arrest was considered complete when more than 90% of cells presented the shmoo, after which the cells were released by filtration, using 10 volumes of medium without the pheromone. The cells were then resuspended into the appropriate fresh medium in the absence of the pheromone.

2.5.5. Microcolony assays

For the microcolony assays, G_1-arrested cells were spotted on YEP plates supplemented with 2% glucose. The cells were micromanipulated in grids using a Nikon dissection microscope. The plates were incubated at the restrictive temperature for the *cdc13-1* allele (32°C) for 24 hours to induce a permanent DNA damage condition. Images were acquired with an upright BX51 Olympus Provis microscope carrying a 10X/0.30 dry long distance UPlanFLN Olympus objective and a Nikon color camera (Digitals Light DS 5MC), using NIS software (Nikon). Based on a similar experiment performed by Toczyski et al. (Toczyski, Galgoczy and Hartwell, 1997), microcolony formation was assessed by quantifying cell bodies, i.e., a cell arrested in metaphase displaying a dumbbell shape was counted as two cell bodies. If the microcolony contained more than three cell bodies, the cells were considered as adapted, thus successfully bypassed the checkpoint-mediated metaphase arrest.

2.5.6. Live cell imaging

2.5.6.1. Budding experiments

For live cell imaging experiments using the budding read-out, G_1-arrested cells were released into appropriate conditions (see Figure legends for details) directly into the microfluidic plates Y04C CellASIC. Imaging was performed with a DeltaVision Elite deconvolution microscope (Applied Precision) equipped with an Olympus IX71 inverted microscope and a CoolSnap HQ2 (Photometrics) CCD camera and driven by SoftWoRx software. Images were acquired with an Olympus U Plan Apo 40X dry objective (NA 0.85) every 15 minutes for 20 hours. The focus plane was maintained throughout the time-lapse using the DeltaVision Autofocus algorithm. In total, more than 100 cells per condition were followed over the whole time-lapse and manually classified into three categories (dumbbell, adapted and dead cells) using the Cell Counter plug-in of the ImageJ software.

2.5.6.2. Spindle morphology experiments

For live cell imaging experiments using the spindle read-out, G_1-arrested cells were released into appropriate conditions (see Figure legends for details) directly into the microfluidic plates Y04C CellASIC. Imaging was performed with a DeltaVision Elite deconvolution microscope (Applied Precision) equipped as described in 2.5.7.1. Images were acquired with an Olympus Plan Apo N 60X oil immersion objective (NA 1.42). Seven z-stacks (0.9 µm step size) were acquired every 15 minutes for 20 hours with FITC filter. Reference images were acquired in DIC. The focus plane was maintained throughout the time-lapse using the Ultimate Focus laser. Images were deconvoluted with SoftWoRx software. In total, more than 100 cells per condition were followed over the whole time-lapse and manually classified into four categories (anaphase, spindle re-joins or abnormal spindles, metaphase break and metaphase arrested) using the Cell Counter plug-in of the ImageJ software. Data were plotted using MS Excel software. The percentages of cells in

each category for each condition were plotted in Prism (GraphPad). For analyses of anaphase duration, they were performed by manually tagging the first point of anaphase spindle elongation (t_1) and the point of interphase microtubules appearance (t_2). The duration of anaphase was calculated by subtracting the two values obtained ($t_2 - t_1$) using MS Excel software. Data were plotted in Prism (GraphPad).

2.5.7. DNA content analyses using flow cytometry

Cell culture samples (1 ml, OD_{600} = 0.2 - 0.4) were collected by centrifugation for 1 minute at 13 000 rpm at RT and incubated O/N at 4°C in 70% ethanol. The cells were then washed with 1 ml 50 mM Tris-HCl pH 7.4 and incubated O/N at 37°C in the same buffer containing 1 mg/ml RNase A. Next, the cells were collected by centrifugation, resuspended in 1 ml 55mM HCl containing 5mg/ml pepsin (Sigma) and incubated at 37°C. After 30 minutes, the cells were collected, resuspended in 50 mM Tris-HCl pH 7.4 and sonicated 3 times for 10 seconds with intervals of 30 seconds using a Bioruptor UCD-300 (Diagenode) water-bath sonicator set on low intensity. Immediately before FACS reading, 100 µl of the cell suspension were added to 1 ml of Sytox Green staining solution and the samples were acquired with FACSCalibur system (Becton Dickinson) operated via the CellQuest software. The data were analyzed with FlowJo Analysis 8.8.6 software.

Sytox green stock solution: 1 mM Sytox green (Invitrogen)

DMSO

Sytox green staining solution: 1 µl/ml 1 mM Sytox green

50 mM Tris-HCl, pH 7.4

2.5.8. Recovery assays

For recovery assay performed using UV-light as source of damage, serial dilutions (1:5) of cell suspensions (from 5 ml overnight cultures grown in YEPD at 23°C) starting from OD_{600}

= 1 were spotted onto YEP plates supplemented with 2% glucose. The cells were then exposed to a single pulse of UV light, ranging from 0 J/m^2 to 120 J/m^2 using a UV Stratalinker 2400 (Stratagene), and the plates were incubated for 2 days at 23°C.

For recovery assays performed using the Haber's system (Vaze *et al.*, 2002), serial dilutions (1:5) of cell suspensions (from 5 ml overnight cultures grown in YEPR at 28°C) starting from OD_{600} = 1 were spotted onto YER plates supplemented with 2% galactose to induce the expression of the Ho endonuclease. Cells were also spotted onto YED plates as control of dilution quality. The cells were then incubated for 2 days at 28°C.

2.5.9. Spot assays

Cells were taken from 5 mL overnight cultures in YEPD at 23°C. Serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto three YEPD plates (one for each temperature) or three YEPD plates supplemented with 1 mM naphthaleneacetic acid (NAA) (one for each temperature) and incubated at 23°C, 25°C or 28°C for 48 hours.

2.6. *In silico* procedures

The *in silico* screen was performed by combining three datasets:

a) *cdc13-1* negative genetic interactions: the gene list was retrieved from TheCellMap website (*http://thecellmap.org/*). *cdc13-1* was used as query; genes with a significant negative genetic interaction were selected (stringent cutoff: score < -0.12, p-value < 0.05, intermediate cutoff: score < -0.08, p-value < 0.05). The final list comprises 374 genes.

b) Decreased resistence to Methyl methanesulfonate ("MMS resistance decreased" gene list): the gene list was retrieved from YeastMine website (*https://yeastmine.yeastgenome.org/yeastmine/begin.do*), using the template Phenotype-Genes and resistance to chemicals as Phenotype Observable. The list was filtered using

Phenotype Qualifier equal to “decreased”, and Phenotype Chemical containing (no concentrations were indicated). The final list comprises 935 genes.

c) exit from mitosis genes: the gene list was retrieved from YeastMine website (*https://yeastmine.yeastgenome.org/yeastmine/begin.do*), using the template GO term-all genes and the following GO terms (and children) as names:

- Mitotic cell cycle
- Mitotic nuclear division
- Mitotic cell cycle process
- Exit from Mitosis

The list has been manually reviewed in order to comprise all FEAR network and MEN genes. The final list comprises 322 genes. The three datasets were intersected using the corresponding tool on YeastMine website (https://yeastmine.yeastgenome.org/).

Table 2.1 **PCR-mediated gene deletions performed.** For each ORF deleted, DNA template, primers used for PCR-based amplifications, and primers used for control of deletion are listed.

ORF	Purpose	Template plasmid	Forward primer	Reverse primer
BNS1	Deletion	pFA6a-KanMX6	BNS1_1_F	BNS1_1_R
BNS1	Control of deletion		BNS1_2_F	BNS1_2_R
MAD1	Deletion	pFA6a-hphMX6	MAD1_F1	MAD1_R1
MAD1	Control of deletion		MAD1_F2	MAD1_R2
SGS1	Deletion	pFA6a-KanMX6	SGS1_1_F	SGS1_1_R
SGS1	Control of deletion		SGS1_2_F	SGS1_2_R
SLK19	Deletion	pFA6a-KanMX6	SLK19_1_F	SLK19_1_R
SLK19	Control of deletion		SLK19_2_F	SLK19_2_R
SPO12	Deletion	pFA6a-hphMX6	SPO12_1_F	SPO12_1_R
SPO12	Control of deletion		SPO12_2_F	SPO12_2_R
SRS2	Deletion	pFA6a-KanMX6	SRS2_1_FW	SRS2_1_REV
SRS2	Control of deletion		SRS2_2_FW	SRS2_2_REV
ZDS1	Deletion	pFA6a-KanMX6	ZDS1-F1	ZDS1-R1
ZDS1	Control of deletion		ZDS1-FC	ZDS1-RC
ZDS2	Deletion	pFA6a-hphMX6	ZDS2-F1	ZDS2-R1
ZDS2	Control of deletion		ZDS2-FC	ZDS2-RC

Table 2.2 **Plasmids used in this study**

Plasmid	Description	Origin
Rp595	pAK011 (pRS306-mCherry-TUB1)	(Khmelinskii *et al.*, 2007)
Rp82	pFA6a-KanMX6-PGal1-3HA	(Longtine *et al.*, 1998)
Rp94	pFA6a-KanMX6	(Longtine *et al.*, 1998)
Rp179	AMp24	Amon lab
	pFA6a-hphMX6	(Hentges *et al.*, 2005)
	pMK43	(Nishimura *et al.*, 2009)

Table 2.3 **Primers used in this study**

Primer	Description	Origin
BNS1_1_F	TAATCAGAGGCTACTACATCATTGCGCTAAAAAAAA GTAACAGATTTGCG CGGATCCCCGGGTTAATTAA	BNS1 deletion
BNS1_1_R	TAACACATACAGCAAAAAAATAGGCCAATTTATGAT CATCATCCGTTAAAGAATTCGAGCTCGTTTAAAC	BNS1 deletion
BNS1_2_F	GCGGAGTACGCATCTTTTGC	BNS1 deletion control
BNS1_2_R	AGATACAGCCAGGGACATTGC	BNS1 deletion control
CFI1_AID_FW	AGAAGCCAAGTGGTGGATTTGCATCATTAATAAAA GATTTCAAGAAAAAACGTACGCTGCAGGTCGAC	CFI1-AID construction
CFI1_AID_FW2	CGTCTAGTATACTTCATGACC	Cfi1 tag control
CFI1_AID_REV	TTTTTACTAGCTTTCTGTGACGTGTATTCTACTGAGA CTTTCTGGTATCAATCGATGAATTCGAGCTCG	CFI1-AID construction
CFI1_AID_REV2	GTAATTATTACCATTGTCTCC	Cfi1 tag control
FOB1-F4	AGAACAATTTAACGATTGTGTGAGTGTGAATTTGTG CTGAGGATAACAGAATTCGAGCTCGTTTAAAC	GAL-FOB1 strain construction
FOB1-FC	TGCGAGGTGATTCCTCATCG	GAL-3HA-FOB1 strain construction
FOB1-R3	TACCGAGTCATCATCATCAAACAACACGTCATTGTA ACGCGGTTTCGTCATGCACTGAGCAGCGTAATCTG	GAL-3HA-FOB1 strain construction
FOB1-RC1	GTGTTGCCATCACCATGTCG	GAL-Fob1 control
MAD1_F1	TAATTCCTAACAATTTTTTCCATCTTAAAATCGAGAG GTAATAGTAAATACGGATCCCCGGGTTAATTAA	Mad1 gene deletion
MAD1_F2	TCGAAATGTAATGAGCAGAGAT	Mad1 gene deletion control
MAD1_R1	TATCATATTATAAAACCGATTACTATTATCTATTAGA AATGTATATACACGAATTCGAGCTCGTTTAAAC	Mad1 gene deletion
MAD1_R2	ACCTACAGACGTGACTTTACCA	Mad1 gene deletion control
OsTir1_FW1	GCCTGCTTCAAACCGCTAAC	URA-OsTir1 amplification
OsTir1_REV2	AAAGCAGGCTGGGAAGCATA	URA-OsTir1 amplification
SGS1_1_F	TACAGATTATTGTTGTATATATTTAAAAAATCATACA CGTACACACAAGGCGGTACGGATCCCCGGGTTAATT AA	SGS1 deletion
SGS1_1_R	AATGCTTGGCGAATGGTGTCGTAGTTATAAGTAACA CTATTTATTTTTCTACTCTGAATTCGAGCTCGTTTAAA C	SGS1 deletion
SGS1_2_F	CGTTTCGAAGTGGATTGCCC	Control of SGS1 deletion
SGS1_2_R	AATGCTTGGCGAATGGTGTC	Control of SGS1 deletion

SLK19_1_F	GTGTCAAGGGGCACCCAGTTAAAAAAGGTTTTGAG CACATATCGTAATTCCGGATCCCCGGGTTAATTAA	SLK19 deletion
SLK19_1_R	TCTCATGACATATTAAGGGAAAAGATAAAATGCAAA AGAAAAAAATGCGTGAATTCGAGCTCGTTTAAAC	SLK19 deletion
SLK19_2_F	GCTTGCCCCCGATAATATGC	SLK19 deletion control
SLK19_2_R	TGTATTGTGCATCTGGACCCC	SLK19 deletion control
SPO12_1_F	AGTAGGAAAACAAAATAACATATACAGTAAGAACA ATAGAAAACGTATTTCGGATCCCCGGGTTAATTAA	SPO12 deletion
SPO12_1_R	GTTTAGTGTAGCATTTGGCTATTTTTGGATGACTAG AAAGGCAGATTTTTGAATTCGAGCTCGTTTAAAC	SPO12 deletion
SPO12_2_F	ATTGCCTCTTCCCATTGTGG	SPO12 deletion control
SPO12_2_R	ACAGCGTGGATATGGACGAG	SPO12 deletion control
SRS2_1_FW	GAGTATCATTCCAATTTGATCTTTCTTCTACCGGTAC TTAGGGATAGCAACGGATCCCCGGGTTAATTAA	SRS2 gene deletion
SRS2_1_REV	AAATTATAAACCGCCTCCAATAGTTGACGTAGTCAG GCATGAAAGTGCTAGAATTCGAGCTCGTTTAAAC	SRS2 gene deletion
SRS2_2_FW	TAGGGTAACGAGACGCGAATG	SRS2 gene deletion control
SRS2_2_REV	AGTTGCCGAATGCTTGGAATC	SRS2 gene deletion control
TRP1_F1	ATGACGCCAGATGGCAGTAG	Control of integration of YIPlac204 in TRP locus and copy number
TRP1_F2	GCATCCGCTTACAGACAAGC	Control of integration of YIPlac204 in TRP locus and copy number
TRP1_R1	ACATTTCCCCGAAAAGTGCC	Control of integration of YIPlac204 in TRP locus and copy number
URA3_F1	CCCCGAAAAGTGCCACCTG	Control of integration of RS306 in URA3 locus and copy number
URA3_F2	GTGGCTGTGGTTTCAGGGTCC	Control of integration of RS306 in URA3 locus and copy number
URA3_R1	CCGATTTCGGCCTATTGGTT	Control of integration of RS306 in URA3 locus and copy number
ZDS1-F1	TTGTGGGTTACATATTTTCAATTCAAAGGAGAATTTA GCTGTCTTTTATACGGATCCCCGGGTTAATTAA	Zds1 gene deletion
ZDS1-FC	GGGGTTTCCTGGTCCTTCAA	Zds1 gene deletion control
ZDS1-R1	GTATGTACGTGTGATGTGTATATGTCTATGTATGCA GCGCTGAAGCCTTTGAATTCGAGCTCGTTTAAAC	Zds1 gene deletion
ZDS1-RC	GACGTCGTGCGGTAGTTTCT	Zds1 gene deletion control
ZDS2-F1	CTTTACATTGATCACGTTTGCACTATAGACTGAATTT AAATTAGAATTTTCGGATCCCCGGGTTAATTAA	Zds2 gene deletion
ZDS2-FC	TAGCGGGACATTTACCAGCG	Zds2 gene deletion control
ZDS2-R1	AAATATGTGGCCTTATATAGGTATCTATCAATCTTGT AAACAGTTATGAGGAATTCGAGCTCGTTTAAAC	Zds2 gene deletion
ZDS2-RC	AGGATGAGATGAGGTCGGCT	Zds2 gene deletion control

Table 2.4 **Bacterial strains used in this study**

Strain	Description
Top10 E. Coli	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ

Table 2.5 **Yeast strains used in this study**

Strain (Ry)	Relevant genotype	Background	Origin
1	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+, rad5-	W303	Visintin lab
1574	MATa, cdc14-1	W303	Visintin lab
2118	MATa, delta-ho, hml::ADE1, hmr::ADE1, ade1-100, leu2-3,112, lys5, trp1::hisG, ura3-52, ade3::GAL10:HO	JKM179	Haber lab
2281	MATa, ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, psi+,	W303 (RAD5 positive)	Foiani lab
6090	MATa, cdc13-1	W303 (RAD5 positive)	This thesis
6092	MATa, cdc5-ad	W303 (RAD5 positive)	This thesis
6378	MATa, cdc13-1, bns1::KanMX6, spo12::HIS3	W303 (RAD5 positive)	This thesis
6387	MATa, bns1::KanMX6, spo12::HIS3	W303 (RAD5 positive)	This thesis
6396	MATa, cdc13-1, slk19::KanMX6	W303 (RAD5 positive)	This thesis
6399	MATa, slk19::KanMX6	W303 (RAD5 positive)	This thesis
6406	MATa, cdc13-1, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
6506	MATa, cdc13-1, bns1::KanMX6, spo12::HIS3, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
6511	MATa, cdc13-1, slk19::KanMX6, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
6659	MATa, cdc13-1, ura3::GAL-SLK19-URA3	W303 (RAD5 positive)	This thesis
6673	MATa, cdc13-1, leu2::GAL-SPO12::LEU2	W303 (RAD5 positive)	This thesis
6676	MATa, cdc13-1, cdc5-ad, leu2::GAL-SPO12::LEU2	W303 (RAD5 positive)	This thesis
6757	MATa, cdc13-1, slk19::KanMX6, leu2::GAL-SPO12::LEU2	W303 (RAD5 positive)	This thesis
6767	MATa, cdc13-1, cfi1::URA3	W303 (RAD5 positive)	This thesis
6769	MATa, cfi1::URA3	W303 (RAD5 positive)	This thesis
6890	MATa, slk19::KanMX6	JKM179	This thesis

7004	MATa, net1-1,	W303 (RAD5 positive)	This thesis
7005	MATa, cdc13-1, trp1::CDC14(TAB6-1)::TRP1	W303 (RAD5 positive)	This thesis
7008	MATa, trp1::CDC14(TAB6-1)::TRP1	W303 (RAD5 positive)	This thesis
7013	MATa, cdc13-1, slk19::KanMX6, cfi1::URA3	W303 (RAD5 positive)	This thesis
7016	MATa, slk19::KanMX6, cfi1::URA3	W303 (RAD5 positive)	This thesis
7019	MATa, cdc13-1, cdc5-ad, cfi1::URA3	W303 (RAD5 positive)	This thesis
7021	MATa, cdc5-ad, cfi1::URA3	W303 (RAD5 positive)	This thesis
8332	MATa, cdc13-1, bns1::KanMX6, spo12::HIS3, trp1::CDC14(TAB6-1)::TRP1	W303 (RAD5 positive)	This thesis
8336	MATa, cdc13-1, slk19::KanMX6, trp1::CDC14(TAB6-1)::TRP1	W303 (RAD5 positive)	This thesis
8350	MATa, cdc13-1, ura3::GAL-CDC5(dN70aa)-HA::URA3	W303 (RAD5 positive)	This thesis
8380	MATa, cdc13-1, bns1::KanMX, spo12::HIS3, ura3::GAL-CDC5(dN70aa)-HA::URA3	W303 (RAD5 positive)	This thesis
8386	MATa, cdc13-1, slk19::KanMX6, ura3::GAL-CDC5(dN70aa)-HA::URA3	W303 (RAD5 positive)	This thesis
8400	MATa, cfi1-AID::KanMX6, ura3::pADH1-OsTIR1-9MYC::URA3	W303 (RAD5 positive)	This thesis
8416	MATa, cdc13-1, net1-1, bns1::KanMX6, spo12::HIS3	W303 (RAD5 positive)	This thesis
8417	MATa, net1-1, bns1::KanMX6, spo12::HIS3	W303 (RAD5 positive)	This thesis
8420	MATa, cdc13-1, net1-1, slk19::KanMX6	W303 (RAD5 positive)	This thesis
8423	MATa, net1-1, slk19::KanMX6	W303 (RAD5 positive)	This thesis
8428	MATa, bns1::KanMX6, spo12::hphMX6	JKM179	This thesis
8431	MATa, slk19::KanMX6, cfi1-AID::KanMX6, ura3::OsTIR1-9MYC::URA3	W303 (RAD5 positive)	This thesis
8436	MATa, cdc5-ad	JKM179	Haber lab
8440	MATa, cdc13-1, bns1::KanMX6, spo12::HIS3, cfi1-AID::KanMX, ura3::OsTIR1-9MYC::URA3	W303 (RAD5 positive)	This thesis
8443	MATa, cdc13-1, slk19::KanMX6, cfi1-AID::KanMX6, ura3::OsTIR-9MYC::URA3	W303 (RAD5 positive)	This thesis
8452	MATa, trp1::CDC14(TAB6-1)::TRP1	JKM179	This thesis
8455	MATa, cdc5-ad, trp1::CDC14(TAB6-1)::TRP1	JKM179	This thesis
8456	MATa, slk19::KanMX6, trp1::CDC14(TAB6-1)::TRP1	JKM179	This thesis
8458	MATa, bns1::KanMX6, spo12::hphMX6, trp1::CDC14(TAB6-1)::TRP1	JKM179	This thesis
8484	MATa, ura3::pADH1-OsTIR1-9MYC::URA3, cfi1-AID::KanMX6	JKM179	This thesis

8491	MATa, sgs1::KanMX6	W303 (RAD5 positive)	This thesis
8496	hmlΔ::ADE1, mataΔ::hisG, hmrΔ::ADE1, leu2-cs, ade3::GAL::HO, ade1, lys5, ura3-52. Segment for repair 30kb from the HO cut site	YMV2	Haber lab
8497	hmlΔ::ADE1, mataΔ::hisG, hmrΔ::ADE1, leu2-cs, ade3::GAL::HO, ade1, lys5, ura3-52. Segment for repair 5kb from the HO cut site	YMV45	Haber lab
8525	MATa, cdc13-1, cdc5-ad	W303 (RAD5 positive)	This thesis
8534	MATa, cdc13-1, bns1::KanMX6, spo12::HIS3, trp1::CDC14(TAB6-1)::TRP1, HTB2-mCherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8592	MATa, srs2::KanMX6	W303 (RAD5 positive)	This thesis
8598	srs2::KanMX6	YMV2	This thesis
8601	srs2::KanMX6	YMV45	This thesis
8603	slk19::KanMX6	YMV2	This thesis
8606	slk19::KanMX6	YMV45	This thesis
8622	bns1::KanMX6, spo12::hphMX6	YMV2	This thesis
8625	bns1::KanMX6, spo12::hphMX6	YMV45	This thesis
8692	MATa, cdc13-1, slk19::KanMX6, trp1::CDC14(TAB6-1)::TRP1, HTB2-mCherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8698	MATa, cdc13-1, trp1::CDC14(TAB6-1)::TRP1, HTB2-mCherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8831	MATa, cdc13-1, cdc14-1, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8838	MATa, cdc13-1, cfi1-AID::KanMX6, ura3::OsTIR1-9MYC::URA3, trp1::GFP-TUB1::TRP1, HTB2-mCherry::HIS3	W303 (RAD5 positive)	This thesis
8841	MATa, cdc13-1, GAL-3HA-FOB1::KanMX6, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8842	MATa, cdc13-1, zds1::KanMX6, zds2::hphMX6, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8843	MATa, cdc13-1, mad1::hphMX6, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8845	MATa, cdc13-1, bns1::KanMX6, spo12::HIS3, mad1::hphMX6, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8846	MATa, cdc13-1, slk19::KanMX6, mad1::hphMX6, HTB2-Cherry::HIS3, ura3::pAFS125-TUB1p-GFPTUB1::URA3	W303 (RAD5 positive)	This thesis
8847	MATa, ura3::pRS306-mCherry-TUB1::URA3	JKM179	This thesis
8848	MATa, slk19::KanMX6, ura3::pRS306-mCherry-TUB1::URA3	JKM179	This thesis
8849	MATa, bns1::KanMX6, spo12::hphMX6, ura3::pRS306-mCherry-TUB1::URA3	JKM179	This thesis

8850	MATa, cdc5-ad, ura3::pRS306-mCherry-TUB1::URA3	JKM179	This thesis
8851	MATa, cdc13-1, net1-1, cdc5-ad	W303 (RAD5 positive)	This thesis
8854	MATa, net1-1, cdc5-ad	W303 (RAD5 positive)	This thesis
8861	MATa, cdc13-1, cfi1-AID::KanMX6, ura3::OsTIR1-9MYC::URA3	W303 (RAD5 positive)	This thesis
8863	MATalpha, bns1::KanMX6, spo12::HIS3, cfi1-AID::KanMX6, ura3::OsTIR1-9MYC::URA3	W303 (RAD5 positive)	This thesis

3. Results

A defect in the checkpoint adaptation response was suggested for FEAR mutants. Work from the Hartwell (Toczyski, Galgoczy and Hartwell, 1997) and Wang (Jin and Wang, 2006) laboratories implicated the activity of the FEAR network in checkpoint adaptation response. The Hartwell laboratory identified a Cdc5 allele (*cdc5-ad*) that is defective in the adaptation process, although it retains kinase activity. Consistently, this mutant does not show any obvious growth defects. However, when the mutant is exposed to an irreparable double strand break (DSB), it cannot switch off the DNA damage checkpoint and cells remain permanently arrested in metaphase (Toczyski, Galgoczy and Hartwell, 1997). Later on, Jin and Wang showed that combining the *cdc5-ad* allele with a mutant in the MEN pathway results in a synthetically lethal interaction (Jin and Wang, 2006). As this genetic interaction is characteristic of mutants of components of the FEAR network, the authors suggested that the *cdc5-ad* allele is defective in FEAR functions, and consistently, they showed that FEAR network mutants (e.g., *spo12* and *slk19*) are defective in the adaptation process following DNA damage elicited by dysfunctional telomeres.

The existence of a crosstalk between the machinery required for exit from mitosis and the DNA damage checkpoint is suggested by two observations: i) the DNA damage checkpoint acts to halt cell cycle progression by directly inhibiting the pathways that control the exit from mitosis, including the FEAR network (Liang and Wang, 2007); and ii) FEAR activity is required for checkpoint adaptation. While several studies aimed at elucidating the role of the DNA damage checkpoint in halting the cell cycle progression have been performed, how the cell cycle machinery influences the DNA damage checkpoint is still poorly understood. In particular, it is still unclear whether adaptation to DNA damage checkpoint is a consequence of an improper inactivation of the checkpoint or, alternatively, a consequence of an improper activation of the cell cycle machinery that, in turn, switches off the checkpoint signaling. Building on these observations, we wished to investigate the

role of the FEAR network in the adaptation response to gain further insights into the crosstalk between the cell cycle machinery and the DNA damage checkpoint.

3.1. Characterization of the adaptation phenotype of the FEAR components Cdc5, Spo12 and Slk19

The FEAR network comprises a number of proteins that together function to regulate the early release of the phosphatase Cdc14. These proteins include the separase Esp1, the separase-binding protein Slk19, the polo-like kinase Cdc5, the nucleolar protein Spo12, the replication fork block protein Fob1, the "zillion different screens" proteins Zds1 and Zds2, the CDK associated with the cyclins Clb1 and Clb2, and, finally, the phosphatase PP2A. Recently, new studies helped in defining the interconnections of FEAR components inside the network, which foresee a three-branched organization with Slk19, Spo12 and Cdc5 as representative members of each branch (Roccuzzo *et al.*, 2015).

To characterize the role of the FEAR network in the adaptation response, strains carrying mutations in Cdc5, Spo12 and Slk19 were employed. For both Spo12 and Slk19, we used null mutations, which result in viable cells with no obvious defects in mitotic exit apart from defects in FEAR-mediated Cdc14 release (Stegmeier, Visintin and Amon, 2002; Visintin, Stegmeier and Amon, 2003). Of note, given that the *SPO12* gene shares 55% similarity with the *BNS1* gene (Grether and Herskowitz, 1999), and Spo12 and Bns1 proteins performs partially overlapping FEAR functions (Visintin, Stegmeier and Amon, 2003), to avoid problems linked to redundant functions, in our analysis we employed double mutant strains carrying the two null mutations (*bns1Δ spo12Δ* double mutant). Regarding the polo kinase, for our studies, we took advantage of the *cdc5-ad* allele that allows to specifically investigate the functions of Cdc5 in the adaptation process. Indeed, unlike loss-of-function mutations, the kinase activity of Cdc5-ad is comparable with its *WT* counterpart (Charles *et al.*, 1998; Serrano and D'Amours, 2016). In unperturbed conditions, cells carrying the *cdc5-*

ad allele display no obvious defects in mitotic exit apart from defects in FEAR-mediated Cdc14 release (Busnelli, Dondi and Visintin, *manuscript in preparation*).

To probe the adaptation phenotype of the mutants of our interest, we employed several approaches, starting from the ones reported in the literature, most of them are based on assessment of the phenotype on the whole population, such as microcolony assays, budding analyses, visualization of the mitotic spindle in fixed cells, DNA content analyses by Flow Cytometry, and analyses of Rad53 phosphorylation (Pabla *et al.*, 2006). Regarding the preliminary link between the FEAR network and the adaptation response, it has been assessed solely by means of microcolony assays with no further characterization (Jin and Wang, 2006). The major limitation of studying adaptation by means of population-based approaches is represented by the process itself. Indeed, adaptation to DNA damage checkpoint *per se* is not a synchronous process, as cells belonging to the same sample exhibit different kinetics of adaptation or can eventually die as a consequence of a strong protracted checkpoint signaling (examples will be provided in the following sections). Taking into account this feature of the adaptation process, for our studies, we recognized the need of more informative techniques and, therefore, introduced single cell analyses by live cell imaging to integrate the finding of investigations at population level. As this approach allows a precise quantification of the rate and timing of adaptation at the single cell level and correlates both the rate and the timing with changes in protein amounts and localization (i.e., by tagging key proteins with fluorescent markers). One limitation of live imaging techniques is that they do not allow the evaluation of protein post-translational modifications (i.e., Rad53 phosphorylation status). For this reason, we also performed synchronous time courses for total protein analyses. As the original observation of a link between the FEAR network and the adaptation response was assessed by means of microcolony assays (Jin and Wang, 2006), we also probed the adaptation phenotype of

FEAR mutants using the same assay, which we exploited as a valuable tool for the quick assessment of the adaptation phenotype of a given strain.

3.1.1. Set-up of the experimental conditions

Before starting to investigate the role of the FEAR network in the adaptation response, we set the conditions for each experimental approach for the assessment of adaptation phenotypes. As the relationship between adaptation and the FEAR network was identified in *cdc13-1* mutant cells (Jin and Wang, 2006), we used the temperature sensitive allele of the *CDC13* gene, namely *cdc13-1*, as a source of DNA damage.

Cdc13 is a telomere-binding protein, and cells carrying the *cdc13-1* allele incubated at restrictive temperatures (>26°C) are subjected to degradation of the C-rich strand of telomeric DNA, resulting in extensive single-stranded DNA that reaches the subtelomeric DNA (Booth *et al.*, 2001). The single-stranded DNA is recognized as a site of DNA damage that triggers a robust Rad9-dependent DNA damage checkpoint response that arrest cells in metaphase (Garvik, Carson and Hartwell, 1995). Therefore, the *cdc13-1* allele can be used to trigger a checkpoint response comparable to the one induced by a proper DSB (Weinert and Hartwell, 1993; Garvik, Carson and Hartwell, 1995).

In all three experimental approaches, *cdc13-1* cells were synchronized in G_1 phase by the addition of alpha-factor pheromone to the medium, and released at the restrictive temperature of 32°C. As the strength of the checkpoint activation increases with increasing temperature, the temperature of the experiment was always carefully monitored over time. Indeed, we found that the adaptation rate of *cdc13-1* cells changed by varying the temperature of the experiment (data not shown), and that 32°C is the ideal temperature for *cdc13-1* cells to adapt to the DNA damage checkpoint.

In microcolony assays, single cells are placed on solid media and kept at the restrictive temperature for the *cdc13-1* allele for 24 hours, the number of cell bodies (separated cells or large buds) per colony is analyzed, hence cells that have bypassed the checkpoint-mediated arrest can be easily distinguished from those that remain arrested (Figure 3.1).

Figure 3.1 **Microcolony assay.** G_1-arrested *cdc13-1* cells (Ry6090) were micromanipulated in order to isolate single cells. The formation of microcolonies (at least three cellular bodies) was evaluated after 24 hours in the damage-inducing condition.

In synchronous time courses, G_1-arrested cells are released in liquid media and samples are collected for analyses of DNA and protein content. When the checkpoint-mediated metaphase arrest is achieved, as assessed by nuclear and cellular morphology, alpha-factor pheromone is added back to the culture to block adapted cells in the following G_1 phase. Indeed, adapted cells would otherwise arrest in metaphase of the subsequent cell cycle, hence they would be confused with the cells that have not adapted at all. Therefore, cells that bypass the checkpoint-mediated arrest have a DNA content equal to 1C (arrested in G_1), while those that remain arrested have a DNA content equal to 2C (arrested in metaphase) (Figure 3.2).

In single cells experiments, G_1-arrested cells are released in a microfluidic chamber for live imaging. As shown in Movie 3.1 and depicted in Figure 3.3, we can follow and quantify every step of the process. Approximately 3 hours after the release, cells arrest in

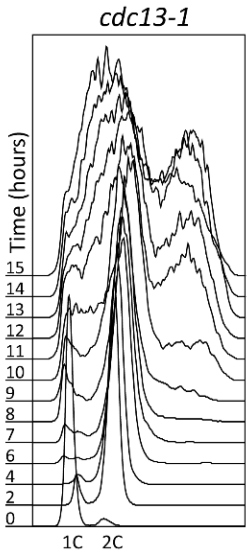

Figure 3.2 **Population analyses**. G_1-arrested *cdc13-1* cells (Ry6090) were synchronously released in a damage-inducing condition. Samples for DNA content analyses were taken every 30 minutes and analyzed by FACS. Once the cells had reached the checkpoint-mediated arrest, alpha-factor pheromone was added back to the culture to arrest adapting cells in G_1 phase. Therefore, at the end of the experiment, cells with a DNA content equal to 1C were considered to have adapted, while cells with a 2C DNA content were considered to be arrested in metaphase. Of note: cells that remain arrested for a long time show larger 1C and 2C peaks. The same larger peaks were observed in cells arrested as a consequence of a single irreparable DSB (Lee *et al.*, 1998). It has been reported that this enlargement is a consequence of artifacts of FACS scanning due to the big size displayed by cells arrested for many hours (Vaze *et al.*, 2002).

metaphase with a dumbbell shape, namely when mother and daughter cells have about the same size. Eventually, a portion of dumbbell cells re-buds. The bud, which emerges at the beginning of S phase, is an indication that the original cell has resumed the cell cycle, thus adapted, and that the daughters have entered the following S-phase. To emphasize that adaptation is not a synchronous process, three categories of cells can be observed within the same population: i) the fraction of cells that re-buds (pink line); ii) the fraction of cells that remains arrested as dumbbells (gray line); and finally, iii) the fraction of cells that eventually dies (green line). On average, cells adapt after about 10 hours from the release, however, the window of time in which cells adapt lasts about 5 hours (beginning at about 8 hours and ending at about 13 hours) (Figure 3.3 B). We noticed that a fraction of adapted cells eventually dies, but for our quantifications, we only considered the first event.

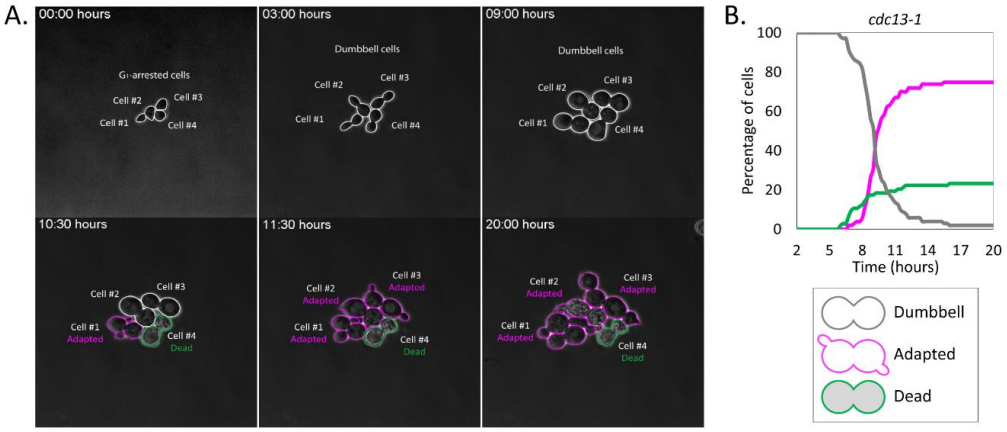

Figure 3.3 **Single cell analyses.** G_1-arrested *cdc13-1* cells (Ry6090) were released in a microfluidic device for cell imaging, and timeframes were acquired every 15 minutes for 20 hours. **A.** Representative timeframes of Movie 3.1 are shown. In each timeframe, we count dumbbell cells (highlighted in white), re-budded cells, our read-out for adaptation (highlighted in pink), and dead cells (highlighted in green). **B.** Quantification of single cell experiments. The percentage of dumbbell cells (gray line), adapted cells (pink line), and dead cells (green line) were scored for each timeframe. A number n ≥ 100 cells was counted.

With these experimental setups, we tested the contribution of the FEAR components to the adaptation process in two damaged conditions, *cdc13-1*-induced damage and DSB lesion (*GAL-HO* model system).

3.1.2. FEAR network components Cdc5, Spo12 and Slk19 are required for checkpoint adaptation to DSB-like lesions

To probe the adaptation defective phenotype of FEAR mutants in *cdc13-1*-induced damage condition, *cdc13-1*, *cdc13-1 cdc5-ad*, *cdc13-1 bns1Δ spo12Δ* (henceforth indicated as *cdc13-1 spo12Δ* mutant), and *cdc13-1 slk19Δ* were examined by microcolony assays (Figure 3.4 A), population analyses (Figure 3.4 B) and single cell experiments (Figure 3.4 C). In all of the three assays, we found that, while *cdc13-1* cells adapt to the checkpoint arrest, all individual FEAR mutants tested are impaired in the adaptation process (mean values: ~73% of *cdc13-1* cells resume the cell cycle compared with 0% of *cdc13-1 cdc5-ad* cells, ~13% of *cdc13-1 spo12Δ* and ~6% of *cdc13-1 slk19Δ* cells). Taken together, in agreement with published results, our data indicate that individual branches of the FEAR network are required for checkpoint adaptation, at least in the absence of the telomere capping protein Cdc13.

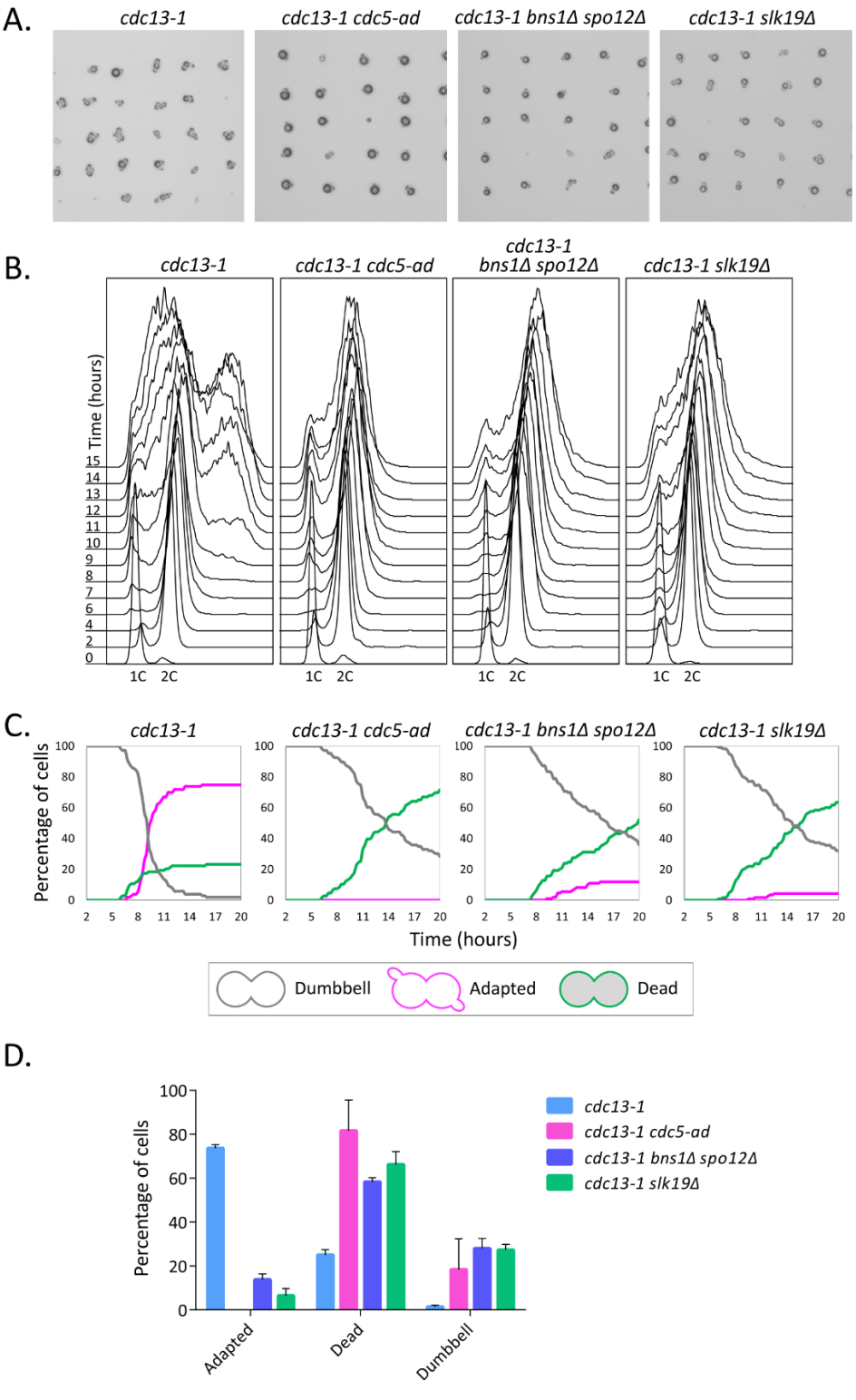

Figure 3.4 **FEAR mutants are defective in adapting to DNA damage checkpoint triggered by unprotected telomeres**. *cdc13-1* (Ry6090), *cdc13-1 cdc5-ad* (Ry8525), *cdc13-1 bns1Δ spo12Δ* (Ry6378), and *cdc13-1 slk19Δ* (Ry6396) cells were arrested in G_1 phase by the addition of alpha-factor pheromone to the culture and analyzed by three approaches. **A.** G_1-arrested cells were micromanipulated into grids on YEPD plates, and incubated at 32°C. 24 hours later, images were acquired using a 10X objective. **B.** Cells were synchronously released at the restrictive temperature of 32°C in YEPD medium. At the indicated time points, cells were collected to determine the DNA content by FACS analysis. **C.** Cells were released at the restrictive temperature of 32°C in YEPD medium in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes for 20 hours. The percentage of dumbbell cells (gray line), adapted cells (pink line), and dead cells (green line) were scored for each timeframe. **D.** For the last time point (20 hours), means and standard deviations deriving from three independent experiments are shown. For each strain, a number n ≥ 100 cells was counted.

3.1.3. FEAR network components Cdc5, Spo12 and Slk19 are required for checkpoint adaptation to a single DSB lesion

Since FEAR-released Cdc14 has been implicated in the resolution of late-segregating chromosomal regions, namely rDNA and telomeres (D'Amours, Stegmeier and Amon, 2004; Machín *et al.*, 2006; Clemente-Blanco *et al.*, 2009, 2011), to exclude the possibility that the adaptation defect highlighted in FEAR mutants in the *cdc13-1* background is the mere consequence of defects in telomere segregation, we decided to assess for FEAR contribution to the adaptation process also in different sources of DSBs.

The observation that the *cdc5-ad* allele of Cdc5 is required for adaptation to the checkpoint triggered by a single DSB induced by overexpressing the endogenous site-specific Ho (HOmothallic switching) endonuclease (Toczyski, Galgoczy and Hartwell, 1997) guided our choice towards this source. To study adaptation to DNA damage checkpoint, Haber and colleagues decided to exploit the Ho endonuclease to induce a single irreparable DSB inside the genome (Lee *et al.*, 1998). To this aim, they engineered a strain with unique features (JKM179 background). More specifically: i) the endonuclease expression is under the control of the inducible galactose promoter (*GAL-HO*); and ii) the homologous loci of the Ho cut site were deleted to prevent homologous recombination, hence the repair of the DSB induced by the Ho endonuclease (Lee *et al.*, 1998). To assess whether the FEAR network activity is required for adaptation in general, we decided to take advantage of this system for our analyses. To this aim, *GAL-HO, GAL-HO cdc5-ad*, *GAL-HO bns1Δ spo12Δ* (henceforth indicated as *GAL-HO spo12Δ* mutant), and *GAL-HO slk19Δ* mutant cells were tested by cell grids (Figure 3.5 A), population analyses (Figure 3.5 B) and single cell experiments (Figure 3.5 C). Similarly to the *cdc13-1* cells, we found that *GAL-HO* cells are able to adapt upon overexpression of *HO* (mean value: about 76%), whereas cells carrying mutations in individual FEAR components were impaired in the process, with *cdc5-ad* cells

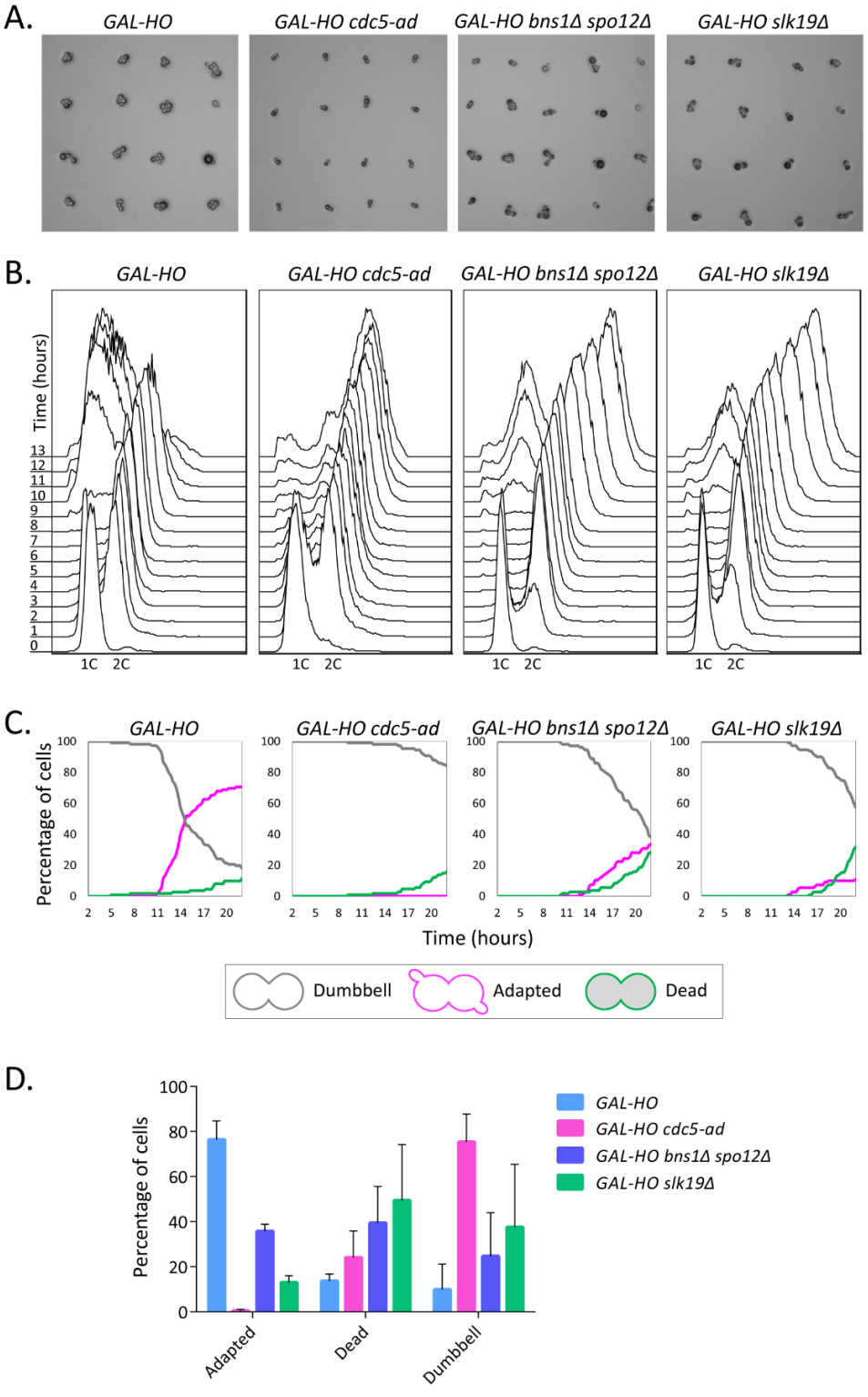

Figure 3.5 **FEAR mutants are defective in the adaptation to DNA damage checkpoint triggered by a single proper DSB**. *GAL-HO* (Ry2118), *GAL-HO cdc5-ad* (Ry8436), *GAL-HO bns1Δ spo12Δ* (Ry8428), and *GAL-HO slk19Δ* (Ry6890) cells were arrested in G_1 phase by the addition of alpha-factor pheromone to the culture and analyzed by three approaches. **A.** Cells were micromanipulated into grids on YEPR/G plates. 24 hours later, images were acquired using a 10X objective. **B.** Cells were synchronously released in YEPR/G medium to induce overexpression of *HO*. At the indicated time points, cells were collected to determine the DNA content by FACS analysis. **C.** Cells were synchronously released in YEPR/G medium in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes for 22 hours. The percentage of dumbbell cells (gray line), adapted cells (pink line) and dead cells (green line) were scored for each timeframe. **D.** For the last time point (23 hours), means and standard deviations deriving from three independent experiments are shown. For each strain, a number n ≥ 100 cells was counted.

exhibiting the strongest phenotype (mean values: ~0.45% of adapting cells *versus* ~35% and 13% in *GAL-HO spo12Δ* and *GAL-HO slk19Δ* mutant cells, respectively). Taken together, our data suggest that the FEAR network is required for adaptation to the DNA damage checkpoint triggered by both proper DSBs and DSB-like lesions.

3.1.4. FEAR network components Cdc5, Spo12 and Slk19 are dispensable for checkpoint recovery

Having assessed for the requirement of FEAR components in the adaptation process, to test whether this requirement is specific for adaptation or general for cell cycle resumption after the inactivation of the DNA damage checkpoint, we tested the contribution of FEAR to the recovery process.

When a cell suffers DNA damage and activates the DNA damage checkpoint, several outcomes are possible: if the damage cannot be repaired, the cell can either adapt or die; alternatively, if the damage is successfully repaired, the cell can recover from the checkpoint-mediated arrest, therefore resuming its cell cycle (Sandell and Zakian, 1993; Toczyski, Galgoczy and Hartwell, 1997; Lee *et al.*, 1998). Since the activity of the FEAR network is dispensable for cell cycle progression in unperturbed conditions (Stegmeier, Visintin and Amon, 2002) but is required for the adaptation response ((Jin and Wang, 2006) and this thesis), we wondered whether and to what extent the FEAR network contributes to checkpoint recovery. To address this question, we performed a recovery assay using UV light as a source of damage (Figure 3.6). UV light harms the DNA both directly, via photochemical reactions, and indirectly, via production of reactive oxygen species (ROS) (reviewed in (Roy, 2017)). UV light induces the formation of covalent linkages in the DNA structure, commonly known as UV photoproducts. Two common UV photoproducts are cyclobutane pyrimidine dimers and (6-4) photoproducts. Pyrimidine dimers are premutagenic lesions that introduce local conformational changes in the DNA structure.

ROS are highly reactive compounds, able to react with the DNA molecule and potentially give rise to multiple forms of oxidative damage (reviewed in (Jena 2012)), including 8-oxoG, modification of bases, intra- and interstrand crosslinks, covalent protein-DNA crosslinks, and DNA strand breaks. DNA adducts, intra- and interstrand crosslinks, and protein-DNA crosslinks are aberrant DNA structures that cause replication arrest, leading to DNA strand breaks (Swift & Golsteyn 2014).

For the recovery assay, *WT*, *cdc5-ad*, *bns1Δ spo12Δ* (henceforth indicated as *spo12Δ* mutant), and *slk19Δ* mutant cells were plated as serial dilutions and exposed to a single pulse of a range of UV light doses (ranging from 0 to 125 J/m^2). As a positive control, *srs2Δ* and *sgs1Δ* cells were employed. Srs2 is a helicase and anti-recombinase factor that counteracts Rad51 nucleoprotein filament formation during DSB repair through HR (Bernstein *et al.*, 2011; Godin *et al.*, 2013). Deletion of the *SRS2* gene causes both recovery and adaptation defects following DSB induction (Vaze *et al.*, 2002). Sgs1 is a member of the RecQ family nucleolar DNA helicases involved in DNA repair and required for checkpoint recovery following DSB induction (Watt *et al.*, 1995). We found that the tested FEAR mutants grew as *WT* cells, suggesting that they are proficient in recovery and exhibit the same cell cycle requirements as cells grown in unperturbed conditions.

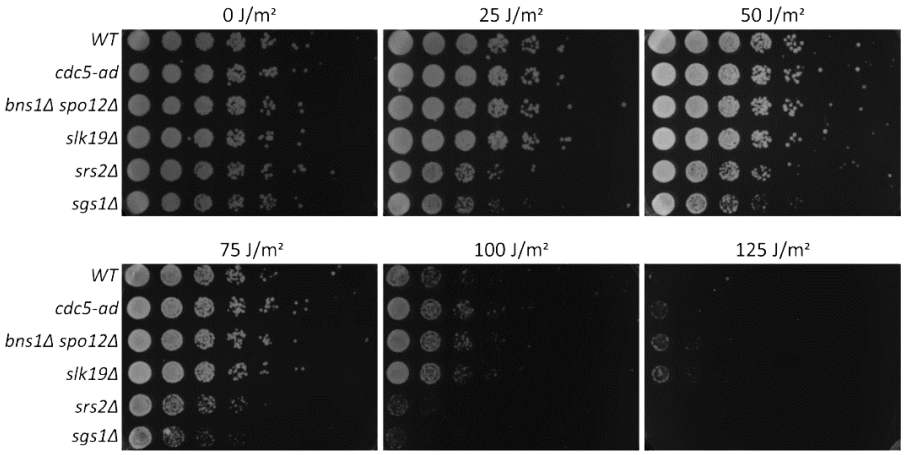

Figure 3.6 **FEAR mutants are proficient in checkpoint recovery following UV-induced DNA damage.** For the *WT* (Ry2281), *cdc5-ad* (Ry6092), *spo12Δ* (Ry6387), *slk19Δ* (Ry6399), *srs2Δ* (Ry8592), and *sgs1Δ* (Ry8491) strains, serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto YEPD plates. The plates were then exposed to a single pulse of UV light, ranging from 0 J/m^2 to 120 J/m^2 and incubated at 23°C. Images were taken after 48 hours of incubation.

In order to directly compare checkpoint adaptation with recovery using the same source of DSB, we also tested the contribution of the FEAR network to checkpoint recovery using the Ho endonuclease. To study checkpoint recovery, Haber and colleagues exploited the Ho endonuclease to induce a single reparable DSB inside the genome (Vaze *et al.*, 2002). The Ho mediates the mating type interconversion, namely a gene conversion event at the *MAT* locus (located on chromosome III), where the *MATa* allele is replaced by the *MATalpha* or *vice versa*. The conversion is achieved through the introduction of a DSB at the *MATa/alpha* locus, followed by repair of the DSB through HR with one of the two donor sequences, HML_{alpha} or HMR_{a}. These heterochromatic regions are located near the left and the right telomere arm, respectively, of Chromosome III, and are kept transcriptionally silenced. It was previously demonstrated that Ho-induced gene conversion repair of DSB lasts about 1 hour once a DSB has been induced, which is a too short time period and does not even allow the activation of the Rad53 kinase (Pellicioli *et al.*, 2001). Therefore, to study checkpoint recovery, it is not possible to exploit the natural homothallic switching machinery, but it requires a system in which the DNA resection would last enough before annealing with the homologous donor sequence, therefore the DSB would remain unrepaired for many hours. As previously estimated, a rate of resection of 4 kb/hour (Fishman-Lobell, Rudin and Haber, 1992) is required to induce a DSB that lasts long enough to trigger the DNA damage checkpoint, which necessitates a homologous sequence situated at 25 kb from the cut site in order to accumulate up to 50 kb single-stranded DNA, an event required for DNA damage checkpoint activation. To this aim, the YMV80 background was engineered with unique features: i) the endonuclease expression is under the control of the inducible galactose promoter (*GAL-HO*); ii) the Ho endonuclease cleavage sites (*HO* cs) at *MAT*, as well as the *HML* and *HMR* loci were deleted; iii) an *HO* cut site was inserted inside the *LEU2* open reading frame (*leu2*::HO cs); and, finally, iv) a 1.3 kb fragment

of the 3' end of *LEU2* gene was inserted in the *HIS4* locus, approximately 25 kb away from the *leu2*:: *HO* cs (Vaze *et al.*, 2002). Based on this set up, Haber and colleagues also created strains in which the homologous donor sequence is situated either 30 kb (YMV2) or 5 kb (YMV45) away from the *leu2*:: *HO* cs (Figure 3.7 A), the latter serving as control in which the repair occurs more rapidly and the checkpoint is activated to varying extents, according to a previous finding in which the checkpoint was not activated during the one hour required for HO-induced mating-type gene switching (Pellicioli *et al.*, 1999).

To assess whether the activity of the FEAR network is required for checkpoint recovery, we decided to take advantage of the Haber's system for our analyses. To this aim, *GAL-HO, GAL-HO bns1Δ spo12Δ,* and *GAL-HO slk19Δ* mutant cells in both the YMV2 (Figure 3.7 B) and the YMV45 backgrounds (Figure 3.7 C) were plated as serial dilutions on YEPR plates supplemented with 2% galactose to induce the Ho expression, and in parallel on YEPD plates to compare the dilution quality. As positive control, *srs2Δ* cells were employed, which were found to be recovery-defective in both the YMV2 and the YMV45 backgrounds (Vaze *et al.*, 2002). In agreement with the results obtained using the UV recovery assay, we found that the tested FEAR mutants grew as *WT* cells, suggesting that they are proficient in recovery and exhibit the same cell cycle requirements as cells grown in unperturbed conditions. The recovery capabilities of *cdc5-ad* mutant cells have not been tested yet. However, data in the literature indicate that *cdc5-ad* mutant cells in the YMV80 background are proficient in checkpoint recovery (Vaze *et al.*, 2002).

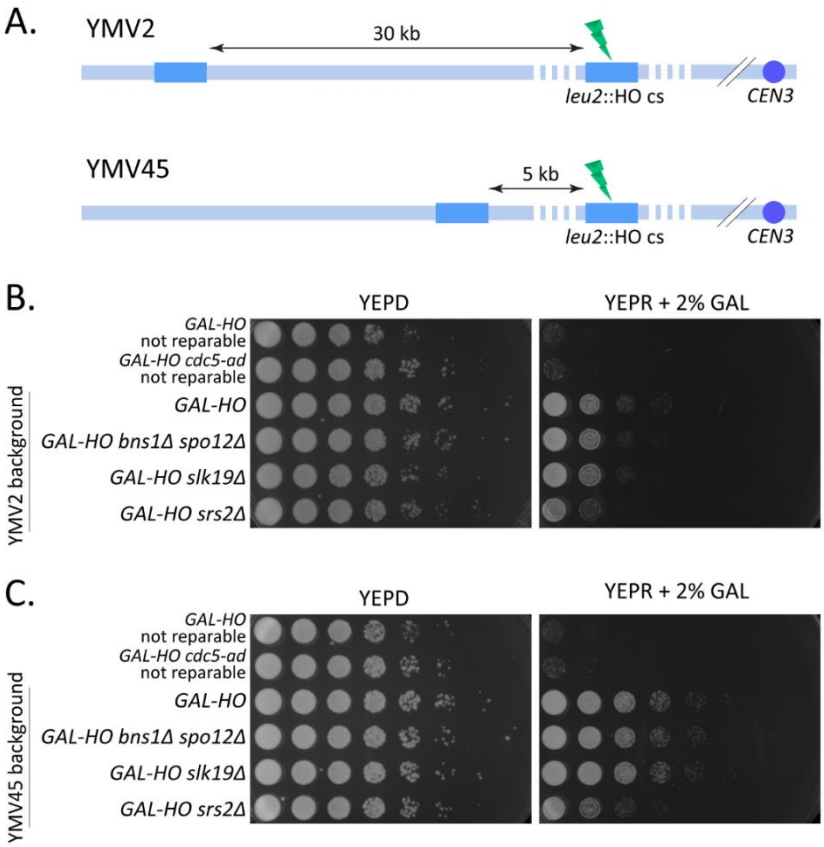

Figure 3.7 **FEAR mutants are proficient in checkpoint recovery following Ho-induced DSB. A.** A graphical representation of YMV2 and YMV45 strain features are shown. The Ho cut site (HO cs) was inserted in the *leu2* locus (*leu2*::HO cs). **B.** and **C.** For *GAL-HO* (irreparable, Ry2118), *GAL-HO cdc5-ad* (irreparable, Ry8436), (B) *GAL-HO* (Ry8496), *GAL-HO spo12Δ* (Ry8622), *GAL-HO slk19Δ* (Ry8603), and *GAL-HO srs2Δ* (Ry8598) strains in the YMV2 background, or (C) *GAL-HO* (Ry8497), *GAL-HO spo12Δ* (Ry8625), *GAL-HO slk19Δ* (Ry8606), and *GAL-HO srs2Δ* (Ry8601) strains in the YMV45 background with serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto YEPR/G plates and YEPD plates and incubated at 23°C. Images were taken after 48 hours of incubation.

The different requirement of individual components in unperturbed cell cycle and checkpoint recovery *versus* a persistent DNA damage condition highlight checkpoint adaptation as a cell cycle with special requirements. Intrigued by these observations, we wondered how the single FEAR components contribute to the adaptation response, and the role of the final effector of FEAR activation, the phosphatase Cdc14, in the adaptation response.

3.2. Dissecting the contribution of single FEAR components and Cdc14 in checkpoint adaptation

3.2.1. FEAR network components Slk19 and Cdc5 play specific roles in the adaptation process

Since the activation of the FEAR network results in a transient release of the phosphatase Cdc14, different hypothesis on the contribution of FEAR to adaptation can be envisioned. It could be mediated: i) solely by Cdc14; ii) by a critical threshold of Cdc14 activity together with the involvement of individual FEAR components; and iii) by additional functions of individual FEAR components independent from Cdc14. Of course, the three possibilities are not mutually exclusive. To discriminate among these possibilities, we started by investigating the role of individual FEAR components in the adaptation response. To this aim, we performed epistasis analyses among FEAR network components. Epistasis analysis represents a genetic tool and is useful for the identification of molecular players involved in a cellular process, and for the genetic dissection of a molecular pathway. With this tool, we wished to genetically dissect the role of individual FEAR components in the adaptation pathway and to highlight possible redundancy among these players. For this analysis, we combined the overexpression of one component with the impairment of another and evaluated the resulting phenotype. If two components have redundant functions, we expect that the overexpression of one of them is able to compensate for the lack of the other one. We assessed the consequences of overexpressing FEAR components in the *cdc13-1* background by single cell analyses. In this experimental setup, cells are synchronously released from a G_1 arrest at the restrictive temperature, and the overexpression is induced 3 hours after the release to avoid interference with cell cycle progression before the checkpoint-mediated arrest is achieved. Since we overexpress FEAR components by means of the galactose promoter, the epistasis analyses cannot be

performed in the *HO* background, where the induction of the Ho endonuclease is mediated by the same promoter.

First of all, we tested if and how high levels of individual FEAR components *per se* were impacting on the adaptation phenotype of the *cdc13-1* single mutant. We found that overexpression of *SPO12* did not affect the adaptation response of *cdc13-1* cells (Figure 3.8 A), while high levels of Slk19 or Cdc5 resulted in different adaptation responses. More specifically, overexpression of *SLK19* (modulated by addition of different amounts of galactose ranging from 0.05-2%) completely abolished the adaptation response in all tested conditions, thus, overexpression of *SLK19* cannot be used for our purposes (Figure 3.8 B). Whether this finding underlines that the adaptation process is sensitive for Slk19 protein quantities, or whether the overexpression of *SLK19* alters mitotic spindle dynamics (in agreement with what has been observed in unperturbed conditions (Visintin, Stegmeier and Amon, 2003)), negatively impacting on adaptation, remains a matter of investigation. In agreement with data in the literature (Donnianni *et al.*, 2010; Vidanes *et al.*, 2010), overexpression of *CDC5* anticipated the timing of adaptation initiation in *cdc13-1* cells (from ~11 hours to ~8 hours), thus, overexpression of *CDC5* can be used in our analyses to assess whether FEAR components play redundant functions in the adaptation response (Figure 3.8 C).

We then analyzed the consequences of high levels of Spo12 and Cdc5 in FEAR mutants. We found that the overexpression of *SPO12* did not rescue the adaptation defect either in the *cdc13-1 cdc5-ad* nor in the *cdc13-1 slk19Δ* mutant cells, therefore suggesting that the function of *SPO12* is not redundant (Figure 3.9).

On the other hand, the overexpression of *CDC5* anticipated the timing of adaptation initiation in *spo12* and *slk19* mutant cells (from ~11 hours to ~7 hours). Moreover, the

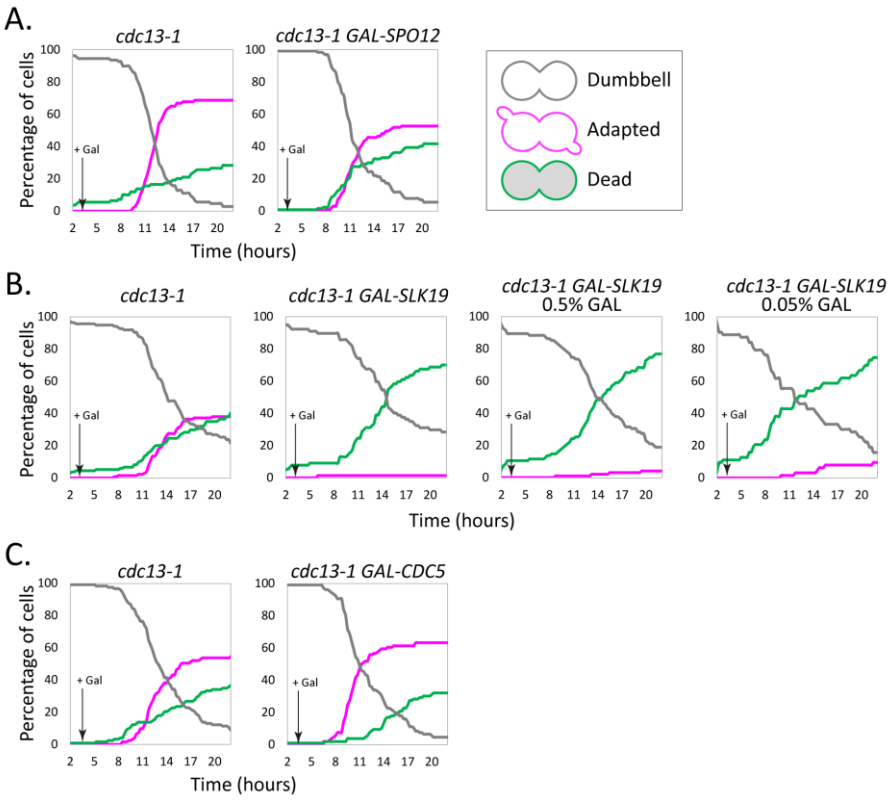

Figure 3.8 **Overexpression of FEAR components causes different effects on checkpoint adaptation. A.**, **B.** and **C.** *cdc13-1* (Ry6090), *cdc13-1 GAL-SPO12* (Ry6673), *cdc13-1 GAL-SLK19* (Ry6659), and *cdc13-1 GAL-CDC5* (Ry8350) strains arrested in G_1 phase were released in YEPR medium at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. After 3 hours, 2% galactose was added to the culture to induce the galactose promoter, unless otherwise specified. Timeframes were acquired every 15 minutes. The percentages of adapted and dead cells were scored for each timeframe. For each condition, a number n ≥ 100 cells was counted.

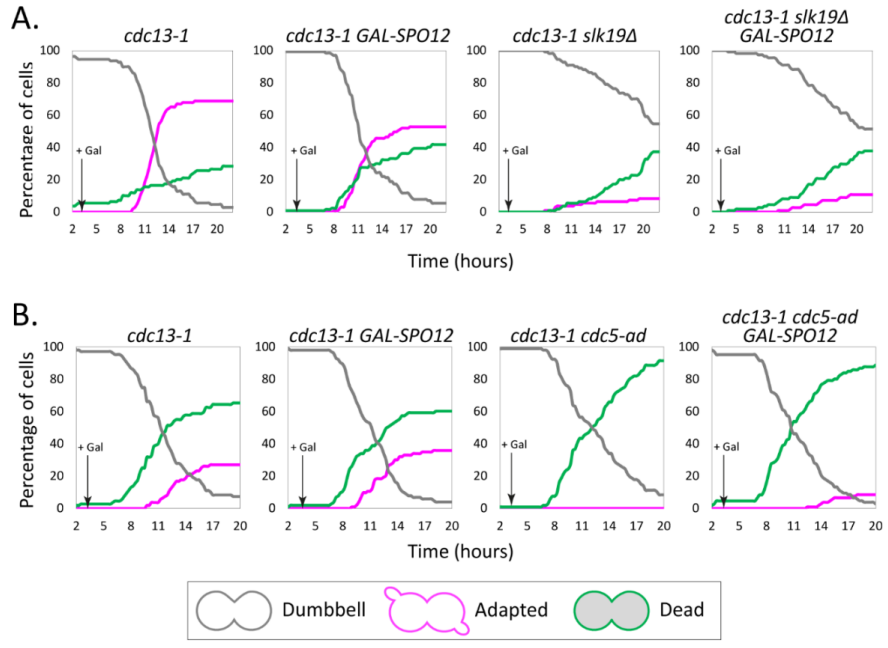

Figure 3.9 **Overexpression of *SPO12* does not alter the adaptation defect of *cdc13-1 cdc5-ad* and *cdc13-1 slk19* cells. A.** and **B.** *cdc13-1* (Ry6090), *cdc13-1 GAL-SPO12* (Ry6673), *cdc13-1 slk19Δ* (Ry6396), *cdc13-1 slk19Δ GAL-SPO12* (Ry6757), *cdc13-1 cdc5-ad* (Ry8525), and *cdc13-1 cdc5-ad GAL-SPO12* (Ry6676) strains arrested in G_1 phase were released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. After 3 hours, 2% galactose was added to the culture to induce the galactose promoter. Timeframes were acquired every 15 minutes. The percentages of adapted and dead cells were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

overexpression of *CDC5* partially rescued the adaptation defect of the other two FEAR components, namely *spo12* and *slk19* mutants (from 10% to 20-30%) (Figure 3.10). Taken together, the observations that: i) in an unperturbed cell cycle, overexpression of *SPO12* is able to rescue for the Cdc14 release defect of *slk19* mutant cells and partially rescue for the defect of *cdc5* mutant cells (Visintin, Stegmeier and Amon, 2003), but it does not rescue the adaptation defect of the same mutants; ii) cells overexpressing *SLK19* are able to exit from mitosis in unperturbed conditions (Visintin, Stegmeier and Amon, 2003), but not in persistent DNA damage conditions; and iii) cells overexpressing *CDC5* show an enhanced adaptation response, also in the absence of other FEAR mutants, suggest that adaptation is not driven solely by the release of Cdc14, and that a different molecular circuitry is required for the exit from mitosis in persistent damage conditions, where Slk19 and Cdc5 play additional specific functions in the process.

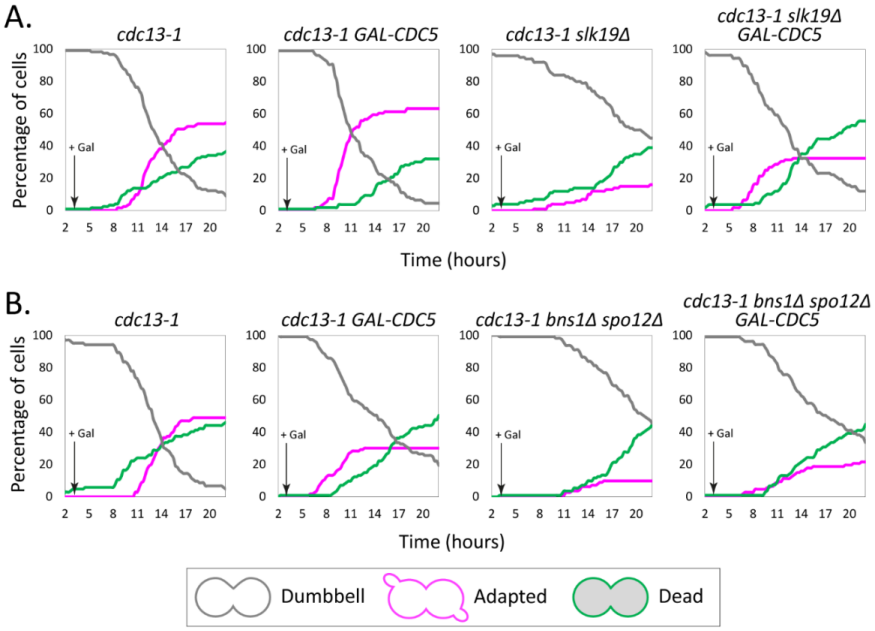

Figure 3.10 **Overexpression of *CDC5* increases checkpoint adaptation in *cdc13-1 spo12* and *cdc13-1 slk19* cells. A.**, and **B.** *cdc13-1* (Ry6090), *cdc13-1 GAL-CDC5* (Ry8350), *cdc13-1 slk19Δ* (Ry6396), *cdc13-1 slk19Δ GAL-CDC5* (Ry8386), *cdc13-1 spo12Δ* (Ry6378), and *cdc13-1 spo12Δ GAL-CDC5* (Ry8380) strains arrested in G_1 phase were released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. After 3 hours, 2% galactose was added to the culture to induce the galactose promoter. Timeframes were acquired every 15 minutes. The percentages of adapted and dead cells were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

While it is already known that Cdc5 contributes to the overcome of the checkpoint signaling (Donnianni *et al.*, 2010; Vidanes *et al.*, 2010), the possible adaptation-specific functions for Slk19 remain matter of investigation.

3.2.2. The release of Cdc14 only partially compensates for the adaptation defect of FEAR mutant cells

In order to understand the contribution of the phosphatase Cdc14 in the adaptation response, we assessed whether FEAR-independent Cdc14 activation could rescue the adaptation defect observed in the mutants of the FEAR network.

Since following the activation of the FEAR network only a partial and transient release of Cdc14 is observed (Stegmeier, Visintin and Amon, 2002), to determine the contribution of FEAR-released Cdc14 to the adaptation phenotype we modulated the activity and the localization of Cdc14 to mimic FEAR-mediated activation of the phosphatase. For these reasons, in order to achieve Cdc14 activation, we took advantage of several strategies (Figure 3.11): we employed a) a dominant allele of Cdc14 (*TAB6-1*) that shows a reduced affinity for its inhibitor Cfi1 and has a reduced phosphatase activity (75% compared to its wild type counterpart) (Shou *et al.*, 2001); b) a thermosensitive allele of Cfi1 (*net1-1*) that results in a nearly-wild type localization of Cdc14 (W Shou *et al.*, 1999), c) an allele of Cfi1 conditional for its degradation (*cfi1-AID*), or d) the deletion of Cfi1 (*cfi1Δ*), that results in a nuclear and, to some extent, cytoplasmic localization of Cdc14 throughout the cell cycle (Visintin, Hwang and Amon, 1999; W Shou *et al.*, 1999).

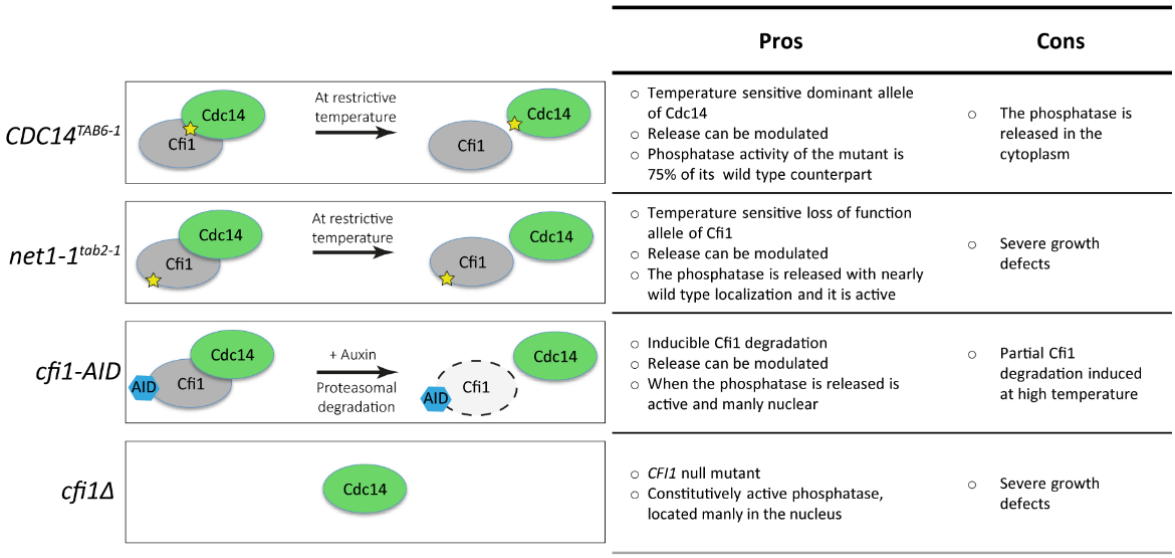

Figure 3.11 **Strategies used to induce a FEAR-independent Cdc14 release.** With the aim of mimicking the partial and nuclear FEAR-release of Cdc14, several mutations were employed. Pros and cons for each mutant are listed.

The Auxin-inducible degradation (AID) system allows for the rapid and reversible proteolysis of proteins of interest, previously fused with the AID tag, upon addition of auxin (naphthaleneacetic acid, NAA, synthetic auxin) to the culture medium (Nishimura *et al.*, 2009). To ensure that the *cfi1-AID* construct worked properly, the kinetics of Cfi1 degradation upon auxin addition and Cdc14 localization were assessed. For degradation kinetics, we performed a synchronous time course at two different temperatures, 23°C (permissive temperature for *cdc13-1*) and 32°C (restrictive temperature for *cdc13-1*), and we compared treated (1mM NAA added at the G_1 release) and untreated conditions (Figure 3.12). At the permissive temperature, we found that already 30 minutes after the addition of auxin Cfi1 protein began to be degraded and completely disappeared 60 minutes after the treatment. At the restrictive temperature, we found that Cfi1 protein began to be degraded 30 minutes after the G_1 release, also in untreated conditions, although Cfi1 was still detectable 120 minutes after the release. These findings suggest that the AID tag behaves as a partial thermosensitive allele.

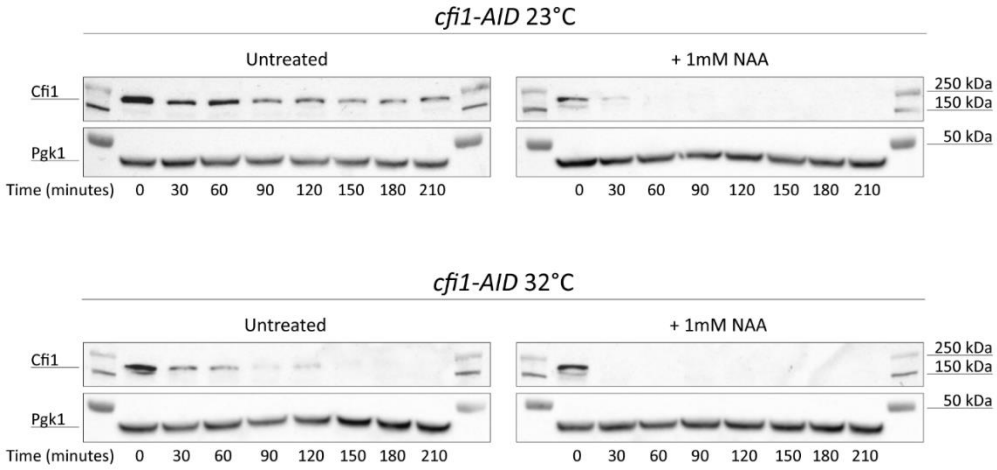

Figure 3.12 **The *cfi1-AID* construct causes Cfi1 protein degradation upon auxin addition and at high temperatures.** *cfi1-AID* (Ry8400) strain arrested in G_1 phase was synchronously released at both 23°C and 32°C in YEPD growth medium in the absence/presence of 1mM NAA. At the indicated time points, cells were collected and Cfi1 protein levels were probed by Western blot analyses with an antibody against the AID tag. Pgk1 protein was used as loading control.

Consistent with the degradation kinetics of Cfi1 were also the kinetics of Cdc14 release (Figure 3.13). Indeed, when we compared the kinetics of Cdc14 release in *wild type*, *cfi1Δ* (where Cdc14 is always released in the nucleus) and *cfi1-AID*, we found that Cdc14 was released in *cfi1-AID* cells upon the addition of 1mM NAA.

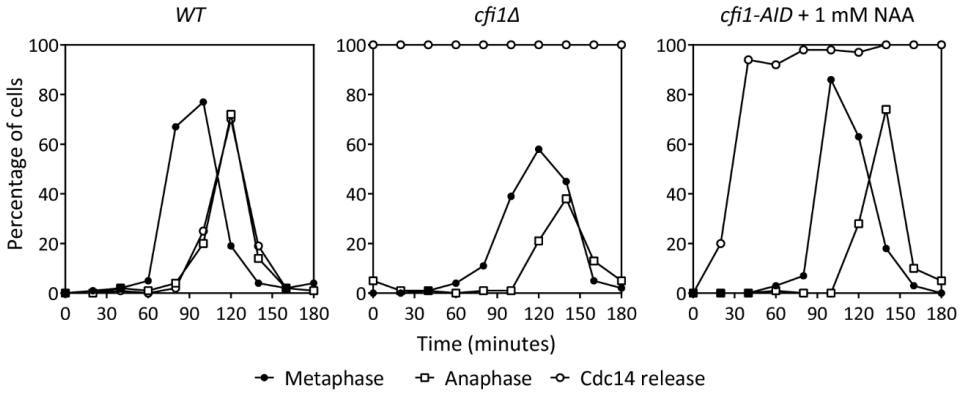

Figure 3.13 **The *cfi1-AID* construct causes Cdc14 release upon auxin addition.** *WT* (Ry2281), *cfi1Δ* (Ry6769), and *cfi1-AID* (Ry8400) strains arrested in G_1 phase were synchronously released in YEPD medium. For the *cfi1-AID* strain, 1mM NAA was added to the growth medium at the release. At the indicated time points, cells were collected and the percentages of cells with metaphase spindles (closed circles), anaphase spindles (open squares), and cells that had released Cdc14 (open circles) were determined.

We then combined *TAB6-1*, *net1-1*, *cfi1-AID*, and *cfi1Δ* alleles with FEAR mutants in the *cdc13-1* background, and screened for genetic interactions by a serial dilution assay. To modulate the release of Cdc14, for the thermosensitive *TAB6-1*, *net1-1*, *cfi1Δ* (partially

thermosensitive), and *cfi1-AID* (partially thermosensitive) alleles, we modulated the temperature from 23°C to 28°C (from permissive to semi-restrictive temperature for the *cdc13-1* allele). We found that *cfi1Δ* (Figure 3.14) and *net1-1* (Figure 3.15) mutant cells show a severe growth defect already at the permissive temperature, in agreement with data in the literature (Shou *et al.*, 2001). Differently, in *TAB6-1* (Figure 3.16) and *cfi1-AID* (Figure 3.17) strains, we found a semi-permissive condition where genetic interactions could be highlighted. We found that the moderate activation of Cdc14 (achieved with both *TAB6-1* and *cfi1-AID* alleles) ameliorated the growth rates of *cdc13-1 spo12Δ* cells, but it had no effect on *cdc13-1* or *cdc13-1 slk19Δ* cells, thus suggesting that Cdc14 can compensate for the lack of Spo12 in these conditions. Whether and how these results are linked to adaptation is unclear. However, since *cfi1Δ* and *net1-1* mutant cells showed a severe growth defect *per se*, and since in our experimental setup we assess adaptation as cell division, we decided to investigate the effect of Cdc14 release in the adaptation response of *TAB6-1* and *cfi1-AID* mutant cells.

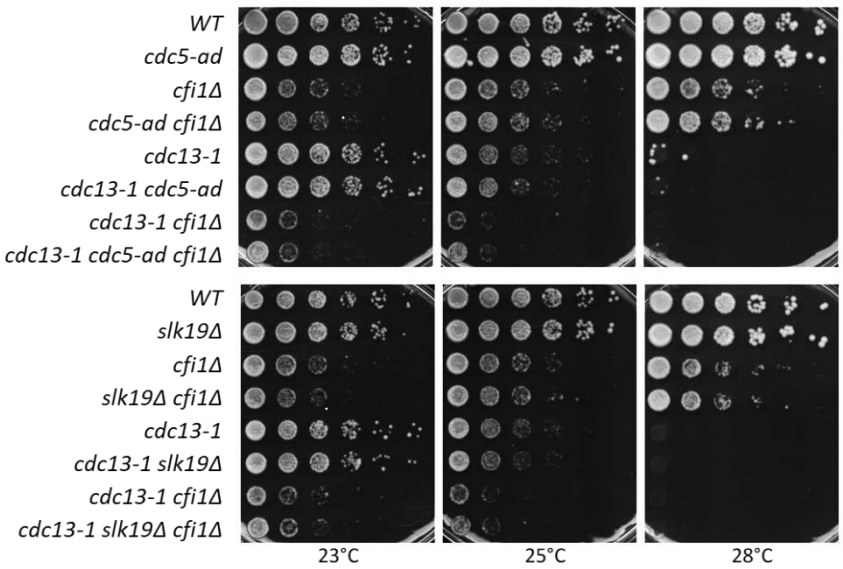

Figure 3.14 ***cfi1Δ* mutant cells show a severe growth defect.** For *WT* (Ry2118), *cdc5-ad* (Ry6092), *cfi1Δ* (Ry6769), *cdc5-ad cfi1Δ* (Ry7021), *cdc13-1* (Ry6090), *cdc13-1 cdc5-ad* (Ry8525), *cdc13-1 cfi1Δ* (Ry6767), *cdc13-1 cdc5-ad cfi1Δ* (Ry7019), *slk19Δ* (Ry6399), *slk19Δ cfi1Δ* (Ry7016), *cdc13-1 slk19Δ* (Ry6396), and *cdc13-1 slk19Δ cfi1Δ* (Ry7013) strains, serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto YEPD plates and incubated at 23°C, 25°C and 28°C. Images were taken after 48 hours of incubation.

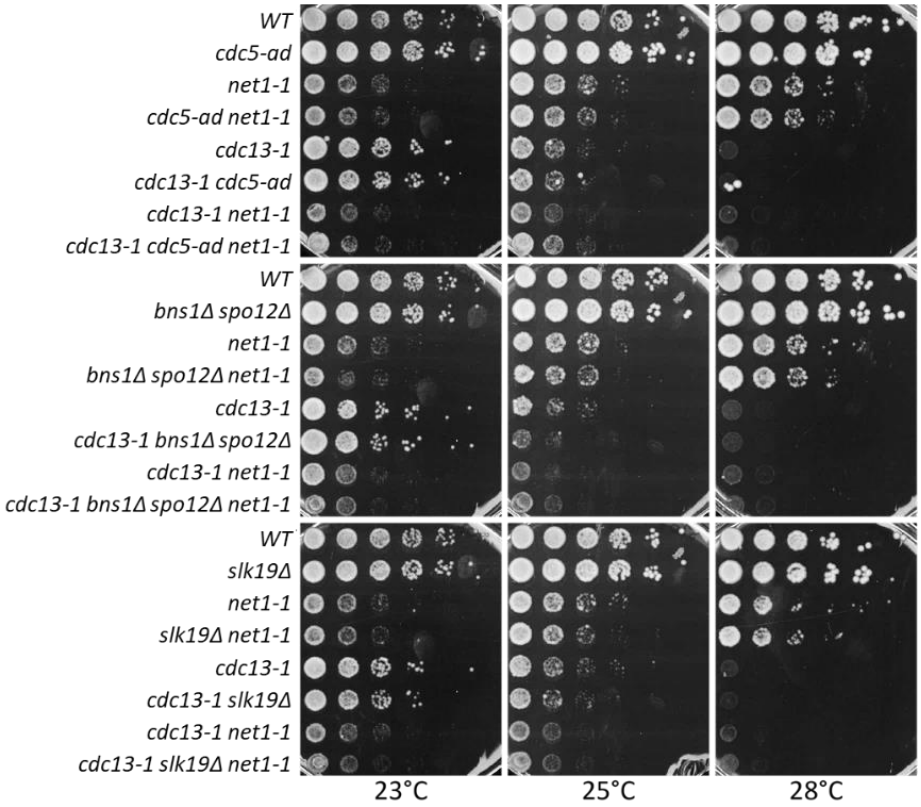

Figure 3.15 ***net1-1^{tab2-1}*** **mutant cells show a severe growth defect.** For *WT* (Ry2281), *cdc5-ad* (Ry6092), *net1-1* (Ry7004), *cdc5-ad net1-1* (Ry8854), *cdc13-1* (Ry6090), *cdc13-1 cdc5-ad* (Ry8525), *cdc13-1 cdc5-ad net1-1* (Ry8851), *spo12Δ* (Ry6387), *spo12Δ net1-1* (Ry8417), *cdc13-1 spo12Δ* (Ry6378), *cdc13-1 spo12Δ net1-1* (Ry8416), *slk19Δ* (Ry6399), *slk19Δ net1-1* (Ry8423), *cdc13-1 slk19Δ* (Ry6396), and *cdc13-1 slk19Δ net1-1* (Ry8420) strains, serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto YEPD plates and incubated at 23°C, 25°C and 28°C. Images were taken after 48 hours of incubation.

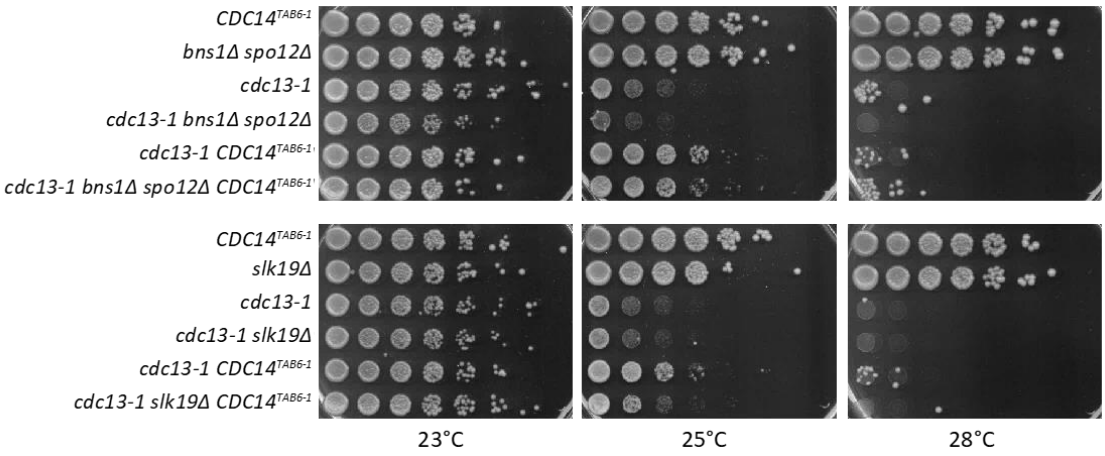

Figure 3.16 **Moderate activation of Cdc14 rescues the growth defect of *cdc13-1 spo12* cells**. For *CDC14^{TAB6-1}* (Ry7008), *spo12Δ* (Ry6387), *cdc13-1* (Ry6090), *cdc13-1 spo12Δ* (Ry6378), *cdc13-1 CDC14^{TAB6-1}* (Ry7005), *cdc13-1 spo12Δ CDC14^{TAB6-1}* (Ry8458), *slk19Δ* (Ry6399*), cdc13-1 slk19Δ* (Ry6396), and *cdc13-1 slk19Δ CDC14^{TAB6-1}* (Ry8336) strains, serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto YEPD plates and incubated at 23°C, 25°C and 28°C. Images were taken after 48 hours of incubation.

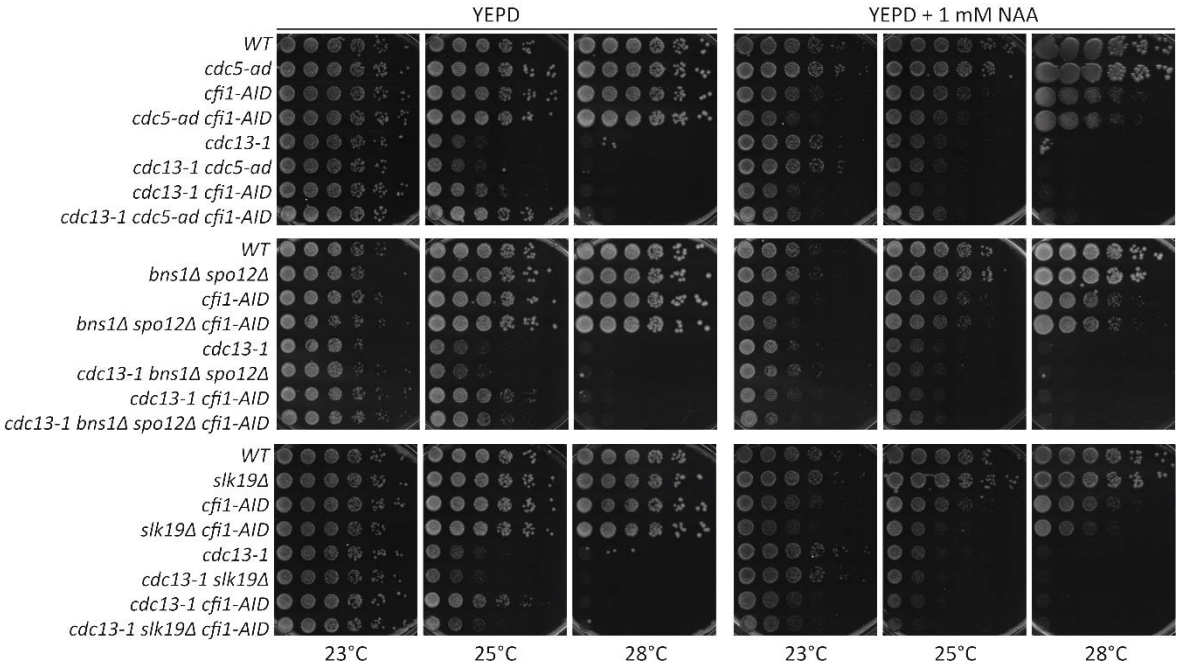

Figure 3.17 **Degradation of Cfi1 rescues the growth defect of *cdc13-1 spo12* cells**. For *WT* (Ry2281), *spo12Δ* (Ry6387), *cfi1-AID* (Ry8400), *spo12Δ cfi1-AID* (Ry8863), *cdc13-1* (Ry6090), *cdc13-1 spo12Δ* (Ry6378), *cdc13-1 cfi1-AID* (Ry8861), *cdc13-1 spo12Δ cfi1-AID* (Ry8440), *slk19Δ* (Ry6399*), slk19Δ cfi1-AID* (Ry8431), *cdc13-1 slk19Δ* (Ry6396), and *cdc13-1 slk19Δ cfi1-AID* (Ry8443), strains, serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto YEPD plates or YEPD plates supplemented with 1mM naphthaleneacetic acid (NAA) and incubated at 23°C, 25°C and 28°C. Images were taken after 48 hours of incubation.

Single cell analyses were performed for the *TAB6-1* allele in both the *cdc13-1* and *HO* backgrounds. In agreement with the results obtained in the serial dilution assays, we found that the adaptation rate was increased in *cdc13-1 spo12Δ* cells that were combined with the $CDC14^{TAB6-1}$ allele, while *cdc13-1, cdc13-1 cdc5-ad*, and *cdc13-1 slk19Δ* cells were not affected (Figure 3.18). The effect of the $CDC14^{TAB6-1}$ allele on *cdc13-1 cdc5-ad* mutant cells has not been analyzed yet. Differently, in the *HO* background, the $CDC14^{TAB6-1}$ allele increased the adaptation rate of *GAL-HO spo12Δ* and *GAL-HO slk19Δ* cells, but it had no effect on *GAL-HO* and *GAL-HO cdc5-ad* cells (Figure 3.19).

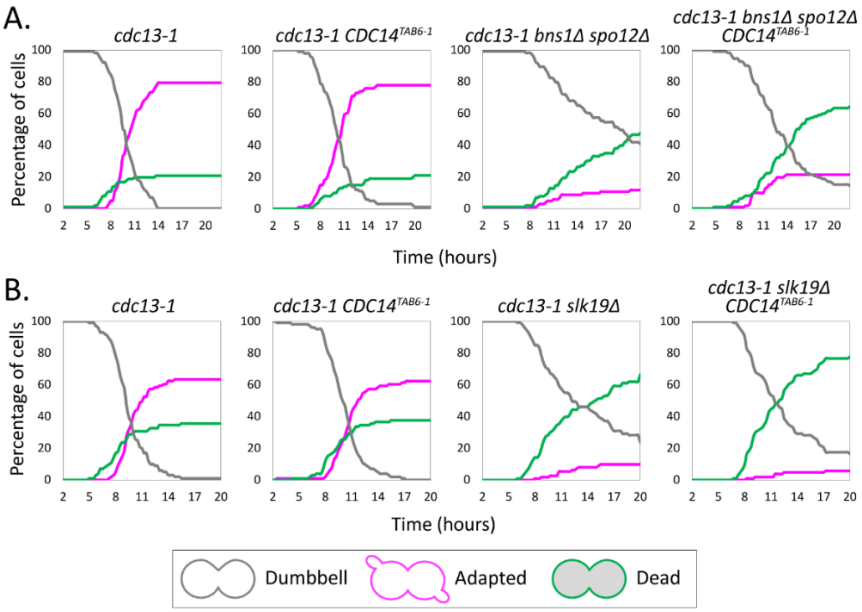

Figure 3.18 **The *CDC14^{TAB6-1}* allele enhances the adaptation response in *cdc13-1 spo12* cells. A.** and **B.** *cdc13-1* (Ry6090), *cdc13-1 CDC14^{TAB6-1}* (Ry7005), *cdc13-1 spo12Δ* (Ry6378), *cdc13-1 spo12Δ CDC14^{TAB6-1}* (Ry8332), *cdc13-1 slk19Δ* (Ry6396), and *cdc13-1 slk19Δ CDC14^{TAB6-1}* (Ry8336) strains arrested in G_1 phase were released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of adapted and dead cells were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

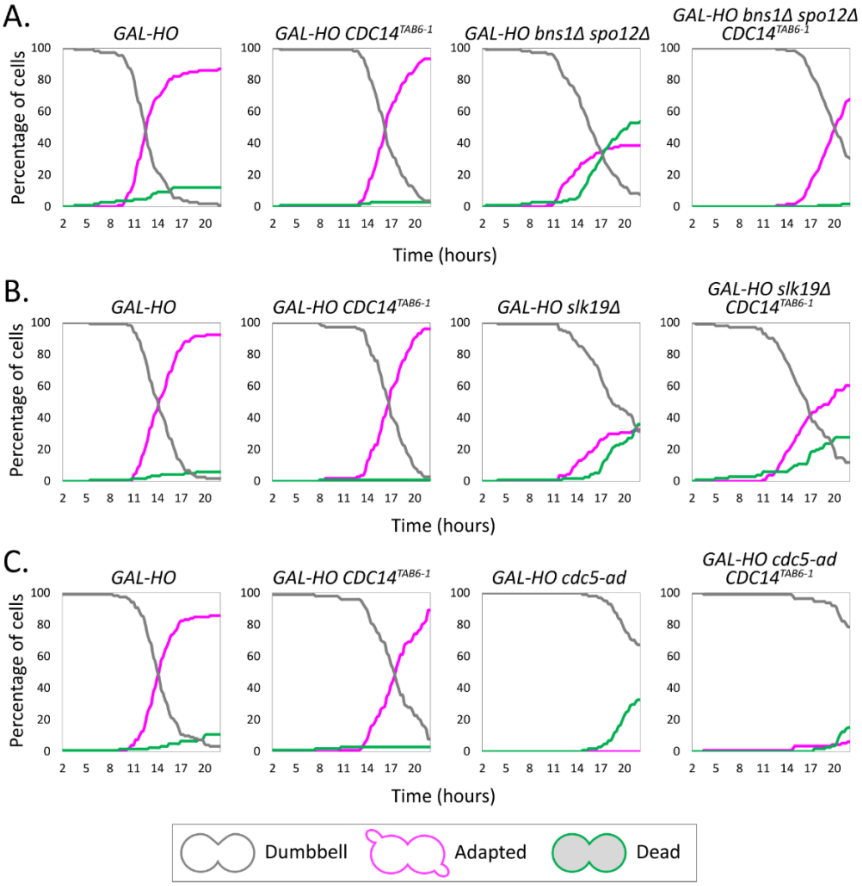

Figure 3.19 **The *CDC14^{TAB6-1}* allele enhances the adaptation response in and *GAL-HO spo12* and *GAL-HO slk19* cells. A.**, **B.** and **C.** *GAL-HO* (Ry2118), *GAL-HO CDC14^{TAB6-1}* (Ry8452), *GAL-HO spo12Δ* (Ry8428), *GAL-HO spo12Δ CDC14^{TAB6-1}* (Ry8458), *GAL-HO slk19Δ* (Ry6890), *GAL-HO slk19Δ CDC14^{TAB6-1}* (Ry8456), *GAL-HO cdc5-ad* (Ry8436), and *GAL-HO cdc5-ad CDC14^{TAB6-1}* (Ry8455) strains arrested in G_1 phase were released in YEPR/G medium in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of adapted and dead cells were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

We then analyzed the consequences of Cfi1 degradation (*cfi1-AID)* on the adaptation phenotype in both the *cdc13-1* and the *HO* backgrounds. First of all, we tested if and how Cfi1 degradation *per se* was impacting on the adaptation phenotype of *cdc13-1* and *GAL-HO* cells. In both backgrounds, we found that Cfi1 degradation caused a severe decrease in the adaptation rate upon treatment with NAA (in *cdc13-1* cells, from ~75% to ~30%; in *GAL-HO* cells, from ~85% to ~50%) (Figure 3.20 and Figure 3.21). Unexpectedly, we found that the adaptation rate decreased also in *cdc13-1* and *GAL-HO* cells upon treatment with NAA (in *cdc13-1* cells, from ~77% to ~39%; in *GAL-HO* cells, from ~86% to ~70%). The causes for such decrease remain unclear.

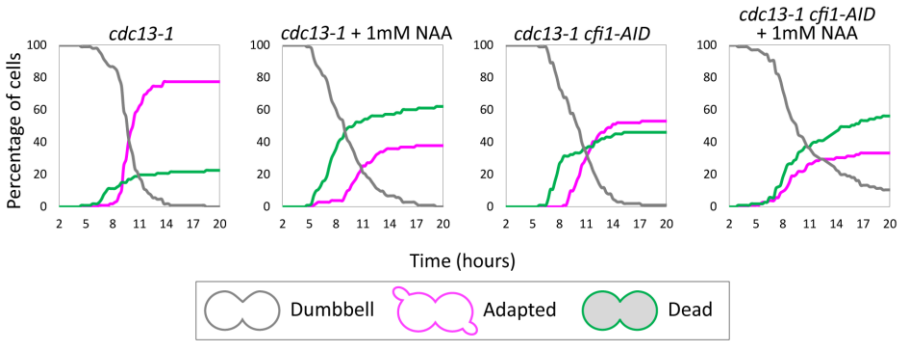

Figure 3.20 **The *cfi1-AID* allele lowers the adaptation rate in *cdc13-1* cells.** *cdc13-1* (Ry6090) and *cdc13-1 cfi1-AID* (Ry8861) strains arrested in the G_1 phase were released at the restrictive temperature of 32°C in the presence of 1mM NAA in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of adapted and dead cells were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

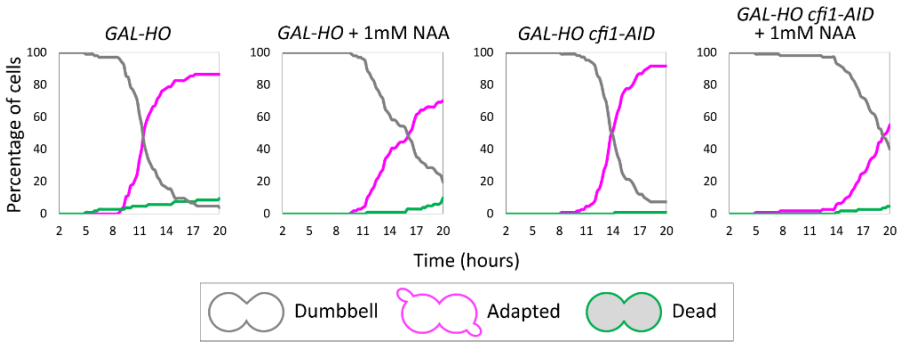

Figure 3.21 **The *cfi1-AID* allele lowers the adaptation rate in *GAL-HO* cells.** *GAL-HO* (Ry2118) and *GAL-HO cfi1-AID* (Ry8484) strains arrested in the G_1 phase were released in YEPR/G medium and 1mM NAA in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of adapted and dead cells were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

Taken together, our findings that moderate Cdc14 release (*TAB6-1* allele) i) slightly ameliorates, but does not completely rescue the adaptation defect of *spo12* mutant cells; ii) does not ameliorate the defect of the *cdc5* mutant cells, and iii) only partially compensates for the defect of *slk19* mutant cells in the *GAL-HO* background, suggest that the phosphatase only partially contributes to the adaptation response in both backgrounds. However, the finding that the *cfi1-AID* allele decreases the adaptation response argues against the results related to the *TAB6-1* allele, which does not impact on the adaptation response in either *cdc13-1* nor *GAL-HO* cells. As cells with perturbed Cfi1 activity, such as *cfi1Δ* and *net1-1* (seen before in the serial dilution assays) show a severe growth defect *per se*, it is tempting to speculate that the abrogation of the adaptation response upon Cfi1 degradation is the consequence of detrimental effects on cell growth in general, and that the phenotype observed in these conditions is not linked to the effect of Cdc14 on the adaptation response. However, a caveat of our approach is that the quantification of the single cell experiments was based on cell morphology, particularly upon bud emergence, hence entry in the S-phase of the following cell cycle. Knowing that fully active, cytoplasmic Cdc14 promotes entry into and maintenance of the G_1 phase of the cell cycle (Visintin *et al.*, 1998), it is possible that by looking at bud emergence we underestimate the percentage of cells that effectively adapted. Indeed, morphologically, it is not possible to distinguish cells arrested in metaphase from cells progressed in anaphase, as they maintain a dumbbell shape, or in the G_1 phase (two cells in the G_1 phase that are in close proximity appear as one single dumbbell cell). To overcome this limitation, we decided to use spindle morphology as a read-out for adaptation. To this aim, alpha-tubulin was tagged with a fluorescent marker to follow changes in the mitotic spindle over time (Figure 3.22). Since we know that the DNA damage checkpoint arrests cells in metaphase (characterized by short bipolar spindles), and that checkpoint adaptation means resuming the cell cycle from

a metaphase arrest, adapting cells are those cells that successfully enter in anaphase (evinced by spindle elongation). This tool allows us to discriminate between dumbbell cells arrested in metaphase and dumbbell cells that successfully adapted, as evinced by spindle elongation. Therefore, this tool allows to assess the adaptation initiation and the rate inside the population more precisely. Indeed, when we analyzed the morphology and the spindle dynamics of *cdc13-1* cells in parallel using both budding and spindle morphology read-outs, we found that only ~45% of the cells rebudded, and, instead, ~85% of the cells had properly entered in anaphase (Figure 3.23). These results indicate that the spindle morphology read-out is an effective tool for assessment of adaptation at single cell level and shows greater sensitivity compared to the budding read-out.

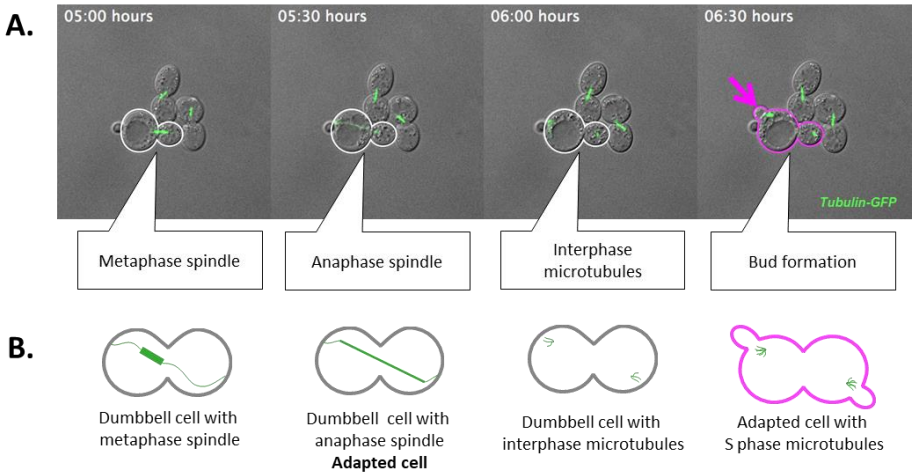

Figure 3.22 **Checkpoint adaptation can be assessed by monitoring spindle elongation.** *cdc13-1 TUB1-GFP* (Ry6406) cells arrested in the G_1 phase were released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. **A.** Representative timeframes of Movie 3.2 and **B.** relative graphical illustrations are shown.

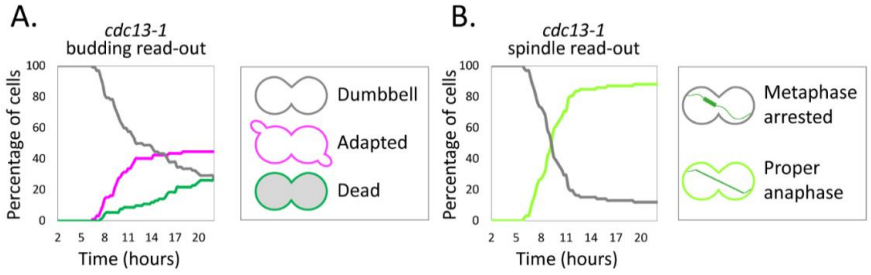

Figure 3.23 **Spindle morphology is a more sensitive tool for assessment of checkpoint adaptation.** *cdc13-1 TUB1-GFP* (Ry6406) cells arrested in the G_1 phase were released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Quantifications with budding read-out and spindle morphology read-out were performed in parallel on the same cells. **A.** The percentage of dumbbell cells (gray line), adapted cells (pink line) and dead cells (green line), and **B.** the percentage of metaphase arrested cells (gray line) and cells that entered in anaphase (light green line) were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

3.3. Characterization of the adaptation phenotype of the FEAR components Cdc5, Spo12 and Slk19 by means of spindle morphology

3.3.1. Analyses of spindle morphology reveal that the FEAR mutants *spo12* and *slk19* show peculiar spindle dynamics

Since we found that a portion of dumbbell cells successfully elongated their spindle, thus adapting to the checkpoint-mediated arrest and entering in anaphase, we analyzed again the phenotype of FEAR mutants by looking at spindle morphology as read-out for adaptation.

We started by analyzing spindle dynamics in the *cdc13-1* background in single cell analyses (Figure 3.24). We found that, while the vast majority of the *cdc13-1* cells entered in anaphase (mean value: ~86%), about half of the *cdc13-1 cdc5-ad* mutant cells entered in anaphase (mean value: ~50%), in contrast with data in the literature (Toczyski, Galgoczy and Hartwell, 1997). Further analyses will be performed on the *cdc13-1 cdc5-ad* mutant cells to exclude the possibility that our strain accumulated suppressor mutations. Moreover, we confirmed that only a small portion of *spo12* and *slk19* mutant cells are able to correctly enter in anaphase (mean value: ~13% in *cdc13-1 spo12Δ* mutant cells and ~6% in *cdc13-1 slk19Δ* mutant cells), in agreement with what was observed in the budding analyses.

Strikingly, in addition to the observation of a portion of cells displaying metaphase spindle, as foreseen for the DDC checkpoint-arrested cells, we identified two additional phenotypes: i) spindle length corresponding to metaphase that at a certain point broke ("metaphase break" category, Figure 3.25 B and Movie 3.4); and ii) spindles that seem to elongate but afterwards become shorter and eventually collapse ("spindle re-joins" category, Figure 3.25 C and Movie 3.5). Interestingly, these phenotypes are shared by both *cdc13-1 spo12Δ* cells (mean value: 45% of cells in the spindle re-joins category) and *cdc13-*

1 slk19Δ cells (mean values: 46% of cells in the spindle re-joins category and 16% in the metaphase break category), but are not present in *cdc13-1* cells.

Additionally, among cells that successfully entered and completed anaphase ("proper anaphase" category), we noticed that a portion of the *spo12* and *slk19* mutant cells took a longer time to reach the G_1 phase compared with *cdc13-1* cells. Given this observation, to highlight and to precisely quantify the difference among the strains, we measured the time from the initial spindle elongation to interphase microtubule appearance in cells that completed anaphase. Consistently, we found that *cdc13-1 spo12* and *cdc13-1 slk19* mutant cells took a longer time to successfully complete anaphase and enter in the G_1 phase than *cdc13-1* cells (mean values: ~30 minutes for *cdc13-1* cells, ~40 minutes for *cdc13-1 cdc5-ad* cells, 112 minutes for *cdc13-1 spo12Δ* cells, and ~62 minutes for *cdc13-1 slk19Δ* cells, Figure 3.24 C).

The reasons for the abnormal spindle dynamics and longer time that is required to complete anaphase observed in the FEAR mutants remain unclear. These defects could underline either defects in bypass of the checkpoint-mediated metaphase arrest, defects in spindle stabilization required for proper anaphase spindle elongation, or defects in chromosome segregation attributable to the link between uncapped telomeres and Cdc14. Knowing that protracted arrest achieved either by the DNA damage checkpoint or by depletion of Cdc20 (data not shown) does not result in the collapse of the spindle suggest that *spo12* and *slk19* mutant cells are defective in proper anaphase execution, and we speculate that the abnormal phenotypes in spindle dynamics are due to unsuccessful attempts to elongate the spindle.

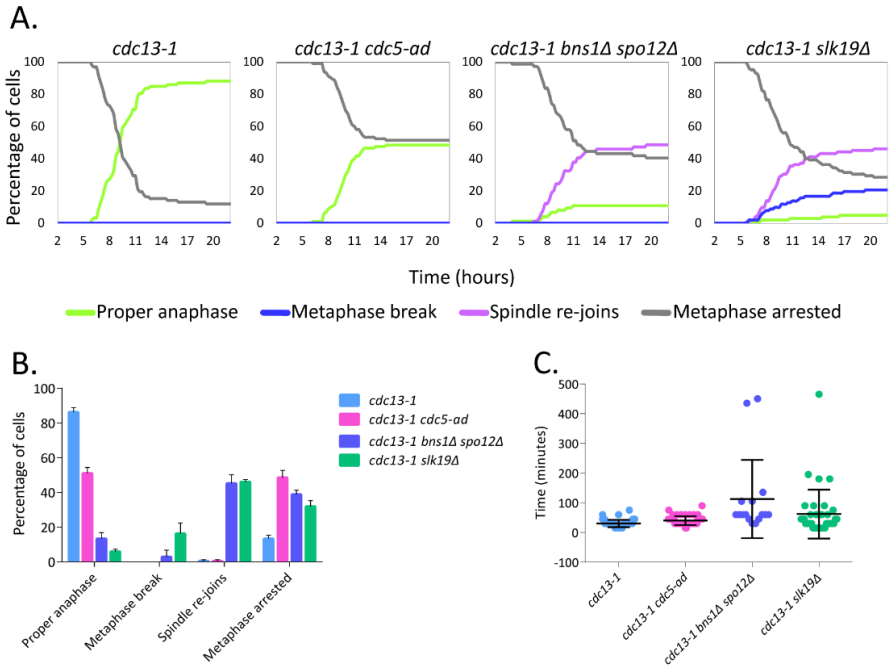

Figure 3.24 ***cdc13-1 spo12* and *cdc13-1 slk19* mutant cells show peculiar spindle dynamics. A.** *cdc13-1* (Ry6406)*, cdc13-1 cdc5-ad* (Ry6401)*, cdc13-1 bns1Δ spo12Δ* (Ry6506), and *cdc13-1 slk19Δ* (Ry6511) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. **B.** For the last time point (23 hours), means and standard deviations deriving from three independent experiments are shown. For each strain, a number n ≥ 100 cells have been counted. **C.** Anaphase duration was measured as the time from initial spindle elongation to interphase microtubule appearance. Exact values, means and standard deviations are shown. For c*dc13-1* cells, a number n ≥ 50 cells were counted, for *cdc13-1 cdc5-ad* n = 48 cells, for *cdc13-1 bns1Δ spo12Δ* n = 16 cells, for *cdc13-1 slk19Δ* n = 37 cells were counted.

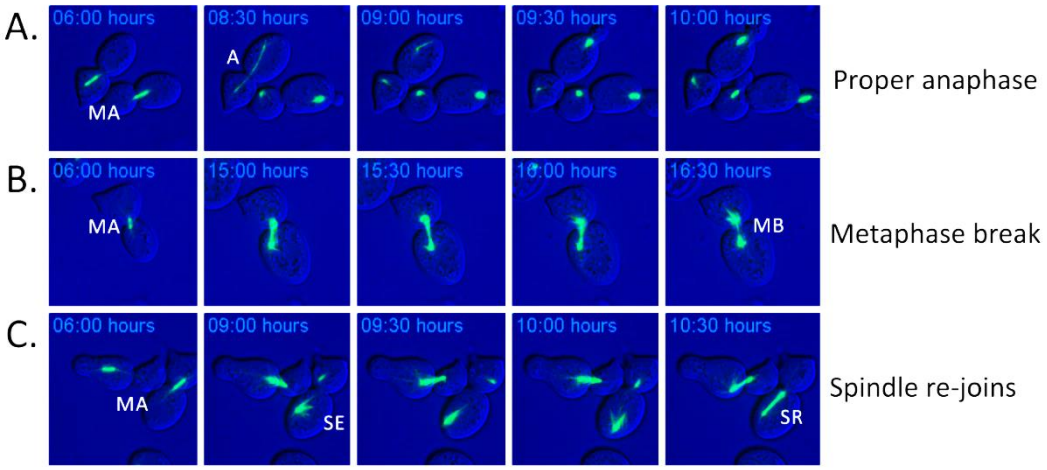

Figure 3.25 ***cdc13-1 spo12* and *cdc13-1 slk19* mutant cells show peculiar spindle dynamics.** *cdc13-1* (Ry6406)*, cdc13-1 bns1Δ spo12Δ* (Ry6506), and *cdc13-1 slk19Δ* (Ry6511) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and next released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Representative fields of the categories **A.** anaphase, **B.** metaphase break, and **C.** spindle re-joins are shown (source movies: Movie 3.3, Movie 3.4 and Movie 3.5). MA: metaphase arrested; A: proper anaphase; MB: metaphase break; SE: spindle elongates; SR: spindle re-joins.

To assess whether the abnormal spindle dynamics observed in the FEAR mutants *spo12* and *slk19* are specific for the *cdc13-1*-induced DNA damage condition, *GAL-HO*, *GAL-HO cdc5-ad*, *GAL-HO spo12*, and *GAL-HO slk19* cells carrying a mCherry-tagged version of alpha-tubulin were analyzed by single cell experiments (Figure 3.26). Strikingly, we observed abnormal spindle dynamics in both *GAL-HO spo12Δ* and *GAL-HO slk19Δ* mutant cells. More specifically, besides the "spindle re-join" phenotype (Figure 3.26 B and Movie 3.7), we also observed cells displaying extremely long anaphase spindles, characterized by spindle poles moving chaotically inside the cell body ("abnormal spindle" phenotype, Figure 3.26 C and Movie 3.8). Additionally, we observed cells properly completing anaphase and entering in the following G_1 phase ("proper anaphase" phenotype, Figure 3.26 A and Movie 3.6), and cells that remained arrested with a stable bipolar metaphase spindle throughout the whole time-lapse ("metaphase arrested" phenotype, Figure 3.26 D and Movie 3.9).

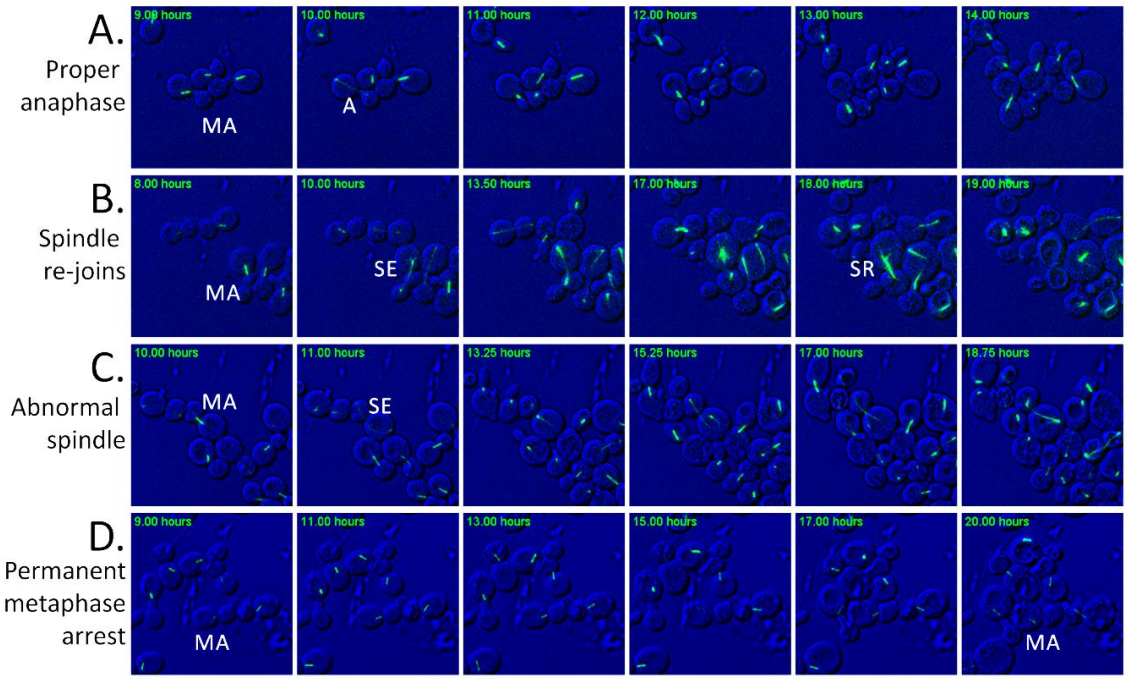

Figure 3.26 ***GAL-HO spo12* and *GAL-HO slk19* mutant cells show peculiar spindle dynamics.** *GAL-HO* (Ry2118)*, GAL-HO cdc5-ad* (Ry8436)*, GAL-HO bns1Δ spo12Δ* (Ry8428), and *GAL-HO slk19Δ* (Ry6890) cells carrying a mCherry-tagged version of α-Tubulin were arrested in the G_1 phase were released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Representative fields of the categories **A.** proper anaphase, **B.** spindle re-joins, **C.** abnormal spindle, and **D.** permanent metaphase arrest are shown (source movies: Movie 3.6, Movie 3.7, Movie 3.8 and Movie 3.9). MA: metaphase arrested; A: proper anaphase; SE: spindle elongates; SR: spindle re-joins.

Similarly to the analyses performed in single cell experiments using the spindle morphology read-out in the *cdc13-1* background, we assessed the spindle dynamics, as previously described (Figure 3.27 A and B), and, for cells that completed anaphase, we assessed the time required for completion of anaphase (measured as the time from initial spindle elongation to interphase microtubule appearance, Figure 3.27 C).

Regarding the comparison of the percentages of cells in each strain for each category (Figure 3.27 B), in agreement with data in the literature, we found that, while the vast majority of *GAL-HO* cells entered in anaphase (mean value: ~98%), almost none of *GAL-HO cdc5-ad* mutant cells bypassed the checkpoint-mediated arrest (mean values: ~91% in the "metaphase arrested" category and ~5% in the "proper anaphase" category). Regarding the *spo12* and *slk19* mutant cells, in contrast with data in the *cdc13-1* background, we found that about half of mutant cells completed anaphase (mean values in the Ho background: ~49% and ~52% for *GAL-HO spo12Δ* cells and *GAL-HO slk19Δ* cells, respectively; mean values in the *cdc13-1* background: ~13% and ~6% for *cdc13-1 spo12Δ* cells and *cdc13-1 slk19Δ* cells, respectively, see Figure 3.24 B). Moreover, when comparing the behavior of the FEAR mutants *spo12* and *slk19* in the two damage conditions tested, we noticed that a great portion of the cells remain arrested in metaphase in the *cdc13-1* background compared to the Ho background (mean values in the Ho background: ~3% and ~1% for *GAL-HO spo12Δ* cells and *GAL-HO slk19Δ* cells, respectively; mean values in the *cdc13-1* background: ~39% and ~32% for *cdc13-1 spo12Δ* cells and *cdc13-1 slk19Δ* cells, respectively). Nevertheless, we found that about half of the *spo12* and *slk19* mutants cells showed abnormal spindle dynamics and that, strikingly, the percentages of cells with abnormal spindles were very similar in the two damage conditions tested (mean values in the Ho background: ~48% and ~46% for *GAL-HO spo12Δ* cells and *GAL-HO slk19Δ* cells,

respectively; mean values in the *cdc13-1* background: ~45% and ~46% for *cdc13-1 spo12Δ* cells and *cdc13-1 slk19Δ* cells, respectively).

Regarding the duration of anaphase (Figure 3.27 C), in agreement with the data observed in the *cdc13-1* background, we found that, while *GAL-HO* cells complete anaphase within about 30 minutes (mean values: 28 minutes in *GAL-HO* cells; 30 minutes in *cdc13-1* cells, see Figure 3.24 C), the FEAR mutants *spo12* and *slk19* require longer time to complete anaphase (mean values in the Ho background: 106 minutes and 117 minutes for *GAL-HO spo12Δ* cells and *GAL-HO slk19Δ* cells, respectively; mean values in the *cdc13-1* background: 112 minutes and 62 minutes for *cdc13-1 spo12Δ* cells and *cdc13-1 slk19Δ* cells, respectively).

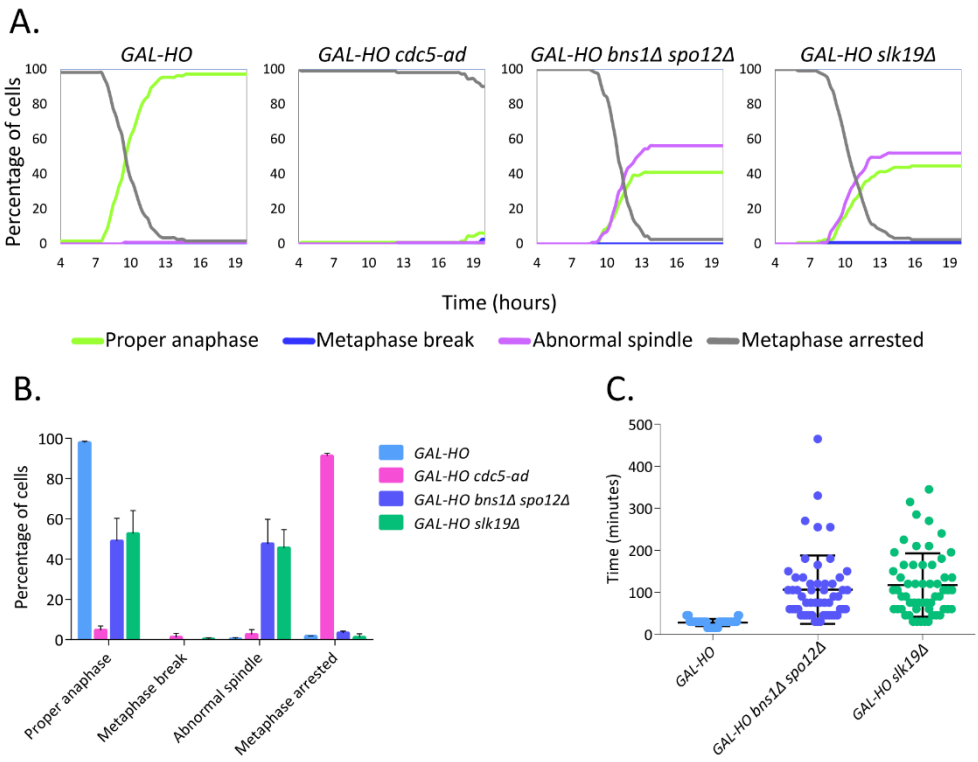

Figure 3.27 ***GAL-HO spo12* and *GAL-HO slk19* mutant cells show abnormal spindle dynamics. A.** *GAL-HO* (Ry8847), *GAL-HO cdc5-ad* (Ry8850), *GAL-HO bns1Δ spo12Δ* (Ry8849), and *GAL-HO slk19Δ* (Ry8848) cells carrying a mCherry-tagged version of α-Tubulin were arrested in the G_1 phase and released in YEPR/G medium in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, abnormal spindle, metaphase break, and metaphase arrested) were scored for each timeframe. **B.** For the last time point (20 hours), means and standard deviations deriving from two independent experiments are shown. For each strain and time point, more than 100 cells were counted. **C.** Anaphase duration was measured as the time from initial spindle elongation to interphase microtubule appearance. Exact values, mean and standard deviation are shown. For each strain, a number n ≥ 100 cells was counted.

Taken together, our data in both damage conditions tested suggest that the FEAR components Spo12 and Slk19 are not required for the bypass of the checkpoint-mediated arrest but for proper anaphase execution following checkpoint switch off. In respect to the polo kinase, our data suggest that Cdc5 is required to turn off the DNA damage checkpoint machinery, in agreement with data in the literature (Donnianni *et al.*, 2010; Vidanes *et al.*, 2010).

When comparing the behavior of the FEAR mutants *spo12 and slk19* in the two backgrounds, we observed a difference in terms of both percentages of cells in the "metaphase arrested" category and the "proper anaphase" category. The reason for this difference remains unclear, but it can be due to: i) intrinsic differences among the two damage conditions, where *cdc13-1* represents a more "global" damage condition, as all telomeres are affected simultaneously, compared to a single proper DSB that, at least in principle, should be more tolerable by cells; or ii) the link between Cdc14 and telomere segregation; or, finally, iii) differences in the two background strains used (with *cdc13-1* cells being in the W303 strain background, and *GAL-HO* cells being in the JKM179 strain background). Of course, the three possibilities are not mutually exclusive. Since the percentage of cells arrested in metaphase in the *cdc13-1* background is greater than that in the Ho background, it is tempting to speculate that these discrepancies can be due to intrinsic differences between the two damage conditions. Nevertheless, to discriminate among these possibilities, we will test the requirement of the FEAR components for proper spindle dynamics using other DSB-inducing systems, and evaluate the consequence of modulating the quantity of the damage. Additionally, to override the differences among the backgrounds, we are backcrossing the *GAL-HO* strain with the W303 strain.

Despite these considerations, we decided to analyze the consequences of ectopic Cdc14 activation on checkpoint adaptation exploiting the spindle morphology read-out.

3.3.2. Analyses of spindle morphology reveal that moderate activation of Cdc14 partially rescues the peculiar spindle dynamics in the FEAR mutants *spo12* and *slk19*

To understand the contribution of Cdc14 in the adaptation response, we repeated our analyses on *cfi1-AID* allele in single cell experiments using spindle morphology as read-out. First, we tested if and how Cfi1 degradation *per se* was impacting on the adaptation phenotype of the *cdc13-1* single mutant. In agreement with the budding read-out, we found that the degradation of Cfi1 caused a severe decrease in the adaptation rate upon treatment with auxin (from ~70% to 10%) (Figure 3.28 A). The same abrogation of the adaptation rate was observed when we modulated the degradation of Cfi1 by changing the concentration of auxin in the growth media (Figure 3.28 B).

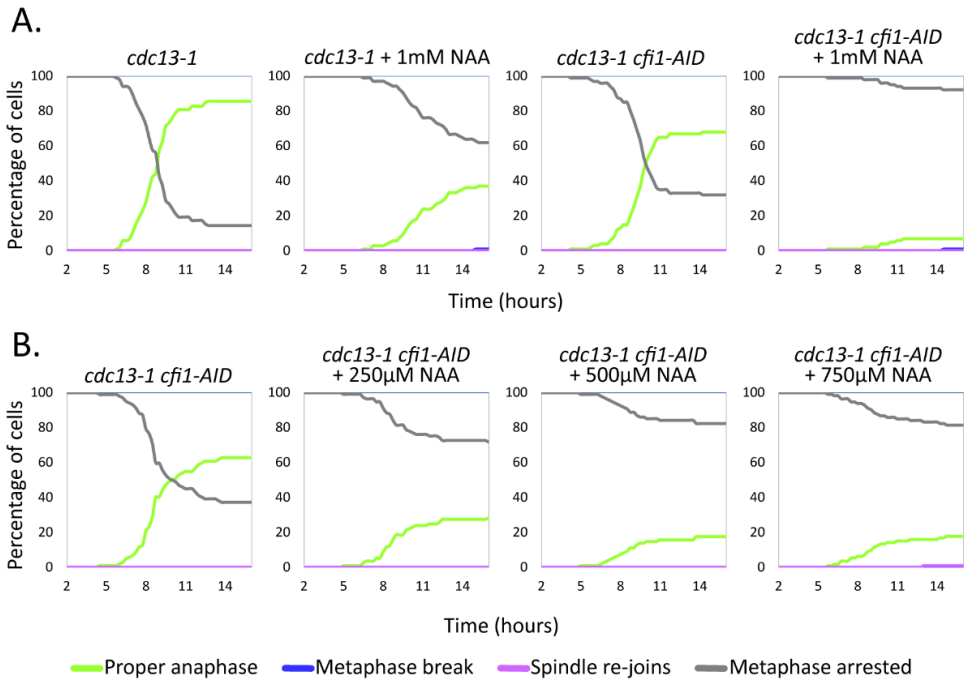

Figure 3.28 **The *cfi1-AID* allele lowers the adaptation rate in *cdc13-1* cells.** *cdc13-1* (Ry8578) and *cdc13-1 cfi1-AID* (Ry8838) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released at the restrictive temperature of 32°C in the presence of **A.** 1mM IAA or **B.** 250 µM, 500 µM, or 750 µM NAA in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. For each condition, a number n ≥ 100 cells was counted.

We then tested the consequences of ectopic Cdc14 activation by exploiting the *TAB6-1* allele on FEAR mutants in the *cdc13-1* background. Similarly to the previous analyses of single cell experiments using spindle morphology as read-out, we assessed the spindle

dynamics (Figure 3.29 A and B, Figure 3.30 A and B), and, for cells that completed anaphase, we assessed the time required for completion of anaphase (measured as the time from initial spindle elongation to interphase microtubule appearance, Figure 3.29 C, Figure 3.30 C).

Regarding the assessment of the spindle dynamics (Figure 3.29 B for *spo12* mutant and Figure 3.30 B for *slk19* mutant), we found that the *TAB6-1* allele increased the percentage of cells that were able to complete anaphase in both *cdc13-1 spo12Δ* and *cdc13-1 slk19Δ* cells (mean values: in the *spo12* mutant, from 13% to 40%; in the *slk19* mutant, from 9% to 41%), while *cdc13-1* cells were not affected (mean values: from 80% to 74%). Interestingly, the *TAB6-1* allele decreased the percentage of cells in the "spindle re-joins" category in both *cdc13-1 spo12Δ* and *cdc13-1 slk19Δ* cells (mean values: in the *spo12* mutant, from 45% to 18%; in the *slk19* mutant, from 37% to 14%).

Regarding the comparison of the duration of anaphase (Figure 3.29 C for *spo12* mutant and Figure 3.30 C for *slk19* mutant), we found that the *TAB6-1* allele decreased the duration of anaphase in both *cdc13-1 spo12Δ* and *cdc13-1 slk19Δ* cells (mean values: in the *spo12* mutant, from 67 to 24 minutes; in the *slk19* mutant, from 86 to 36 minutes), while *cdc13-1* cells were not affected (mean values: from 20 to 22 minutes). These results suggest that moderate Cdc14 activation compensates for the absence of both Spo12 and Slk19. However, the *TAB6-1* allele does not perfectly recapitulate the phenotype of *cdc13-1* cells. The partial rescue observed can be due to FEAR-mediated Cdc14 release that is not perfectly mimicked by the *TAB6-1* allele and/or to specific functions of Spo12 and Slk19 in the mitotic exit that cannot be compensated for by the phosphatase.

Taken together, our data on the alterations of Cfi1 activity (for *cfi1Δ* see Figure 3.14, for *net1-1* see Figure 3.15, and for *cfi1-AID* alleles see Figure 3.20, Figure 3.21, and Figure 3.28), on the *TAB6-1* allele suggest that impairment of Cfi1 functions has severe consequences on

cell viability, both in unperturbed and DNA damage condition, and that high levels of Cdc14 are not detrimental for adaptation. Moreover, the partial rescue observed in *spo12* and *slk19* mutant cells suggests Cdc14 as the final effector of the FEAR network for exit from mitosis in persistent DNA damage conditions.

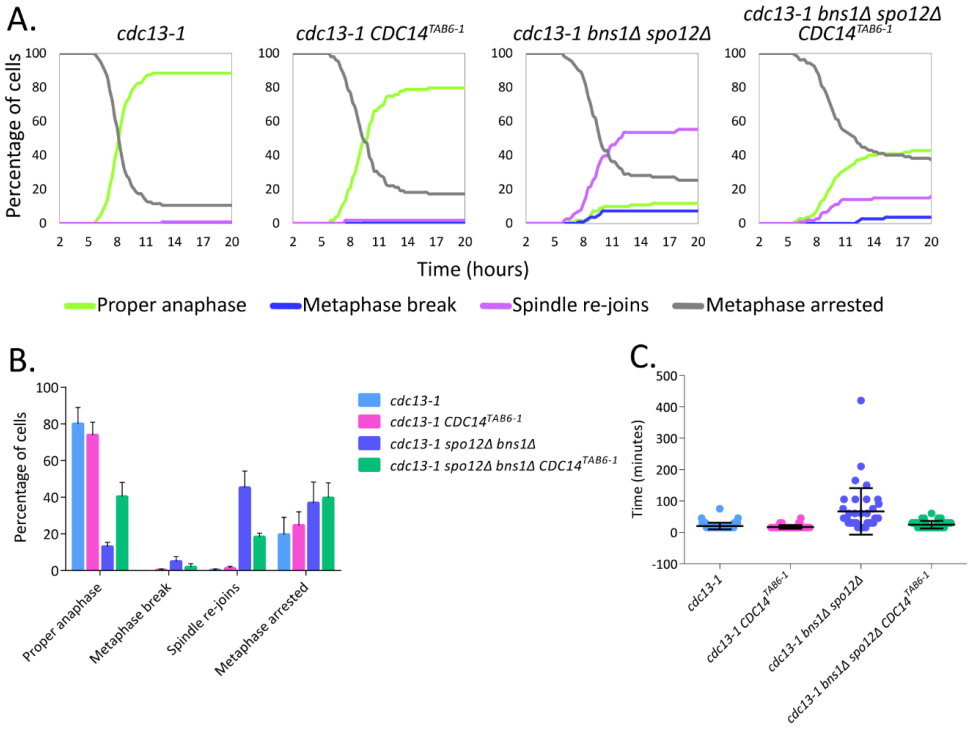

Figure 3.29 **The *CDC14^{TAB6-1}* allele enhances the adaptation response in *cdc13-1 spo12Δ* cells.** *cdc13-1* (Ry6406), *cdc13-1 CDC14^{TAB6-1}* (Ry8698), *cdc13-1 bns1Δ spo12Δ* (Ry6506), and *cdc13-1 bns1Δ spo12Δ CDC14^{TAB6-1}* (Ry8534) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. **B.** For the last time point (20 hours), means and standard deviations deriving from three independent experiments are shown. For each strain, a number n ≥ 100 cells was counted. **C.** Anaphase duration was measured as the time from initial spindle elongation to interphase microtubule appearance. Exact values, means and standard deviations are shown. For each strain, a number n ≥ 50 cells was counted, except for *cdc13-1 bns1Δ spo12Δ*, for which a number n = 38 cells was counted.

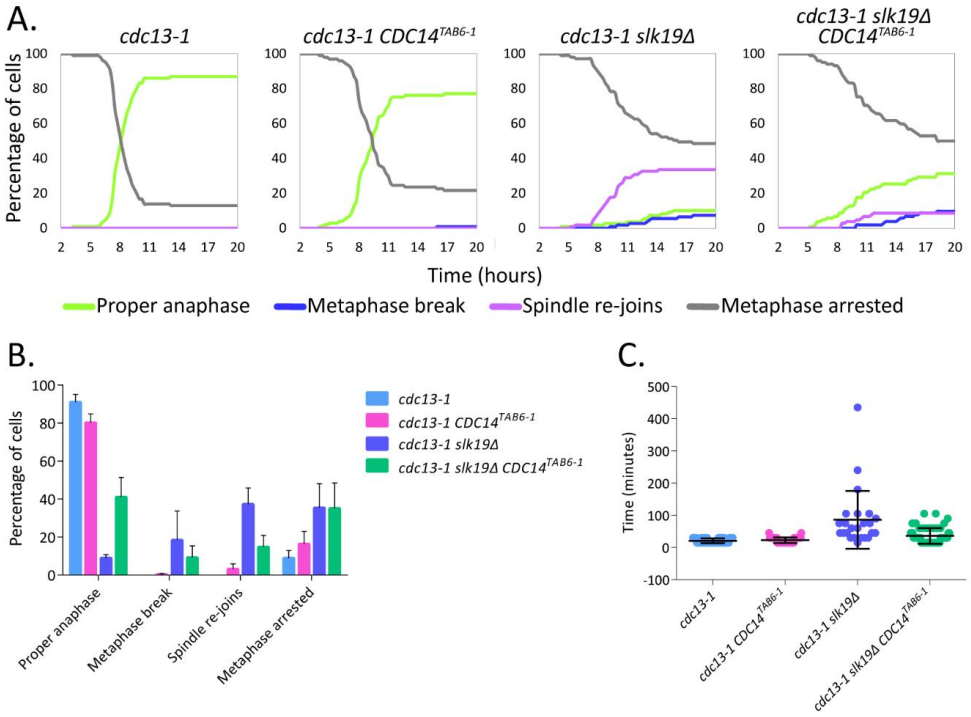

Figure 3.30 **The *CDC14^{TAB6-1}* allele enhances the adaptation response in *cdc13-1 slk19Δ* cells. A.** *cdc13-1* (Ry6406), *cdc13-1 CDC14^{TAB6-1}* (Ry8698), *cdc13-1 slk19Δ* (Ry6511), and *cdc13-1 slk19Δ CDC14^{TAB6-1}* (Ry8534) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. **B.** For the last time point (20 hours), means and standard deviations deriving from three independent experiments are shown. For each strain, a number n ≥ 100 cells was counted. **C.** Anaphase duration was measured as the time from initial spindle elongation to interphase microtubule appearance. Exact values, means and standard deviations are shown. For each strain, a number n ≥ 50 cells was counted, except for *cdc13-1 slk19Δ*, for which 24 cells were counted.

3.3.3. Impairment of the functions of Cdc14 recapitulates the peculiar spindle dynamic defects observed in the FEAR mutants *spo12* and *slk19*

So far, our data clearly indicate that the functions of the FEAR network are specifically required for exit from mitosis in case of persistent DSB conditions, and suggest Cdc14 as the final effector of the FEAR network for exit from mitosis in persistent DNA damage conditions. However, the possible role of Cdc14 in the adaptation response remains unclear. The peculiar phenotype in spindle dynamics observed in *spo12* and *slk19* mutant cells in both the *cdc13-1* and the *GAL-HO* backgrounds would point to specific functions of Cdc14 in mitotic spindle dynamics required for the exit from mitosis in case of a persistent DNA damage, although we cannot exclude the possibility that Spo12 and Slk19 may play FEAR-independent function in these conditions. We reasoned that if Cdc14 is the final

effector of the FEAR network in controlling mitotic spindle dynamics, inactivation of the phosphatase should recapitulate the same peculiar spindle phenotypes observed in the FEAR mutants *spo12 and slk19*. To this aim, we analyzed the phenotype of *cdc13-1* and *cdc13-1 cdc14-1* mutant cells in single cell experiments using spindle morphology as read-out. Usually, experiments with strains carrying the *cdc14-1* allele are performed at 37°C. However, the *cdc14-1* strain shows severe growth defects already at 30°C, demonstrating that the protein is inactive already at this temperature (Figure 3.31).

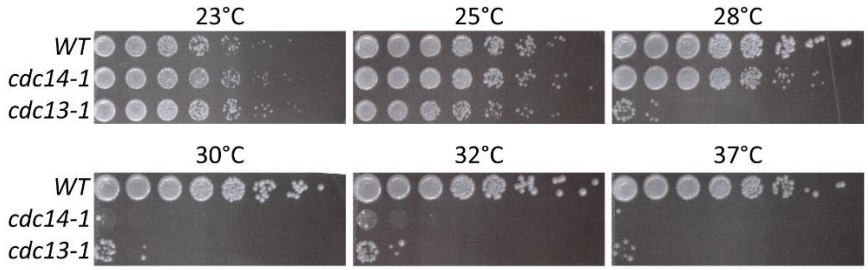

Figure 3.31 **The Cdc14-1 protein is inactive at 30°C.** For *WT* (Ry1), *cdc14-1* (Ry1574), and *cdc13-1* (Ry6090) strains, serial dilutions (1:5) of yeast cell suspensions starting from OD_{600} = 1 were spotted onto YEPD plates and incubated at 23°C, 25°C, 28°C, 30°C, 32°C, and 37°C. Images were taken after 24 hours of incubation.

As before, we assessed the spindle dynamics (Figure 3.32 A and B), and, for cells that completed anaphase, we assessed the time required for completion of anaphase (measured as the time from initial spindle elongation to interphase microtubule appearance, Figure 3.32 C).

Regarding the percentages of cells in each strain for each category, we found that *cdc13-1 cdc14-1* cells showed abnormal spindle phenotypes as well, with percentages very close to the ones displayed by *spo12* and *slk19* mutant cells (mean values: ~40% in *cdc13-1 cdc14-1* cells, ~45% in *cdc13-1 spo12Δ* cells, and ~37% in *cdc13-1 slk19Δ* cells, see Figure 3.24 B). Regarding the duration of anaphase, we found that, while *cdc13-1* cells enter in anaphase and in the following G1 phase within about 30 minutes (mean values: 26 minutes), in *cdc13-1 cdc14-1* cells this process lasts longer, similarly to what we observed for *spo12* and *slk19*

mutant cells (mean values: 77 minutes in *cdc13-1 cdc14-1* cells, 112 minutes in *cdc13-1 spo12Δ* cells, and 62 minutes in *cdc13-1 slk19Δ* cells, see Figure 3.24).

Taken together, our data concerning the moderate activation (*TAB6-1* allele) and inactivation (*cdc14-1* allele) of Cdc14 suggest that the activity of the FEAR network is required for proper mitotic spindle dynamics in persistent DNA damage conditions and point to Cdc14 as the final effector of the FEAR network in this process.

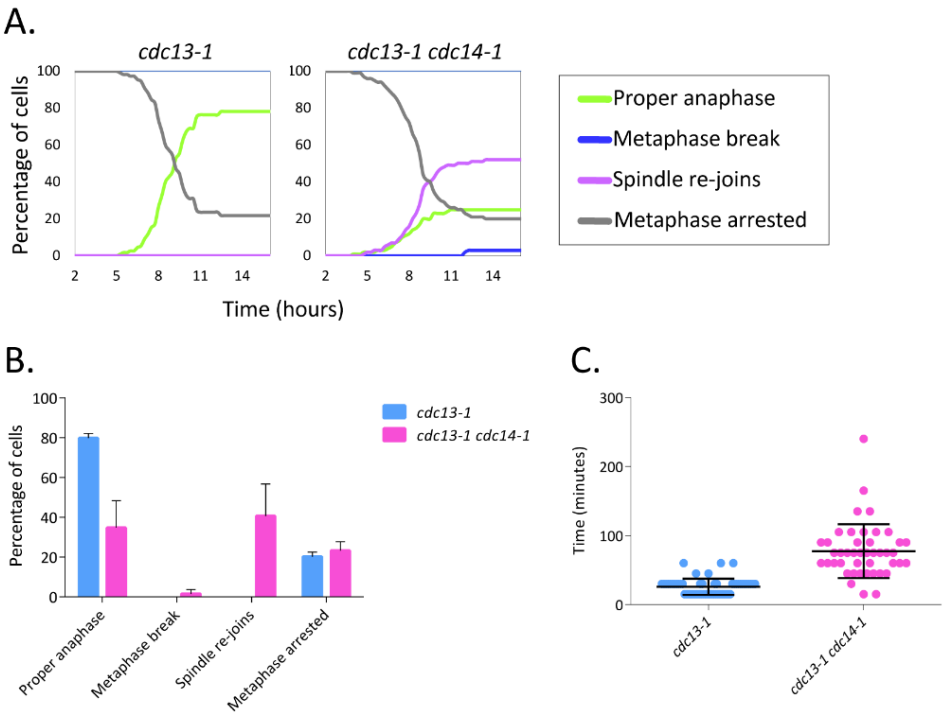

Figure 3.32 **Inactivation of Cdc14 recapitulates the same spindle dynamic phenotype observed for *spo12* and *slk19* mutants. A.** *cdc13-1* (Ry6406) and *cdc13-1 cdc14-1* (Ry8831) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. **B.** For the last time point (16 hours), means and standard deviations deriving from two independent experiments are shown. For each strain, a number n ≥ 100 cells was counted. **C.** Anaphase duration was measured as the time from initial spindle elongation to interphase microtubule appearance. Exact values, means and standard deviations are shown. For the *cdc13-1* strain, a number n = 55 cells was counted, and for *cdc13-1 cdc14-1* strain, a number n = 44 cells was counted.

To gain further insight into the role of Cdc14 in persistent DNA damage conditions, we plan to assess the effect of both moderate activation (*TAB6-1* allele) and impairment (*cdc14-1* allele) of the phosphatase in the Ho background by means of spindle morphology read-out. Nevertheless, we have already an indication that Cdc14 may play the same role in both

backgrounds, as we found that the *CDC14*$^{TAB6\text{-}1}$ allele slightly increased the adaptation rate of *GAL-HO spo12Δ* and *GAL-HO slk19Δ* cells (assessed by budding read-out, see Figure 3.19).

3.3.4. Impairment of the functions of SAC does not impact on the FEAR mutants *spo12* and *slk19* in *cdc13-1*-induced damage conditions

By comparing the phenotypes associated with spindle dynamics in FEAR mutants both in *cdc13-1* and Ho-induced DNA damage, we noticed that a great portion of the cells remain arrested in metaphase in the *cdc13-1* background compared with the Ho background (see Figure 3.24 for data concerning the *cdc13-1* background, and Figure 3.27 for data concerning the Ho background). Since it has been reported that FEAR mutants *spo12* and *slk19* partially activate the spindle assembly checkpoint (SAC) (Stegmeier, Visintin and Amon, 2002), to exclude the possibility that these cells remain arrested in metaphase due to the activation of the SAC, we combined the FEAR mutants with the deletion of *MAD1* gene, a component of the SAC, and assessed at the resulting phenotype. The SAC inhibits the metaphase-to-anaphase transition until all chromosomes are correctly attached by microtubules emanating from opposite poles of the mitotic spindle (reviewed in (Akera and Watanabe, 2016). The SAC exerts its checkpoint functions by keeping the APC/C cofactor Cdc20 inhibited, which is responsible for the APC/C activation and consequent targeting of the securin Pds1 and mitotic cyclin Clb2 for proteasomal degradation. Briefly, the core components of the SAC include Mad1, Mad2, Mad3, Mps1, Bub1, Bub3, and Ipl1. At unattached kinetochores, Ipl1, Mps1, Bub1, and Bub3 are responsible for the recruitment of Mad1. In turn, Mad1 recruits Mad2 and induces its conformational change to assemble the mitotic checkpoint complex (MCC). Deletion of the *MAD1* gene results in cells that are unable to activate the SAC in case of improperly attached kinetochores (Hardwick, 1995).

We analyzed the consequences of the inactivation of Mad1 in *cdc13-1* cells, as well as in FEAR mutants *cdc13-1 spo12Δ* and *cdc13-1 slk19Δ* cells by looking at spindle morphology (Figure 3.33). We found that the percentage of cells that successfully enter in anaphase was not affected in any of the cell types tested in combination with the deletion of *MAD1* (in *cdc13-1* cells, from ~83% to 77%; in *cdc13-1 spo12Δ* cells, from ~22% to 19%; in *cdc13-1 slk19Δ* cells, and from ~26% to 19% in *cdc13-1 slk19Δ* cells). Moreover, we also found that the inactivation of Mad1 did not impact on the percentage of cells that remained arrested in metaphase (in *cdc13-1* cells, from ~16% to 22%; in *cdc13-1 spo12Δ* cells, from ~31% to 27%; and in *cdc13-1 slk19Δ* cells, from ~40% to 44% in *cdc13-1 slk19Δ* cells), therefore indicating that the persistent metaphase arrest is not linked to the activation of SAC in these cells.

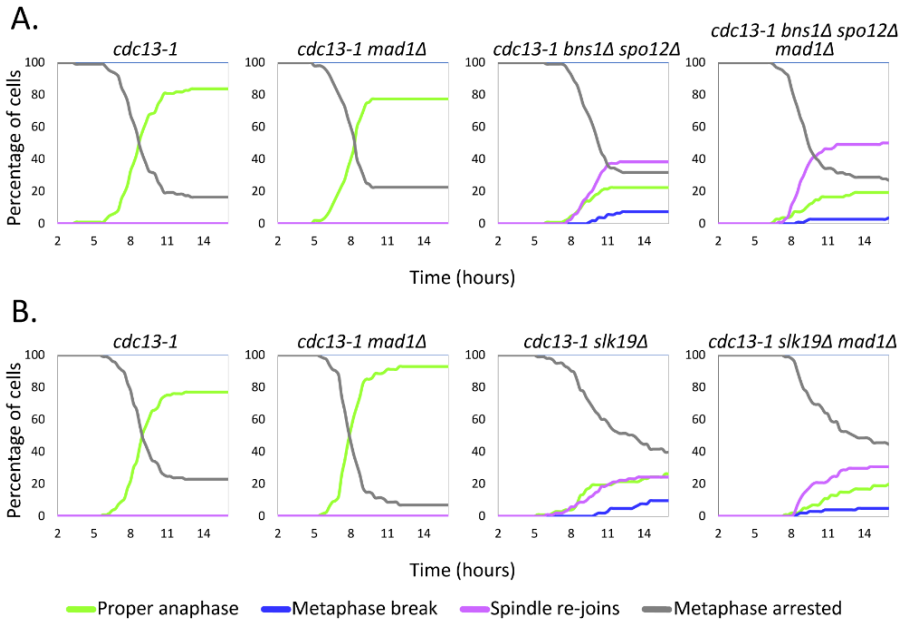

Figure 3.33 **Inactivation of the SAC has no effects on *cdc13-1 spo12* and *cdc13-1 slk19* mutant cells. A.** and **B.** *cdc13-1* (Ry6406), *cdc13-1 mad1Δ* (Ry8843), *cdc13-1 bns1Δ spo12Δ* (Ry6506), *cdc13-1 bns1Δ spo12Δ mad1Δ* (Ry8845), *cdc13-1 slk19Δ* (Ry6511), and *cdc13-1 slk19Δ mad1Δ* (Ry8846) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. For each strain, a number n ≥ 100 cells was counted.

3.3.5. *zds1Δ zds2Δ* cells show a mild defect in anaphase progression in persistent DNA damage conditions

So far, the analyses of *spo12* and *slk19* mutant cells revealed common features in spindle dynamics for exit from mitosis in persistent DNA damage conditions, but suggested that specific functions may be played by individual FEAR components. We were curious to assess whether the same phenotypes are shared by other members of the network. To this aim, we tested the behavior of other three FEAR components, Zds1/Zds2 (members of the Slk19 branch) that work together to counteract the phosphatase $PP2A^{Cdc55}$, which is an inhibitor of the FEAR network, and the overexpression of Fob1, an inhibitor of the FEAR network that acts both by keeping the Cdc14 sequestration machinery in the nucleolus (Toyn and Johnston, 1993; Stegmeier, Visintin and Amon, 2002; Buonomo *et al.*, 2003a; Tomson *et al.*, 2009; Bairwa *et al.*, 2010), and by inhibiting Spo12 (Stegmeier, Visintin and Amon, 2002; Tomson *et al.*, 2009).

We started by analyzing the spindle dynamics of *zds1 zds2* mutant cells and of the overexpression of *FOB1* in the *cdc13-1* background in single cells (Figure 3.34). We found that, while *cdc13-1 zds1Δ zds2Δ* mutant cells showed a mild defect in anaphase progression upon persistent DNA damage (from ~87% in *cdc13-1* cells to ~46% in *cdc13-1 zds1Δ zds2Δ* cells), the overexpression of *FOB1* resulted in a strong adaptation defect, with almost the whole population of cells arrested in metaphase at the end of the experiment (from ~61% in *cdc13-1* cells to ~9% in *cdc13-1 zds1Δ zds2Δ* cells). However, no cells showing the "spindle re-joins" dynamics were found. Analyses of anaphase duration revealed that *cdc13-1 zds1Δ zds2Δ* mutant cells require slightly longer time than *cdc13-1* cells to complete anaphase (from 27 minutes in *cdc13-1* cells to 33 minutes in combination with *zds1 zds2* deletions). These data suggest that the components of the FEAR network could play different roles in anaphase progression in persistent DNA damage conditions, which is somehow surprising as it was found that impairment of Zds1/Zds2 functions in unperturbed

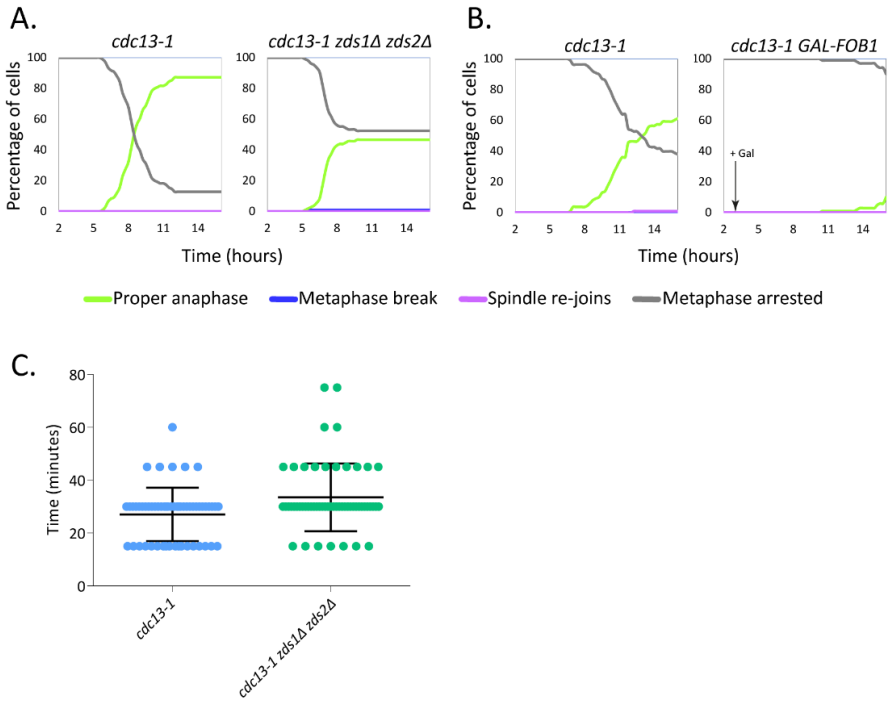

Figure 3.34**. Impairment of FEAR functions by mutating *ZDS1 ZDS2* genes or overexpressing the FEAR inhibitor *FOB1* impairs anaphase entry upon persistent DNA damage. A.** *cdc13-1* (Ry6406) and *cdc13-1 zds1Δ zds2Δ* (Ry8842) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released in YEPD medium at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. **B.** *cdc13-1* (Ry6406) and *cdc13-1 GAL-FOB1* (Ry8841) strains carrying a GFP-tagged version of α-Tubulin were arrested in the G_1 phase and released in YEPR at the restrictive temperature of 32°C in a microfluidic device for time-lapse imaging. Three hours after the release, 2% galactose was added to the medium. Timeframes were acquired every 15 minutes. The percentages of cells in each category (anaphase, spindle re-joins, metaphase break, and metaphase arrested) were scored for each timeframe. **C.** For strains in (A), anaphase duration was measured as the time from initial spindle elongation to interphase microtubule appearance. Exact values, means and standard deviations are shown. For each strain, a number n ≥ 50 cells was counted.

conditions resulted in a defect of the FEAR network that was comparable to the one of *spo12* mutant cells (Queralt and Uhlmann, 2008c). On the other hand, the adaptation defect observed in cells overexpressing *FOB1* is consistent with data in the literature, which indicate that high levels of Fob1 antagonize FEAR-mediated Cdc14 activation (Stegmeier *et al.*, 2004). However, such a strong defect is somehow surprising, given the phenotypes observed in other FEAR mutants in persistent DNA damage conditions. In the literature, the consequences of high levels of Fob1 on FEAR-mediated Cdc14 activation was probed only in combination with mutation of *MAD1*, a spindle assembly checkpoint protein (Stegmeier *et al.*, 2004). Therefore, it is possible that high levels of Fob1 have a negative effect *per se* on anaphase progression, in agreement with our data. To clarify the phenotype associated

with high levels of Fob1, we will combine the overexpression of *FOB1* with *mad1Δ* mutation and assess the resulting phenotype.

3.4. Is the checkpoint still active in *spo12* and *slk19* mutant cells after a prolonged damage condition?

Since single cell experiments revealed that a portion of *spo12Δ* and *slk19Δ* mutant cells show an initial spindle lengthening followed by a shortening, we wondered whether *spo12Δ* and *slk19Δ* mutant cells have a defect in checkpoint turn off. To this aim, we performed a time-course experiment of FEAR mutants in the *cdc13-1* background and we assessed the phosphorylation status of Rad53, bearing in mind the caveat that adaptation is not a synchronous process. Rad53 together with Chk1 are the effector kinases of the DDC, which are responsible for the checkpoint-mediated cell cycle arrest (Liang and Wang, 2007). When the checkpoint is active, Rad53 becomes hyperphosphorylated (Pellicioli *et al.*, 1999; Ma *et al.*, 2006). DDC inactivation, following checkpoint recovery or adaptation, leads to dephosphorylation of Rad53 (Leroy *et al.*, 2003), hence the phosphorylation status of Rad53 is used as a proxy for the DDC activation.

In *cdc13-1* cells, Rad53 phosphorylation increased, and after about 8 hours it began to gradually decrease, however, it did not reach a complete dephosphorylation state, probably because of a residue of non-adapting/dead cells in the population (Figure 3.35). In agreement with data in the literature (Pellicioli *et al.*, 2001), in *cdc13-1 cdc5-ad* cells, Rad53 phosphorylation increased and remained stable throughout the time-course. Interestingly, in *cdc13-1 spo12Δ* and *cdc13-1 slk19Δ* cells, Rad53 phosphorylation increased with the same kinetics as wild type and *cdc5-ad* cells, however, it began to decrease at about the same time and reached the same dephosphorylation state as *cdc13-1* mutant cells. Despite the limits of a population analysis discussed above, these results unambiguously indicate that *cdc13-1 spo12Δ* and *cdc13-1 slk19Δ* cells have already

switched off the DDC, but somehow are impaired in cell cycle progression. Intrigued by these observations, we wanted to assess whether these results hold true also in the Ho background.

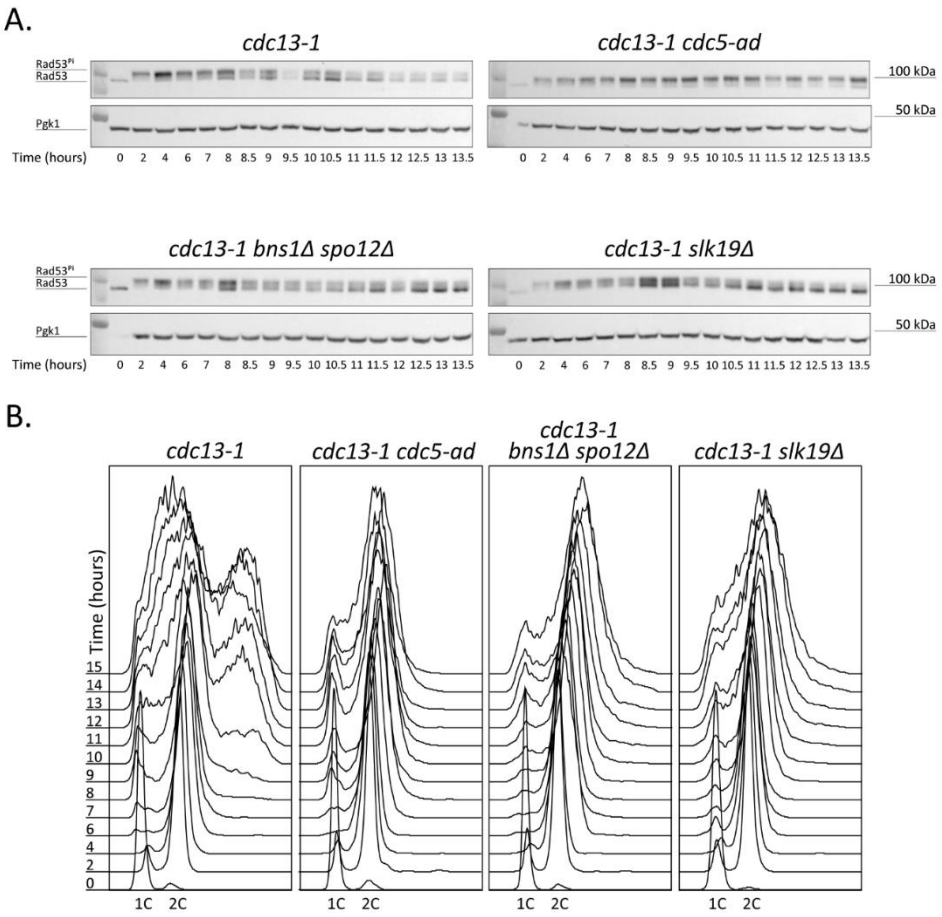

Figure 3.35 ***cdc13-1 spo12Δ* and *cdc13-1 slk19Δ* mutant cells are proficient in the DDC switch off but are impaired in the exit from mitosis.** c*dc13-1* (Ry6090)*, cdc13-1 cdc5-ad* (Ry8525)*, cdc13-1 bns1Δ spo12Δ* (Ry6378), and *cdc13-1 slk19Δ* (Ry6396) strains arrested in the G_1 phase were synchronously released at the restrictive temperature of 32°C in YEPD medium. At the indicated time points, cells were collected to determine **A.** Rad53 phosphorylation status by Western blot analyses (Pgk1 was used as loading control), and **B.** the DNA content by FACS analyses.

To this aim, we performed a time-course experiment of FEAR mutants in the Ho background (Figure 3.36), and similarly to what we observed in *cdc13-1* cells, we found that, after the release and induction of the DSB, the Rad53 phosphorylation increased in all of the tested strains. Although the DNA content analyses do not allow to appreciate the bypass of metaphase arrest and the entrance in the following G_1 phase, the phosphorylation began to gradually decrease after about 10 hours in *GAL-HO* cells, while it remained stable throughout the time course in *GAL-HO cdc5-ad* cells. Instead, in both *GAL-*

HO spo12Δ and *GAL-HO slk19Δ* cells, the phosphorylation began to gradually decrease at the same time as that of *GAL-HO* cells. Taken together, our data indicate that the activity of Spo12 and Slk19 is dispensable for DDC switch off but is implicated in other cell cycle events required for anaphase progression in persistent DSB conditions.

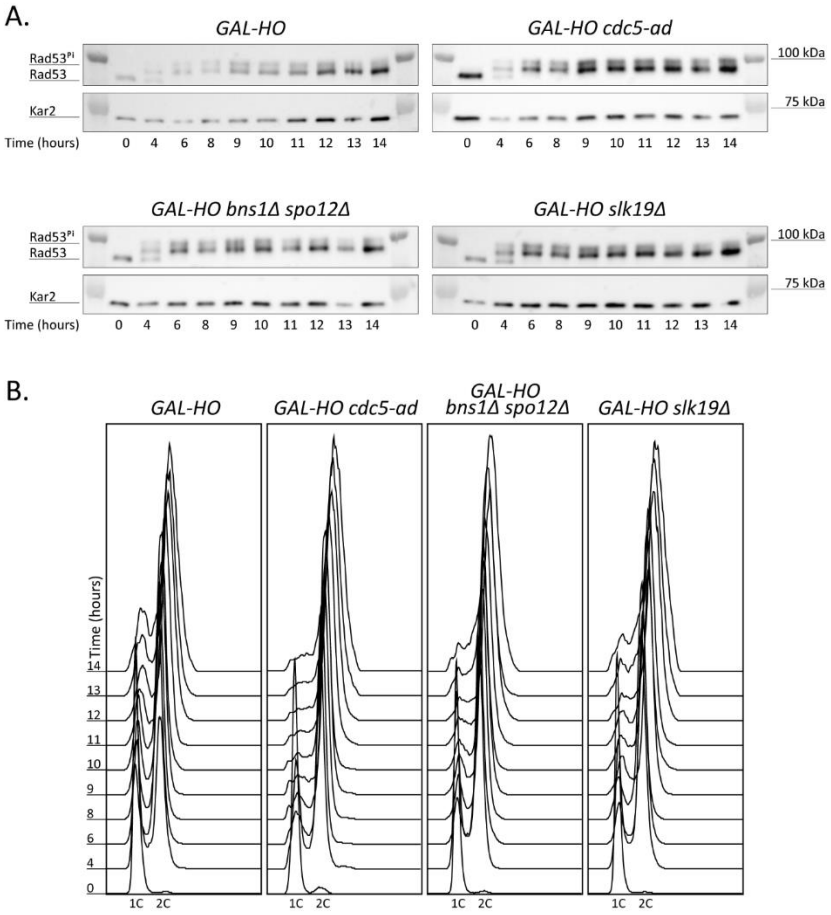

Figure 3.36 ***GAL-HO spo12Δ* and *GAL-HO slk19Δ* mutant cells are proficient in the DDC switch off but are impaired in the exit from mitosis.** *GAL-HO* (Ry2118), *GAL-HO cdc5-ad* (Ry8436), *GAL-HO bns1Δ spo12Δ* (Ry8428), and *GAL-HO slk19Δ* (Ry6890) strains arrested in the G_1 phase were synchronously released at the restrictive temperature of 32°C in YEPD medium. At the indicated time points, cells were collected for determination of **A.** Rad53 phosphorylation status by Western blot analyses (Pgk1 was used as loading control), and **B.** the DNA content by FACS analyses.

These results, together with the results obtained in the recovery assay, highlight checkpoint adaptation as the initiation of a specialized cell cycle, and as such require a peculiar molecular circuitry for the exit from mitosis, different from the one required in unperturbed conditions. Intrigued by these observations, we wished to investigate the peculiar molecular circuitry required for the exit from mitosis after checkpoint adaptation.

3.5. *In silico* screen identifies several cell cycle regulators, including FEAR components, in the adaptation process

Although adaptation to DNA damage checkpoint was observed for the first time in yeast back in 1993 (Sandell and Zakian, 1993), the molecular mechanism underlining this process remains elusive. To gain insights into how the FEAR network and, more in general, players involved in the exit from mitosis contribute to the adaptation response, we aimed at identifying new molecular players involved in this process. We reasoned that a cell cycle component involved in the adaptation response should have a role in cell resistance to a variety of DNA damaging agents. For these reasons, we decided to perform an *in silico* screen, taking advantage of data already reported in the literature. We created three datasets representing: a) genes found to have a genetic interaction with Cdc13, b) genes that confer resistance to methyl methanesulfonate (MMS), a DNA damaging drug, and c) genes involved in the exit from mitosis.

The dataset for *cdc13-1* synthetic genetic interactions (a) was retrieved from The Cell Map (*TheCellMap.org)*, a database collecting quantitative genetic interaction data from Synthetic Genetic Array (SGA) experiments (Usaj *et al.*, 2017). SGA is a high-throughput technique that allows systematic analyses of genetic interactions. Since we are interested in finding players that actively promote the adaptation process, we considered only genes that showed a negative genetic interaction with the *cdc13-1* allele, namely genes that when mutated show a worse growth fitness in combination with *cdc13-1* compared with the two single mutants. The datasets generated for (b) and (c) were retrieved from YeastMine (*yeastmine.yeastgenome.org*), a database that collects and integrates data from several sources, including annotations from the literature. We interrogated this database to retrieve: b) all genes annotated in the literature that confer resistance to MMS, namely those genes that when mutated confer a worse growth fitness in the presence of the drug;

and c) all genes associated with the exit from mitosis. The lists were intersected to find common elements that represent potential players in the adaptation response (Figure 3.37). We found a total of 131 candidate genes, of which only 15 were common in the three datasets (Table 3.1), and thus represent our favorite candidates; 28 genes were common in both the *cdc13-1* negative genetic interactions (a) and the exit from mitosis (c) lists (Table 3.2), and 88 genes were common in both the decreased resistance to MMS (b) and the exit from mitosis (c) lists (Table 3.3). Interestingly, among the identified genes, many FEAR components were represented, including Spo12, Fob1, Clb2, and Cdc14. Although this approach allows for the identification of novel candidates, it also has clear limitations, especially for false negative genes, as it is built on observations coming from the literature, implying that genes that never correlate with a certain phenotype will not be represented in the final list of candidates. Among the 131 identified candidates, the most interesting genes will be validated using the approaches presented in this thesis. Similar analyses will be performed to gain insights into negative regulators of the adaptation response.

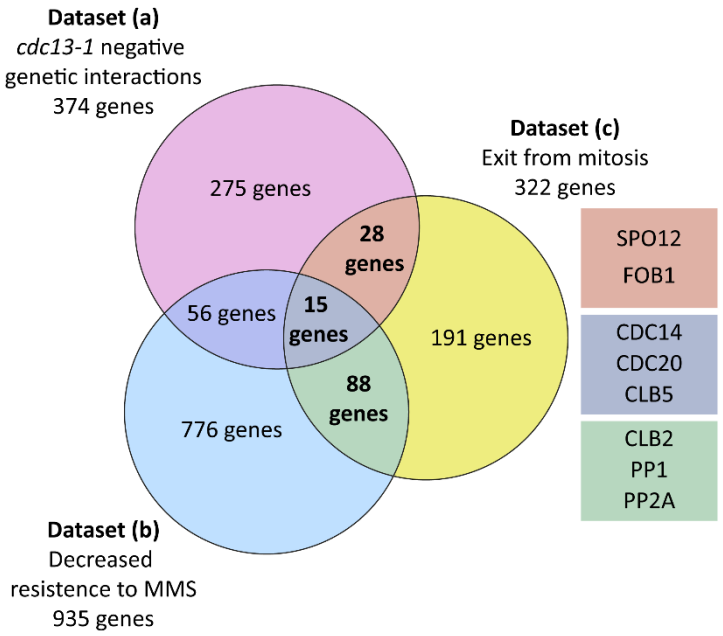

Figure 3.37 **In silico screen for identification of candidates in the adaptation response.** The screen was performed by combining three datasets: a) genes that show a negative genetic interaction with *cdc13-1*, b) genes that are important for conferring resistance to methyl methanesulfonate (MMS), and c) genes involved in the exit from mitosis. The three datasets were intersected in order to identify common elements. 131 candidates were identified, of which 15 are present in all of the three lists, 28 are present in the list (a) and in the list (c), and 88 are present in the list (b) and in list (c). FEAR components identified in the lists are shown.

Table 3.1 **Genes in common among *cdc13-1* negative genetic interactions, decreased resistance to MMS and exit from mitosis gene lists**

Systematic Gene Name	Standard Gene Name	Gene Name
YBR170C	NPL4	Nuclear Protein Localization
YCL061C	MRC1	Mediator of the Replication Checkpoint
YHR129C	ARP1	Actin-Related Protein
YHR191C	CTF8	Chromosome Transmission Fidelity
YFR028C	CDC14	Cell Division Cycle
YDR014W	RAD61	RADiation sensitive
YGL116W	CDC20	Cell Division Cycle
YGR092W	DBF2	DumbBell Former
YGR211W	ZPR1	Zinc finger PRotein
YMR048W	CSM3	Chromosome Segregation in Meiosis
YNL262W	POL2	POLymerase
YPL008W	CHL1	CHromosome Loss
YPR120C	CLB5	CycLin B
YPR135W	CTF4	Chromosome Transmission Fidelity
YPR141C	KAR3	KARyogamy

Table 3.2 **Genes in common among *cdc13-1* negative genetic interactions and exit from mitosis gene lists**

Systematic Gene Name	Standard Gene Name	Gene Name
YAL040C	CLN3	CycLiN
YAL041W	CDC24	Cell Division Cycle
YBR135W	CKS1	Cdc28 Kinase Subunit
YER149C	PEA2	PEAnut shmoo mutant
YHR061C	GIC1	GTPase Interactive Component
YHR152W	SPO12	SPOrulation
YIL046W	MET30	METhionine requiring
YIL131C	FKH1	ForK head Homolog
YKL052C	ASK1	Associated with Spindles and Kinetochores
YFL009W	CDC4	Cell Division Cycle
YDR110W	FOB1	FOrk Blocking less
YDR159W	SAC3	Suppressor of ACtin
YDR184C	ATC1	Aip Three Complex
YDR247W	VHS1	Viable in a Hal3 Sit4 background
YDR424C	DYN2	DYNein
YGL060W	YBP2	Yap1-Binding Protein
YGR040W	KSS1	Kinase Suppressor of Sst2 mutations
YGR058W	PEF1	Penta-EF-Hand protein
YJR059W	PTK2	Putative serine/Threonine protein Kinase
YLR212C	TUB4	TUBulin
YLR330W	CHS5	CHitin Synthase-related
YMR168C	CEP3	CEntromere Protein
YNL127W	FAR11	Factor ARrest
YNL164C	IBD2	Inhibition of Bud Division 2

YPL017C	IRC15	Increased Recombination Centers
YPL018W	CTF19	Chromosome Transmission Fidelity
YPL174C	NIP100	Nuclear ImPort
YPL253C	VIK1	Vegetative Interaction with Kar3p

Table 3.3 **Genes in common among decreased resistance to MMS and exit from mitosis gene lists**

Systematic Gene Name	Standard Gene Name	Gene Name
YAL021C	CCR4	Carbon Catabolite Repression
YBR088C	POL30	POLymerase
YBR160W	CDC28	Cell Division Cycle
YCL016C	DCC1	Defective in sister Chromatid Cohesion
YER016W	BIM1	BInding to Microtubules
YER111C	SWI4	SWItching deficient
YER133W	GLC7	GLyCogen
YHL027W	RIM101	Regulator of IME2
YHR030C	SLT2	Suppressor of the LyTic phenotype
YHR158C	KEL1	KELch repeat
YIL031W	ULP2	UbL-specific Protease
YIL126W	STH1	SNF Two Homolog
YIL153W	RRD1	Resistant to Rapamycin Deletion
YIR006C	PAN1	Poly(A)-binding protein-dependent poly(A) riboNuclease
YKL049C	CSE4	Chromosome SEgregation
YKL108W	SLD2	Synthetically Lethal with Dpb11-1
YKR072C	SIS2	SIt4 Suppressor
YFL039C	ACT1	ACTin
YFL008W	SMC1	Stability of MiniChromosomes
YFR027W	ECO1	Establishment of COhesion
YFR040W	SAP155	Sit4 Associated Protein
YDL003W	MCD1	Mitotic Chromosome Determinant
YDL017W	CDC7	Cell Division Cycle
YDL047W	SIT4	Suppressor of Initiation of Transcription
YDL056W	MBP1	MluI-box Binding Protein
YDL074C	BRE1	BREfeldin A sensitivity
YDL134C	PPH21	Protein PHosphatase
YDL155W	CLB3	CycLin B
YDL164C	CDC9	Cell Division Cycle
YDL188C	PPH22	Protein PHosphatase
YDL225W	SHS1	Seventh Homolog of Septin
YDR002W	YRB1	Yeast Ran Binder
YDR054C	CDC34	Cell Division Cycle
YDR099W	BMH2	Brain Modulosignalin Homolog
YDR217C	RAD9	RADiation sensitive
YDR253C	MET32	METhionine requiring
YDR260C	SWM1	Spore Wall Maturation
YDR318W	MCM21	MiniChromosome Maintenance
YDR363W	ESC2	Establishment of Silent Chromatin
YGL058W	RAD6	RADiation sensitive

YGL071W	AFT1	Activator of Ferrous Transport
YGR041W	BUD9	BUD site selection
YGR140W	CBF2	Centromere-Binding Factor
YGR188C	BUB1	Budding Uninhibited by Benzimidazole
YGR245C	SDA1	Severe Depolymerization of Actin
YJL047C	RTT101	Regulator of Ty1 Transposition
YJL074C	SMC3	Stability of MiniChromosomes
YJL090C	DPB11	DNA Polymerase B (II)
YJL194W	CDC6	Cell Division Cycle
YJR017C	ESS1	ESSential
YJR072C	NPA3	Nucleolar Preribosomal Associated
YJR076C	CDC11	Cell Division Cycle
YJR090C	GRR1	Glucose Repression-Resistant
YJR135C	MCM22	MiniChromosome Maintenance
YLR079W	SIC1	Substrate/Subunit Inhibitor of Cyclin-dependent protein kinase
YLR182W	SWI6	SWItching deficient
YLR226W	BUR2	Bypass UAS Requirement
YLR234W	TOP3	TOPoisomerase
YLR319C	BUD6	BUD site selection
YLR353W	BUD8	BUD site selection
YMR032W	HOF1	Homolog Of cdc Fifteen
YMR078C	CTF18	Chromosome Transmission Fidelity
YMR190C	SGS1	Slow Growth Suppressor
YMR198W	CIK1	Chromosome Instability and Karyogamy
YMR308C	PSE1	Protein Secretion Enhancer
YNL031C	HHT2	Histone H Three
YNL078W	NIS1	Neck protein Interacting with Septins
YNL084C	END3	ENDocytosis defective
YNL152W	INN1	required for INgressioN
YNL225C	CNM67	Chaotic Nuclear Migration
YNL233W	BNI4	Bud Neck Involved
YNL273W	TOF1	TOpoisomerase I-interacting Factor
YNL330C	RPD3	Reduced Potassium Dependency
YOL004W	SIN3	Switch INdependent
YOL006C	TOP1	TOPoisomerase
YOL133W	HRT1	High level expression Reduces Ty3 transposition
YOR014W	RTS1	Rox Three Suppressor
YOR144C	ELG1	Enhanced Level of Genomic instability
YOR198C	BFR1	BreFeldin A Resistance
YOR326W	MYO2	MYOsin
YOR372C	NDD1	Nuclear Division Defective
YPL020C	ULP1	UbL-specific Protease
YPL024W	RMI1	RecQ Mediated genome Instability
YPL116W	HOS3	Hda One Similar
YPL194W	DDC1	DNA Damage Checkpoint
YPR119W	CLB2	CycLin B
YPR122W	AXL1	AXiaL budding
YER014C-A	BUD25	BUD site selection

4. Discussion

When a DNA lesion is sensed, the DNA damage checkpoint is activated and halts cell cycle progression by directly inhibiting the pathways that control exit from mitosis (Liang and Wang, 2007). While the molecular events that lead to DDR activation and cell cycle arrest have been intensively studied and relatively well characterized, the molecular events that lead to checkpoint adaptation, that is exiting from mitosis with damaged DNA, are less well understood.

Originally identified in *S. cerevisiae*, checkpoint adaptation was long considered to occur only in unicellular organisms with the rationale that it would provide cells with an additional chance to repair DNA lesions in the following generation, therefore it was considered a beneficial process for cell fitness and preservation of the species. Conversely, as checkpoint adaptation could promote genomic instability, it was considered unlikely to occur in multicellular organisms (Lupardus and Cimprich, 2004; Yoo *et al.*, 2004), until 2006, when Syljuåsen and collaborators published the first report of checkpoint adaptation in human cells (Syljuåsen *et al.*, 2006). We now know that when challenged by damaged DNA most mammalian cells die by apoptosis, including a large fraction of cells that initially adapted. Yet, a small but biologically significant number of cells that undergo checkpoint adaptation survive (Kubara *et al.*, 2012; Swift and Golsteyn, 2016). Given that these cells progress through mitosis with faulty DNA, it is likely that they introduce changes to their genome. Since it has been reported that treatment-resistant tumors are often characterized by a complex genome organization (McLendon *et al.*, 2008; Muzny *et al.*, 2012), to understand how genomic complexity originates, it is essential to provide insights for the design of successful treatments for patients. With this in mind, understanding the molecular mechanisms that drive checkpoint adaptation, and learning how cells that have adapted to the DNA damage checkpoint survive are fundamental questions to be addressed.

The molecular mechanism causing adaptation, as well as the players involved in this process, remains largely unknown. Interestingly, a role for Polo-like kinase 1 (homolog of *S. cerevisiae* Cdc5) was observed both in *S. cerevisiae* and in human cells, suggesting the existence of an evolutionarily conserved mechanism (Syljuåsen *et al.*, 2006). Elucidating the role of Plk1 in regulating checkpoint adaptation is of special interest, as the polo kinase is frequently found overexpressed in a variety of cancers (reviewed in (Liu, Sun and Wang, 2017)).

In the work presented in this thesis, we integrated different approaches, including genetics, single cell analyses, and fluorescence microscopy techniques to address this question in budding yeast.

6.1. Checkpoint adaptation rewires the cell cycle machinery

The activation of kinases Rad53 and Chk1 is at the core of the checkpoint-mediated cell cycle arrest (Pellicioli *et al.*, 1999; Sanchez *et al.*, 1999; Ma *et al.*, 2006). When the checkpoint is active, Rad53 becomes hyperphosphorylated (Pellicioli *et al.*, 1999; Ma *et al.*, 2006). Checkpoint inactivation, following checkpoint recovery or adaptation, leads to dephosphorylation of Rad53 (Leroy *et al.*, 2003), this event is in part mediated by the protein phosphatases Ptc2 and Ptc3 (Leroy *et al.*, 2003; Heideker, Lis and Romesberg, 2007). In addition, the polo-like kinase Cdc5 contributes to the loss of Rad53 hyperphosphorylation and the consequent resumption of the cell cycle (Donnianni *et al.*, 2010; Vidanes *et al.*, 2010), through a mechanism independent from Ptc2 and Ptc3 (Vidanes *et al.*, 2010). It has been proposed that this event is mediated by a yet to be identified phosphatase (Vidanes *et al.*, 2010). Given that, in *S. cerevisiae*, Cdc5 is central for the activation of the CDK-counteracting phosphatase Cdc14, we hypothesized that the unknown phosphatase may be Cdc14 itself. In agreement with this hypothesis is the finding that mutants in components of the FEAR network (including Spo12, Slk19 and Cdc5 itself),

a pathway deputed to the activation of Cdc14 at anaphase onset, were also reported to be defective in the adaptation process (Toczyski, Galgoczy and Hartwell, 1997; Jin and Wang, 2006). Building from these observations, we tested the contribution of FEAR network components to the adaptation process.

The role of the FEAR network in the adaptation process was first described in *cdc13-1* mutant cells (Jin and Wang, 2006), in which a robust DNA damage checkpoint response is triggered by telomeres being perceived as DSB. Given that the FEAR network activity is implicated in telomere segregation (Clemente-Blanco *et al.*, 2011), to identify master regulators of the adaptation process, rather than factors specific for the *cdc13-1*-induced lesion, we decided to study adaptation also in presence of another DNA damage-inducing agent, namely the Ho endonuclease.

Given the exception of Cdc5, our data indicate that FEAR mutants are proficient in switching off the checkpoint but cannot exit mitosis. This essential requirement for the FEAR network activity appears to be unique for exit from mitosis with damaged DNA, since it is not observed either in unperturbed conditions (Stegmeier, Visintin and Amon, 2002; Roccuzzo *et al.*, 2015), nor for exit from mitosis following recovery, namely after DNA lesions have been repaired (both UV light-induced damage and DSB repair were tested). In unperturbed conditions, exit from mitosis is prevented only upon simultaneous inactivation of all the three branches of the FEAR network (Roccuzzo et al. 2015). However, since this essential requirement for individual components has been highlighted for the meiotic cell cycle as well (Buonomo *et al.*, 2003b; Marston, Lee and Amon, 2003), that is a specialized cell cycle in which one round of replication is followed by two divisions, we propose that mitosis in presence of DNA lesions elicits a specialized cell cycle with unique requirements.

What are the special features of this cell cycle? Looking at the problem from a naïve point of view, the difference between unperturbed *versus* persistent DNA damage conditions is basically the presence of an irreparable lesion. Therefore, one possibility is that the unique cell cycle requirements are a mere consequence of the persistent DNA damage condition *per se*. Once a lesion is sensed and the DNA damage response is activated, the checkpoint effectors mediate and coordinate a plethora of responses to deal with the damage and promote its repair (reviewed in (Finn, Lowndes and Grenon, 2012)). Following repair of the lesion, the cell cycle progression requires that all these responses are reverted. A similar mechanism can be envisioned also for the adaptation process. Therefore, a defect in reversing the DDC-mediated cellular responses could result in mitotic arrest. Little is known about the components and the mechanisms that mediate the reversal of DDC-mediated responses. Some proteins involved in reversal of DDC-mediated responses are found to be both recovery and adaptation-defective, examples of those include the phosphatases Ptc2 and Ptc3 (Leroy *et al.*, 2003) and the DNA repair proteins Sae2 and Srs2 (Vaze *et al.*, 2002; Baroni *et al.*, 2004; Clerici *et al.*, 2006), and some have been found to be important for checkpoint adaptation alone, including Cdc5-ad, CK2, Tid1 and others, suggesting that an additional layer of regulation exists. A second possibility is that the cell cycle machinery is rewired to overcome the DDR-mediated cellular responses instead of specific components of the recovery machinery.

A consequence of a persistent DNA lesion is the establishment of additional cohesion between sister chromatids in the region surrounding a DSB. This additional recruitment of the cohesin complex occurs in a DNA damage checkpoint-dependent manner, and it contributes to DNA repair (reviewed in (Marston, 2014)). Therefore, one challenge in exit from mitosis with damaged DNA is the removal of this extra cohesion.

As one of the first activities promoted by the DNA damage response is the attempt to repair the lesion, we expect that these cells enter mitosis with DNA intertwines, namely unresolved recombination intermediates that require to be resolved for chromosome segregation to occur. Of note, these are resolved by the cell cycle-regulated backup mechanisms that intervene in anaphase, namely the resolvases Mus81-Mms4 and Yen1. Interestingly, the activity of these two resolvases is regulated in a cell cycle-dependent manner by Cdc5 and Cdc14, respectively (Matos and West, 2014).

Last but not least, it is the observation that post-translational modifications other than phosphorylation mediated by the DDR may be involved in the process for exit from mitosis in persistent DNA damage conditions. Indeed, SUMOylation has recently emerged as a critical factor in multiple processes triggered in response to DNA damage (reviewed in (Jalal, Chalissery and Hassan, 2017)). SUMO has been shown to play important roles at multiple levels in DNA repair, including DSB repair, localization of recalcitrant DSB and telomeres at the nuclear periphery, and activity of telomere-associated proteins. Interestingly, many cell cycle components showed an increased SUMOylation upon MMS-induced DNA damage, including Cfi1 and Fob1, two inhibitors of the FEAR network (Gillies *et al.*, 2016). The SUMO signal is removed by SUMO proteases, Ulp1 and Ulp2. It was shown that Ulp2 protease is required for cell division following termination of the DNA damage checkpoint, and, importantly, *ulp2Δ* cells show aberrant mitotic spindles following checkpoint overcome in persistent DNA damage conditions (Schwartz, Felberbaum and Hochstrasser, 2007), similar to the ones identified for FEAR components.

To understand at molecular level the specific requirement of cell cycle components following checkpoint adaptation, we will investigate all the above-mentioned possibilities. To this aim, we will take advantage of the results from the *in silico* screen to reinterpret

them in the light of these speculations to identify potential molecular targets and processes that can explain the peculiarities of the cell cycle in the context of irreparable DNA lesions.

6.2. FEAR mutants exhibit faulty anaphases

In general, *spo12* and *slk19* mutants showed faulty anaphases. From our analyses we could distinguish two distinct defects: a) anaphase spindle elongation; and, in the case of cells that manage to elongate their spindles, b) longer anaphases. On top of these general defects, we observed background specific differences as well. More specifically, the two backgrounds differed in the fraction of cells arrested in metaphase ("metaphase arrested" category, prevalent in *cdc13-1* background cells) and in the fraction of cells undergoing proper anaphase ("proper anaphase" category, prevalent in Ho background cells). The reasons for these differences remain unclear. They can reflect: i) intrinsic differences among the two damage inducing conditions, with *cdc13-1* representing a more severe condition, as all telomeres are affected simultaneously, compared to a single proper DSB that, at least in principle, should be more tolerable by cells; or ii) a link between Cdc14 and telomere segregation; or, finally, iii) intrinsic differences among the two strain background used, with *cdc13-1* cells being in the W303 strain background, and *GAL-HO* cells being in the JKM179 strain background. Of course, the three possibilities are not mutually exclusive. As in *cdc13-1* background the percentage of metaphase arrested cells is greater than in the Ho, it is tempting to speculate that these discrepancies can be due to differences in the strength of the checkpoint elicited by the two systems. As in *cdc13-1* cells all telomeres are affected simultaneously, we hypothesize that this condition can have a major impact on cells in terms of viability. This speculation is also supported by three observations: a) in experiments concerning the budding read-out, both *cdc13-1* and FEAR mutants cells in the *cdc13-1* background showed a higher percentage of dead cells than in the Ho (i.e. for *spo12* mutant, about 58% in the *cdc13-1* background and 40% in the Ho); b) increasing the

temperature results in a greater percentage of dead cells in the *cdc13-1* mutant (data not shown); and, finally, c) impairing the checkpoint mediator protein Rad9 or the exonuclease Exo1, involved in checkpoint activation following uncapped telomeres, in *cdc13-1* cells results in cell growth at higher temperature (the maximal temperature for *cdc13-1 RAD9 EXO1* cells growth is 25°C, while for *cdc13-1 rad9 EXO1* cells is 28°C, and for *cdc13-1 RAD9 exo1* cells is 27°C), indicating that the activation DNA damage checkpoint is responsible for the lower viability of *cdc13-1* cells at restrictive temperature (Garvik, Carson and Hartwell, 1995; Maringele and Lydall, 2002b; Zubko, Guillard and Lydall, 2004; Addinall *et al.*, 2008).

Proper anaphase execution requires chromosome separation and segregation, stability and elongation of the anaphase spindle at anaphase onset, and, finally, disassembly of the anaphase spindle in late anaphase. The metaphase spindle breaks and spindles elongating and then re-joining phenotypes call for defects in chromosome segregation that can originate from defects in chromosome separation (i.e cohesion removal, resolution of DNA intertwines) or in spindle elongation. Differently, the longer anaphase phenotype calls for failures in anaphase spindle disassembly, hence mitotic CDK inactivation. Interestingly, FEAR components, including Cdc14 have been implicated in several of the processes mentioned. Indeed, FEAR-released Cdc14 activates important players for: a) resolution of residual cohesion between segregating DNA; b) dynamics and stability of the anaphase spindle; and c) activation of the MEN network, required for cells to exit from mitosis.

Regarding the resolution of residual cohesion between segregating DNA, FEAR-released Cdc14 has been implicated in rapid cohesin loss by removing stabilizing CDK-dependent phosphorylations on the securin Pds1, accelerating its proteolysis (Holt et al. 2008). Furthermore, Cdc14 activity removes cohesin-independent linkages to allow segregation of the telomeres and the rDNA (D'Amours et al. 2004; Sullivan et al. 2004), and finally, it

plays a role in the resolution of homologous recombination intermediates (Blanco, Matos and West, 2014; Eissler *et al.*, 2014).

Of particular importance for anaphase spindle stabilization and elongation is the spindle midzone, where a number of spindle motor proteins and microtubule-associated proteins (MAPs) associates to mediate sliding and stabilization of interphase microtubules during anaphase. FEAR-released Cdc14 activity contributes to dynamics and stability of the anaphase spindle by dephosphorylating several components of the midzone to enable their association with the spindle (Pereira and Schiebel, 2003; Higuchi and Uhlmann, 2005; Khmelinskii *et al.*, 2007).

Finally, Cdc14 released by the FEAR network directly promotes the activation of the MEN, therefore self-sustaining its own release in late anaphase through a positive feedback loop (Jaspersen and Morgan, 2000; Pereira *et al.*, 2002; Stegmeier, Visintin and Amon, 2002; Bardin, Boselli and Amon, 2003). MEN-released Cdc14 promotes anaphase spindle disassembly by activating the APC/C^{Cdh1} complex that targets for degradation the components of the spindle midzone (Hildebrandt and Hoyt 2001; Juang et al. 1997).

To get insights on whether the defects in anaphase spindle dynamics are due to one or more of such processes, we plan to characterize the defects observed in *spo12* and *slk19* mutants by measuring the speed of spindle elongation. As anaphase spindle elongation is characterized by two phases, one initial fast elongation, followed by a second slow elongation phase, by measuring the speed of spindle elongation, we have an indication on whether the defects in anaphase spindle dynamics are due to one or more of such processes. If we find that there is a defect in initial spindle elongation, mechanisms for chromosome separation completion and spindle stability and elongation will be investigated first, while if we find that the defect is in disassembly, the mechanisms regulating this process will be investigated instead (Figure 4.1).

Having highlighted abnormal spindle dynamics in FEAR mutants in persistent DNA damage conditions, we asked whether individual FEAR components have specific FEAR-independent roles in this process, or whether is the final effector of the FEAR network, Cdc14, that mediates these functions (Figure 4.1). Regarding possible FEAR-unrelated functions of individual components, for Cdc5 the answer is more obvious. Besides the well-known role in promoting the inhibition of the checkpoint effector kinase Rad53 (Donnianni *et al.*, 2010; Vidanes *et al.*, 2010), Cdc5 phosphorylates cohesin subunit Scc1 to promote the efficient cleavage of the cohesin ring by the separase Esp1, that allows for sister chromatids separation (Ciosk *et al.*, 1998; Alexandru *et al.*, 2001; Hornig and Uhlmann, 2004). In anaphase, Cdc5 promotes both transient and full release of Cdc14 via its activity in FEAR and MEN networks, respectively (Visintin, Stegmeier and Amon, 2003). Recently, a new role for Cdc5 in anaphase spindle elongation was highlighted, where, together with Cdc14, regulates the activity of the motor protein Cin8 (Roccuzzo *et al.*, 2015), therefore promoting sister chromatid segregation. Given these considerations, we speculate that in persistent DNA damage conditions, Cdc5 may coordinate the checkpoint overcome with the other important events required for exit from mitosis, including the activation of Cdc14.

Less obvious is to envision a Cdc14-independent contribution for Spo12 and Slk19. Besides its role in promoting the release of Cdc14, nothing is known for Spo12 in terms of enzymatic activity. On the other hand, Slk19 has been already implicated in spindle stabilization (Khmelinskii *et al.*, 2007) and kinetochore clustering (Richmond *et al.*, 2013), therefore suggesting an additional contribution of Slk19 in spindle dynamics in persistent DNA damage conditions. This speculation is also supported by the observation that moderate Cdc14 activation did not perfectly compensate for the spindle phenotype in *slk19* mutants, and that *zds1Δ zds2Δ* cells (components belonging to the Slk19 branch) did not recapitulate the defect observed in *slk19Δ* cells. Taken together, these findings would argue that specific

functions in the adaptation process may be played by individual FEAR components, or at least by Slk19. To get a clear answer on specific functions of single components *versus* Cdc14 activity in exit from mitosis following checkpoint adaptation, additional FEAR network components will be tested to highlight coherence or different functions among components belonging to the same branch. In respect to the role of single components, we plan to test also the contribution of a peculiar molecular circuitry at the core of the FEAR network regulation, recently highlighted in our laboratory. The major mitotic cyclin Clb2 promotes Cdc14 release as part of the FEAR network. However, we found that to mediate its FEAR functions, Clb2 has to be previously phosphorylated by Cdc5 (Busnelli, Dondi and Visintin, *manuscript in preparation*). Interestingly, we found that Cdc5-ad protein variant, although kinase proficient (Charles *et al.*, 1998; Serrano and D'Amours, 2016), is defective in Clb2 phosphorylation, and that the *clb2-AA* mutant (where the Cdc5 phosphorylation sites were mutated into the non-phosphorylatable amino acid alanine) is defective in the first wave of Clb2 degradation occurring at the metaphase to anaphase transition. As in unperturbed conditions the first wave of mitotic cyclin degradation does not impair exit from mitosis, we ask whether it becomes essential for cell cycle resumption following checkpoint overcome, therefore providing insights on the possible special molecular circuitry required for exit from mitosis in persistent DNA damage conditions.

Nevertheless, given the observation that: a) *CDC5* overexpression partially compensated for the absence of other FEAR components (assessed by bud formation); b) *CDC5* overexpression is sufficient to induce Cdc14 release in unperturbed conditions (Visintin, Stegmeier and Amon, 2003); c) both *spo12Δ* and *slk19Δ* cells showed the same abnormal spindle phenotype; d) the abnormal spindle phenotype was partially restored by moderate Cdc14 activation, achieved by the *TAB6-1* allele; e) the release mediated by the *TAB6-1* allele is not equivalent to the one mediated by FEAR, as in this mutant Cdc14 is localized

also in the cytoplasm; and, finally, f) *cdc14-1* cells showed abnormal spindle phenotype as well, we speculate that the main contribution of FEAR in proper anaphase spindle elongation is via Cdc14.

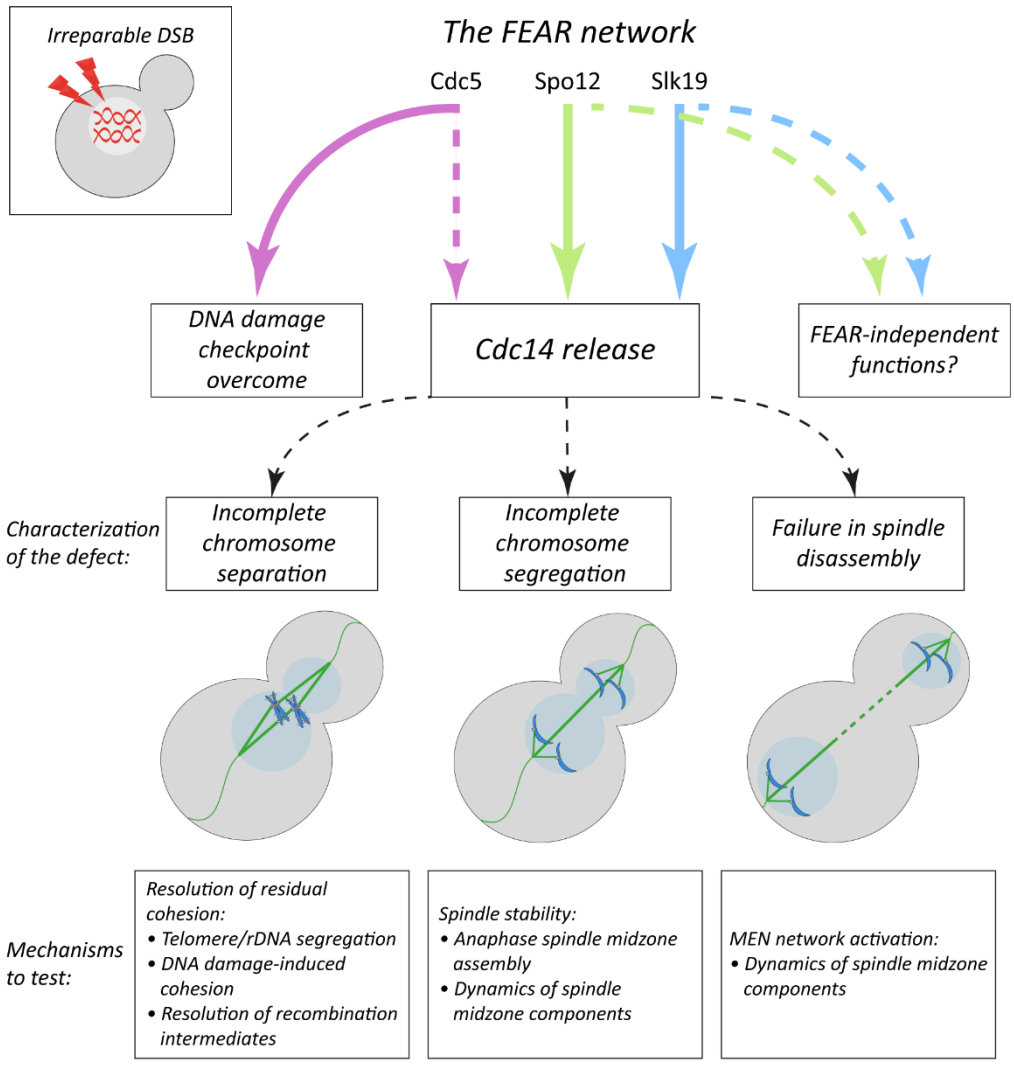

Figure 4.1 Working model depicting the mechanisms that will be tested to elucidate the contribution of the FEAR network to cell cycle progression in persistent DNA damage conditions.

Our investigations will continue by characterizing in more details the defect spindle dynamics. For instance, if our analyses will point to defects in chromosome separation as causing factors, we will test for the presence of residual DNA cohesion by looking at cohesin complex exploiting a GFP-tagged version of the cohesin subunit Scc1 in single cell analyses. Moreover, we will test for complete rDNA and telomere segregation by looking at specific loci on sister chromatids exploiting *TetR-GFP/tetO* system in which an array of *tetO*

operators have been integrated in a specific locus of interest and it is bound by a GFP-tagged version of TetR repressor (Michaelis, Ciosk and Nasmyth, 1997). With this system, we will analyze the segregation of three loci: centromere (*CENXV*), a locus in the middle of chromosome arm (*HIS3*) and a telomere (*TELXV*), all located on the long arm of chromosome XV (Renshaw *et al.*, 2010).

Moreover, in order to understand whether the activities of FEAR network and Cdc14 are required for reversal of some specific post-translational modifications, we need to correlate the activation status of the DNA damage checkpoint (i. e. phosphorylation of Rad53) with the activation of the FEAR network. Given the above-mentioned limits of population-based approaches, we plan to create a fluorescent biosensor to assess post-translational modification in single cell analyses (Oldach and Zhang, 2014). Based on genetically encoded biosensors developed for assessment of kinase activity in live-cell imaging, we plan to design biosensors for the most important DNA damage kinases, namely Mec1, Rad53, and Chk1. The first question that we will answer with this tool is if and how FEAR-activated Cdc14 is directly or indirectly involved in the reversal of some specific DDC-mediated phosphorylation event, therefore providing a mechanism for the crosstalk between the cell cycle and the DDR machineries.

6.3. What do we really consider as checkpoint adaptation?

The original definition of checkpoint adaptation provided by Hartwell and colleagues foresee checkpoint adaptation as the result of three consecutive steps: a) a cell cycle arrest following detection of DNA lesions; b) overcoming of the checkpoint-mediated arrest; and c) re-entering in the cell cycle in presence of DNA lesions (Toczyski, Galgoczy and Hartwell, 1997). Consistently, checkpoint adaptation-defective mutants identified so far, including *cdc5-ad, sae2Δ, tid1Δ* and others, show a persistent Rad53 phosphorylation upon irreparable DNA damage induction and remain in a permanent arrested state as a consequence of the inability

to overcome the DNA checkpoint signaling. However, FEAR mutants were classified as adaptation-defective by looking at the ability of cells of entering in a new cell cycle in persistent DNA damage conditions (probed by microcolony assay) (Jin and Wang, 2006). Assessing adaptation through bud emergence or microcolony formation implies that these cells have to accomplish three consecutive but distinct processes: a) checkpoint overcome; b) resuming the cell cycle to exit from mitosis; and c) entering in a new cell cycle, reaching the S phase, when the bud emerges. Therefore, probing the adaptation phenotype by looking at bud emergence without evaluating the checkpoint activation state does not allow to assess whether the overcome of the checkpoint and the following exit from mitosis really occurred.

Checkpoint adaptation is considered a beneficial process for cell fitness and preservation of the species, as it represents an additional chance to fix an irreparable DNA lesion in the following generation. Given these considerations, we ask what checkpoint adaptation should be considered like: should it be intended solely as the overcome of the checkpoint (consistent with the original definition) or should it be intended as the result of two processes, namely the switch off of the checkpoint and reentering in the cell cycle? As our findings indicate that the overcome of the checkpoint *per se* is not sufficient to drive exit from mitosis, hence the survival of cells, and suggest a rewiring of a special cell cycle for cells to adapt, we envision the adaptation process as a more complex mechanism that cannot be fully described solely by overcoming of the checkpoint-mediated arrest.

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