Centrosome scaffolds and combinatorial PBD binding as drivers of functional diversity in the budding yeast Polo-like kinase Cdc5

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ABSTRACT

Upon exposure to DNA damage, eukaryotic cells preserve genome integrity by coordinating DNA repair with transient, checkpoint-mediated cell cycle arrest. When moderate levels of irreparable chromosomal lesions persist, cells may override this arrest through DNA damage adaptation, a process driven by the Polo-like kinase 1 (PLK1) in humans and its conserved ortholog Cdc5 in budding yeast. While Polo-box domain (PBD)-mediated enrichment of Cdc5 at spindle pole bodies (SPBs) –the yeast equivalent of centrosomes– is known to be required for adaptation, the molecular basis by which SPBs mediate this response has remained unclear. In this thesis, we demonstrate that SPBs actively contribute to DNA damage adaptation by functioning as supramolecular signaling hubs for Cdc5. We identify three core SPB components –Nud1, Spc110, and Spc72– as critical mediators of Cdc5's docking to both the nuclear and cytoplasmic domains of the organelle, a step essential for Cdc5-dependent adaptation. Upon recruitment, Cdc5 exploits the nuclear envelope (NE) protein Mps3 and the outer plaque component Cnm67 to propagate a phospho-signal necessary for full adaptation. Mutations disrupting Cdc5's interactions with these SPB targets impair adaptation without affecting microtubule-organizing center (MTOC) activity, indicating that SPB signaling –rather than canonical MTOC function– is essential for this response. This specificity is further supported by the observation that generic MTOC disruption does not recapitulate the defect. Finally, through structural and biochemical analyses, we show that Cdc5 expands its substrate repertoire through dual, non-competitive PBD-driven binding. Point mutations that selectively impair an alternative hydrophobic interface –distinct from the canonical phosphopeptide-binding pocket– induce an early anaphase arrest, underscoring the functional significance of combinatorial PBD interactions in Cdc5-regulated mitotic events. Together, these findings reveal how PLKs integrate spatially defined SPB signaling with versatile substrate recognition to coordinate mitotic progression under both normal and genotoxic cycling conditions, thereby safeguarding genome stability with adaptive flexibility.

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LIST OF ABBREVIATIONS

3D: Three-dimensional

4-NQO: 4-nitroquinoline 1-oxide

AKAPs: A-kinase anchoring proteins

ALT: Alternative lengthening of telomeres

AMPK: AMP-activated protein kinase

AP: Apyrimidinic/apurinic site; Anterior–posterior axis

APC: Anaphase-promoting complex

ATP: Adenosine triphosphate

BCC: Breast cancer cell

BER: Base-excision repair

BI: Budding index

BIR: Break-induced replication

C-Mad2: Closed Mad2

CAK: CDK-activating kinase

CAR: Contractile actin ring

CASP2: Cell-death effector caspase-2

CDC: Cell division cycle

CDK: Cyclin-dependent kinase

CFS: Common fragile site

°C: Degree Celsius

CIN: Chromosomal instability

CKII: Casein kinase II

CMG: Cdc45–MCM–GINS

CO: Crossover

COFS: Cerebro-oculo-facio-skeletal (syndrome)

CS: Cockayne syndrome

CPC: Chromosomal passenger complex

CPD: Cyclobutane pyrimidine dimer

CRADD: CASP2 and RIPK1 domain-containing adaptor with death domain

DAPI: 4’,6-diamidino-2-phenylindole

DAPs: Distal appendages

DDICA: DNA damage-induced centrosome amplification

DDR: DNA damage response

DNA: Deoxyribonucleic acid

DRC: DNA replication checkpoint

DSB: Double-strand break

DSC: De Sanctis-Cacchione (syndrome)

dsDNA: Double-stranded DNA

DVL: Dishevelled

EM: Electron microscopy

EMT: Epithelial-to-mesenchymal transition

ER: Endoplasmic reticulum

FA: Fanconi anemia

FEAR: Cdc fourteen early anaphase release

G1: First gap phase

G2: Second gap phase

GAP: GTPase-activating protein

GEF: Guanine-exchange factor

GFP: Green fluorescent protein

GSC: Germline stem cell

Gwl: Greatwall

HBOC: Hereditary breast and ovarian cancer

HJ: Holliday junction

HOG: High osmolarity glycerol pathway

HR: Homologous recombination

hrs: Hours

HSP: Heat shock protein

HSR: Heat shock response

HU: Hydroxyurea

hyperPP: Hyperphosphorylated

ICL: Interstrand crosslink

IDLs: Insertion/deletion loops

IL: Intermediate layer

IR: Ionizing radiation

KD: Kinase-dead

KPO4: Potassium phosphate

MCC: Mitotic checkpoint complex

ME: Mitotic exit

MEN: Mitotic exit network

MGMT: O^6-methylguanine-DNA methyltransferase

MiDAS: POLD3-dependent mitotic DNA synthesis

MMR: Mismatch repair

MMS: Methyl methanesulfonate

MPF: Mitosis-promoting factor

MRX: Mre11–Rad50–Xrs2

MSI: Microsatellite instability

MT: Microtubule

MTOC: Microtubule-organizing center

NABs: Nucleus-associated bodies

NC: Nuclear–centrosomal

NCO: Non-crossover

NE: Nuclear envelope

NEBD: Nuclear envelope breakdown

NEKs: NIMA-related kinases

NER: Nucleotide-excision repair

NHEJ: Non-homologous end joining

NLS: Nuclear localization signal

NRF2: Nuclear factor erythroid 2-related factor 2

NuMA: Nuclear mitotic apparatus protein

nMTs: Nuclear microtubules

O-Mad2: Open Mad2

OD: Optical density

PARP: Poly(ADP-ribose) polymerase

PBD: Polo-box domain

PCP: Planar cell polarity

PCNT: Pericentrin

PCM: Pericentriolar material

PIDD1: p53-induced death domain-containing protein 1

PKA: Protein kinase A

PLK: Polo-like kinase

PP: Phosphorylated

PP2A: Protein phosphatase 2A

Pre-RCs: Pre-replication complexes

PROTAC: Proteolysis-targeting chimera

PTM: Post-translational modification

RB: Retinoblastoma

RCD: Regulated cell death

RENT: Regulator of nucleolar silencing and telophase

RLS: Replicative lifespan

ROS: Reactive oxygen species

RSR: Replication stress response

r.m.s.: Root mean square

SAC: Spindle assembly checkpoint

SAPK: Stress-activated protein kinase

SCID: Severe combined immunodeficiency

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

sDAPs: Subdistal appendages

SEM: Standard error of the mean

SI: Supplemental information

SIN: Septum initiation network

SPB: Spindle pole body

SPoC: Spindle position checkpoint

ssDNA: Single-stranded DNA

STD: Saturation-transfer difference

STOC: Signal transduction organizing center

STR: Saturation transfer reference

SUN: Sad1–UNC-84

TEV: Tobacco etch virus

TMZ: Temozolomide

Ts: Temperature-sensitive

TTD: Trichothiodystrophy

UV: Ultraviolet light

unPP: Unphosphorylated

WT: Wild-type

XFEPS: XPF-ERCC1 progeroid syndrome

XP: Xeroderma pigmentosum

YEPD: Yeast extract peptone dextrose

YEPG: Yeast extract peptone galactose

zPLK1: Zebrafish Polo-like kinase 1

SCIENTIFIC CONTRIBUTIONS

Manuscript #1 (Chapter II): **Langlois-Lemay L**, D'Amours D. Yeast centrosomes act as organizing centers to promote Polo kinase-mediated adaptation to persistent DNA damage. Proc Natl Acad Sci U S A. 2025 Jul 8;122(27):e2414426122. doi: 10.1073/pnas.2414426122. Epub 2025 Jul 2. PMID: 40601630.

[LLL and DD conceived and designed yeast experiments; LLL performed all experiments; LLL prepared all figures; LLL and DD wrote the paper.]

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*L.L.-L. and D.D. designed the experiments corresponding to Figures 3.6, 3.7, and 3.S4. L.L.-L. performed and analyzed these experiments and prepared Figures 3.6, 3.7, 3.S4, and Table 3.S2.]

Manuscript #3 (review article)*: **Langlois-Lemay L**, D'Amours D. Moonlighting at the Poles: Non-Canonical Functions of Centrosomes. Front Cell Dev Biol. 2022 Jul 14;10:930355. doi: 10.3389/fcell.2022.930355. PMID: 35912107; PMCID: PMC9329689.

[LL-L and DD'A made a substantial and direct contribution to the work.]

*Reproduced verbatim in **Chapter I – General introduction: Sections 1.3.1. and 1.7.**

1 CHAPTER I: GENERAL INTRODUCTION

1.1 Pioneers of the cell cycle

The depiction of a cell, the most fundamental unit of life, originally took place in 1665 when British scientist Robert Hooke, equipped with a compound microscope, sought to examine a thin piece of cork under high magnification. Hooke witnessed endless rows of ''tiny pores'', which he analogously compared to the compartmentalized architecture of traditional monastic cells (Robert Hooke 1665; Uzbekov and Prigent 2022). Since then, the term ''cell'' has been universally adopted to represent the building block of all living organisms, a pivotal finding that has played an instrumental role in advancing our understanding of how cellular mechanisms influence biological processes. In 1882, Walther Flemming was the first to successfully distinguish the different stages of the cell cycle using cytological staining techniques (Flemming, W 1882). This seminal work led to the characterization of filaments found within the nucleus, eventually termed ''chromosomes'' by Heinrich Wilhelm Gottfried von Waldeyer-Hartz (von Waldeyer-Hartz, W. 1888), fluctuating in shape and size prior to cell division. In 1953, James Watson and Francis Crick recognized the deoxyribonucleic acid (DNA) structure as the molecule carrying genetic information and dictating the development, function, and reproduction of a living organism (Watson and Crick 1953b; 1953a). It was only in the 1960s that the notion of the cell cycle as a universal four-stage process (G1, S, G2, M) was globally accepted as a fundamental framework across eukaryotic species.

Given the central role of the cell division cycle in ensuring the faithful segregation of duplicated DNA to daughter cells and its critical impact on genome stability, researchers worldwide began investigating the regulatory mechanisms governing the cell cycle. In 1967, Canadian zoologist Yoshio Masui was the first to demonstrate that maturation-promoting factors (MPFs, or mitosis-promoting factors) could spontaneously drive isolated oocytes to the following stage of their ongoing cell division cycle (Masui 1967). Concomitantly, geneticist and Nobel laureate Leland Hartwell explored the processes driving cell cycle progression using the budding yeast *Saccharomyces cerevisiae*, a eukaryotic unicellular system showing exceptional qualities

as a model organism for fundamental cell cycle studies (See section 1.8 for more details). Hartwell isolated a range of Cell Division Cycle ''*cdc*" thermosensitive mutants, including alleles of *CDC28* (yeast homolog to human CDK1), that would consistently arrest at specific stages of the cell cycle (Hartwell 1967; Hartwell et al. 1970; 1973; 1974). In parallel, Paul Nurse's pioneering work in fission yeast yielded crucial insights on the regulation of cell cycle progression, notably through the identification of *cdc2* as a master regulator of cell cycle transitions (Nurse et al. 1976; Nasmyth and Nurse 1981; Nurse and Bissett 1981). From that point onwards, the existence of driving forces behaving as molecular clocks leading cells through cell cycle transitions became evident. Tim Hunt's seminal discovery of the cyclin molecule in 1982 –stemming from his work on sea urchin eggs– transformed our understanding of cell cycle regulation by demonstrating that the periodic oscillation of cyclin levels, governed by precisely coordinated synthesis and degradation, drives critical cell cycle transitions (Evans et al. 1983). In 1987, Paul Nurse's identification of CDK1 as the human homolog of fission yeast Cdc2 significantly advanced our understanding of cell cycle regulation by revealing the evolutionary conservation of these mechanisms across species (Lee and Nurse 1987), a conceptual advance he further articulated in subsequent reviews (Nurse 1990; 2000; 2002). Building on this conceptual framework, Hartwell and Weinert later introduced the notion of conserved cell cycle checkpoints as intrinsic quality control systems overseeing to the accuracy of essential cellular processes and working in concert with the molecular forces driving the cell cycle (Hartwell and Weinert 1989).

While these pivotal discoveries have profoundly shaped our current understanding of the cell cycle and its regulatory features, many of the molecular mechanisms capable of modulating cell cycle progression under both normal and strenuous cycling conditions remain poorly understood. The catastrophic consequences associated with cell cycle dysregulation and genome instability –including oncogenic transformation– are severely detrimental to organismal homeostasis and long-term survival. A comprehensive understanding of the regulatory processes designed to maximise survival under fluctuating

cycling conditions is, therefore, crucial to effectively mitigate the adverse consequences of inappropriate cell division at the onset of human disease.

1.2 Fundamentals of the eukaryotic cell cycle

1.2.1 Overview of the eukaryotic cell cycle

The eukaryotic cell cycle encompasses an organized and meticulously regulated series of events facilitating the duplication and distribution of genetic material into two daughter cells. This division cycle is composed of two main states: Interphase, where cells undergo cell growth and chromosome duplication to prepare for cell division, and mitosis, where cells equally distribute duplicated DNA into daughter nuclei. Both interphase and mitosis can be dissected into discrete sub-phases. (Figure 1.1.).

Interphase typically comprises a regulatory G1 (Gap 1) phase, where metabolically active cells increase in size and commit –pending favorable growth conditions– to cell division. The G1 phase is followed by an S phase (synthesis), in which DNA and other cellular components are replicated and chromosomes are duplicated. In most higher eukaryotes, chromosome duplication is closely followed by a second regulatory phase, termed G2 (Gap 2), where cell growth and supervisory processes take place to ensure the accuracy and success of the upcoming round of cell division. However, the G2 phase is not a prerequisite among all eukaryotes and certain species, including the budding yeast *Saccharomyces cerevisiae,* closely link the end of chromosome duplication with the onset of mitosis, with no distinct G2 phase.

During mitosis, eukaryotic cells undergo five distinct chromosomal events. In prophase, the amorphous chromatin condenses into concrete rod-shaped chromosome with identical sister chromatids joined at the centromere. The microtubule-organizing centers (MTOCs), termed centrosomes in higher eukaryotes and spindle pole bodies (SPBs) in yeast, migrate to opposite poles –of the cell in higher eukaryotes, and of the nucleus in yeast– to organize the formation of the mitotic spindle. In eukaryotic species undergoing open mitosis, nuclear envelope breakdown occurs during prometaphase, enabling

spindle microtubules (MTs) to connect with kinetochore-bound sister chromatids. In contrast, certain species –including budding yeast– undergo closed mitosis, maintaining an intact nuclear envelope throughout division and thereby lacking a classical prometaphase stage (Taddei and Gasser 2012). In these organisms, SPBs remain firmly embedded within the nuclear envelope, and nucleate MTs on both the cytoplasmic and nuclear faces of the organelle. Once attached to spindle fibers, sister chromatids migrate toward the cell center. During metaphase, sister chromatids align at the metaphase plate, located perpendicular to the spindle axis of the cell, where they are inspected for tension and bi-orientation – two essential features for successful chromosome segregation (Cleveland et al. 2003; Bunning and Gupta Jr. 2023; Li et al. 2024). The alignment of chromosomes on the metaphase plate is not an absolute requirement for eukaryotic mitosis, as the budding yeast *Saccharomyces cerevisiae* typically enters anaphase with scattered chromosomes (Straight et al. 1997; Morgan 2007). In anaphase A, sister chromatids are pulled toward opposite poles of the cell as kinetochore MTs shorten. During anaphase B, interpolar MTs, extending from the spindle poles, push against each other, further elongating the spindle axis of the cell. This sets the stage for telophase, during which daughter chromosomes gradually decondense to ultimately reach the amorphous interphase DNA state. Nuclear envelopes begin to form around each set of segregated chromosomes, and the mitotic spindle disassembles. Cytokinesis then allows for physical cell separation through actomyosin ring constriction and septum deposition (Bi et al. 1998). Once complete, the cell division cycle restarts as the newly formed daughter cells go back to the interphase state.

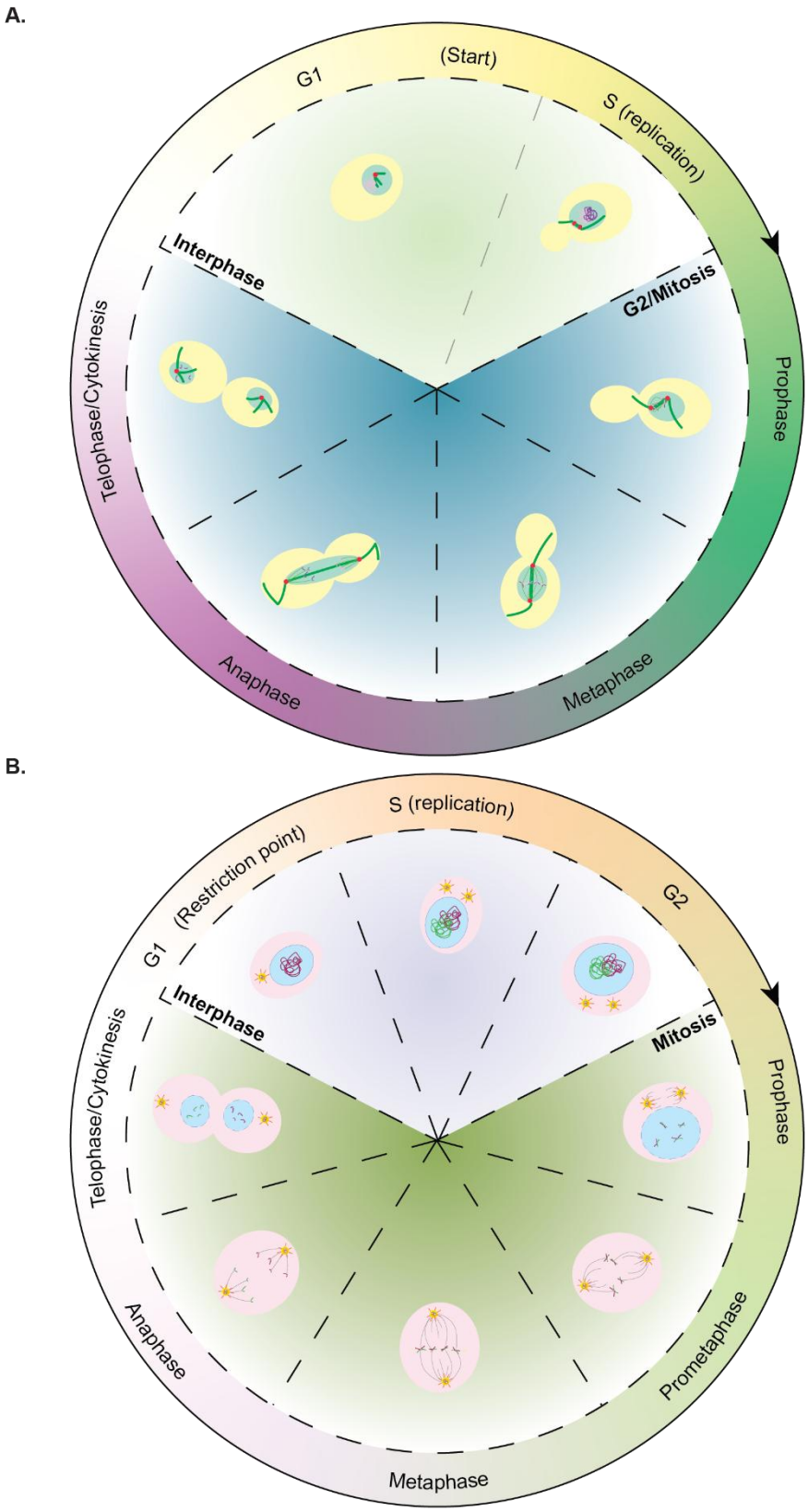

Figure 1.1. Diagrams illustrating the cytological changes that take place as budding yeast and human cells progress through one complete division cycle. **(A)** Budding yeast cell cycle. **(B)** Human cell cycle.

1.2.2 Mechanisms of cell cycle progression: Cyclin-CDK complexes

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that integrate intra- and extracellular signals to drive cell cycle progression. The CDK-mediated regulation of cell cycle transitions requires the sequential association of CDKs with their activating partners, termed cyclins, to form cyclin-CDK complexes (Pluta et al. 2024; Pellarin et al. 2025; Pines 1995). These complexes serve as pivotal molecular switches that drive the ordered and irreversible progression of the eukaryotic cell cycle. In line with the functional specialization of the human genome, more than 20 CDKs –CDK1 to CDK20– are ubiquitously expressed and differentially regulated across tissues (Pellarin et al. 2025; Malumbres et al. 2009; Cao et al. 2014). These CDKs can be divided into three subfamilies: cell cycle regulation, transcription regulation, and atypical CDKs with uncommon functions or regulatory mechanisms (Malumbres 2014). Akin to CDKs, human cyclins have undergone significant diversification throughout evolution, resulting in a family comprising nearly 30 distinct cyclins (Ma et al. 2013; Cao et al. 2014). While many human CDKs and their associated cyclin-CDK complexes exhibit varying degrees of functional redundancy, CDK1 is essential for cell viability, and its absence is associated with embryonic lethality in mouse embryos (Santamaría et al. 2007). In budding yeast, cyclin-CDK complexes are fewer and less complex than their human equivalents, and the Cdc28 kinase is the sole CDK governing cell division (Liu and Kipreos 2000). Nine cyclins interact with Cdc28 to regulate cell cycle transitions: three G1 cyclins (Cln1, Cln2, Cln3) and six B-type cyclins (Clb1-Clb6) (Barberis 2021; Zhang et al. 2023; Tyers et al. 1993; Wittenberg et al. 1990; John et al. 2001). CDK function is conserved from yeast to humans, and fundamental research conducted in budding and fission yeast has been pivotal in understanding the mechanisms of cyclin-CDK complexes in cell cycle regulation (Wittenberg et al. 1990; Nurse 2000; Archambault et al. 2004; Cross et al. 2002). In fact, the first demonstration of cross-species CDK conservation showed that human CDK1 can complement fission yeast *cdc2* mutants (Lee and Nurse 1987). Soon after, similar complementation was also demonstrated in budding yeast, underscoring the significant

degree of functional conservation observable between these kinases across species (Wittenberg and Reed 1989).

The typical bilobal core structure of CDKs comprises an N-terminal lobe that supports ATP binding, and a C-terminal lobe that facilitates substrate phosphorylation. An important aspect of cyclin-CDK interactions lies in the conformational rearrangement of the T-loop, a key C-terminal activation segment, that occurs following the binding of a cyclin partner to its corresponding CDK. This repositioning of the T-loop generates a more accessible catalytic cleft and facilitates key phosphorylation events required for full CDK activation (Jeffrey et al. 1995). These phosphorylation events, occurring within the T-loop, partly rely on a CDK-activating kinase (CAK) (Liu and Kipreos 2000). In budding yeast, the kinase Cak1 exerts this stimulatory phosphorylation on the conserved residue T169 of Cdc28 across all cell cycle transitions (Kaldis et al. 1996; Sutton and Freiman 1997). In conjunction with Cak1 activating function, inhibitory phosphorylation events also take place during cell division to regulate Cdc28 mitotic activity and prevent inappropriate CDK function. Specifically, the kinase Swe1 and the phosphatase Mih1 play opposing roles in modulating Cdc28 activity, ensuring the precise control of the cell cycle (Sia et al. 1998; Russell and Nurse 1987). In addition to phosphorylation-based regulation, cyclin-CDK complexes are also modulated by stoichiometric inhibitors. For example, in G1 phase, the CDK inhibitor Sic1 binds to Clb-Cdc28 complexes, thereby preventing premature entry into S phase (Schwob et al. 1994). Collectively, these regulatory mechanisms ensure the precise coordination of cell cycle transitions while preventing erratic CDK activation, both of which are critical for successful cell division.

In budding yeast early G1, Cdc28 associates with cyclin Cln3 to form the Cln3-Cdc28 complex. This event initiates the activation of transcription factors required for Cln1 and Cln2 expression, which accumulation further activates Cdc28 in a positive feedback loop to irreversibly commit cells to a round of cell division (Dirick et al. 1995; Skotheim et al. 2008). High levels of Cln1/Cln2-Cdc28 complexes stimulate bud emergence and triggers the onset of SPB duplication (Moffat and Andrews 2004; Lew and Reed 1993).

Then, S phase cyclins Clb5 and Clb6 bind to Cdc28, stimulating DNA replication via substrate phosphorylation and thereby ensuring genome duplication (Epstein and Cross 1992; Kühne and Linder 1993; Schwob and Nasmyth 1993). The subsequent accumulation and binding of Clb3 and Clb4 to Cdc28 in late S phase induces SPB separation and spindle formation, setting the stage for mitosis (Surana et al. 1991; Fitch et al. 1992). In budding yeast, elevated Clb1 and Clb2 levels during early M phase drive the formation of Clb1/Clb2-Cdc28 complexes, which indirectly promote spindle assembly, chromosome condensation, and sister chromatid separation at the metaphase-to-anaphase transition through downstream effectors (Chee and Haase 2010). In contrast, in higher eukaryotes, Cyclin B-CDK1 can directly phosphorylate mitotic effectors to trigger chromosome condensation and spindle assembly (Kimura et al. 2001; Abe et al. 2011; Guo et al. 2019). Following DNA segregation, Clb1/Clb2-Cdc28 complexes activate the anaphase-promoting complex (APC), consequently instigating their own degradation (Surana et al. 1993; Rudner and Murray 2000; Rudner et al. 2000). This self-regulatory mechanism, combined with sustained Sic1-mediated inhibition of mitotic Cdc28 following APC-dependent Clb1/2 destruction, ensures the irreversible passage through cytokinesis.

While CDK1/Cdc28, in complex with cyclins, constitutes a central driving force in cell cycle progression (Enserink and Kolodner 2010), a range of other regulatory proteins play equally critical roles in coordinating cell cycle events. Members of the polo-like kinase (PLK) family represent key downstream effectors essential to support and fine-tune CDK-driven cell cycle events in a meticulously controlled spatiotemporal manner (See Figure 1.2. for schematic domain organization of budding yeast Cdc5 and human PLK1) (Archambault and Glover 2009). To justly appreciate the function of CDKs in cell cycle regulation, one must first achieve a comprehensive understanding of PLK-mediated signal amplification and its downstream effects on cellular homeostasis (Archambault and Glover 2008).

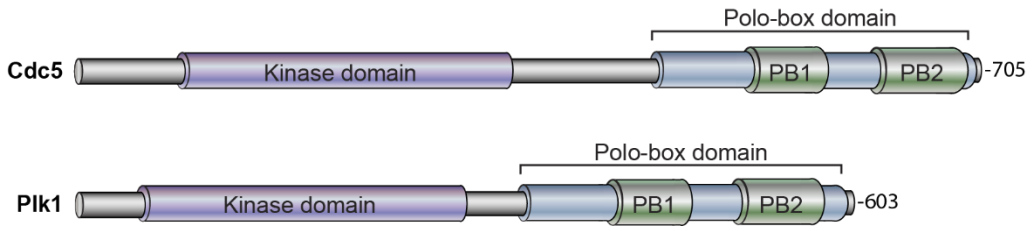

Figure 1.2. Schematic representation of budding yeast Cdc5 and human PLK1 protein domains. Top panel: Cdc5. Bottom panel: PLK1. Linear domain architecture shown to scale. Protein length is shown on the right side of each structure. PB1: Polo-box 1. PB2: Polo-box 2.

1.3 Cell cycle regulation by polo-like kinases (PLKs)

1.3.1 The Polo-like kinase family

*Section 1.3.1 has been reproduced verbatim from my previously published review article: [Langlois-Lemay L, D'Amours D. Moonlighting at the Poles: Non-Canonical Functions of Centrosomes. Front Cell Dev Biol. 2022 Jul 14;10:930355. doi: 10.3389/fcell.2022.930355. PMID: 35912107; PMCID: PMC9329689.]

The polo-like kinase (PLK) family, comprised of PLK1-PLK5 in humans, are serine/threonine kinases that regulate fundamental aspects of cell cycle progression (Zitouni et al., 2014; Iliaki et al., 2021). Within this family, polo-like kinase 1 (PLK1) is arguably the most prominent effector of cell cycle events. PLK1 and its functional homologs in budding and fission yeasts (Cdc5 and Plo1, respectively) require phosphorylation by Cdk1/Cdc28/Cdc2 kinases and/or Aurora kinases for full activation *in vivo*. Following this initial activation stage, PLK1 and its yeast counterparts play crucial roles in the regulation of mitotic entry, spindle assembly, chromosome condensation, sister chromatid segregation, cytokinesis, and adaptation to DNA damage (Toczyski et al., 1997; St-Pierre et al., 2009; Ratsima et al., 2011; Zitouni et al., 2014). Importantly, PLK1 function is also essential for centrosome maturation and aberrant PLK1 activity can lead to serious diseases in humans, including cancer (Liu et al., 2017).

All PLKs share a C-terminal polo-box domain (PBD) and a highly conserved multi-domain structure with an N-terminal kinase domain (KD) that harbors a T-loop with an activating phosphorylation site

(Rodriguez-Rodriguez et al., 2016; reviewed in; Serrano and D'Amours, 2016). To recognize pre-phosphorylated substrates including CDK1/Cdc28 targets, members of the PLK family use their PBD as a signal amplification module to locate and hyperphosphorylate aforementioned targets. However, the distinctive tripartite architecture of PLK4 PBD differs from the PBDs described across other PLK members and was shown to operate in a phospho-independent manner, making PLK4 PBD an exception on that matter (Slevin et al., 2012). The PBD also behaves as a subcellular targeting domain that allows PLKs to recognize and bind specific structures –such as centrosomes and SPBs– and promote specialized cell cycle functions (Colicino and Hehnly, 2018).

The other members of the PLK family perform distinct but sometimes overlapping functions in cell biology. PLK2, involved in centriole duplication, is dynamically expressed throughout the cell cycle and peaks at the G1/S transition of the cell cycle (Warnke et al., 2004). Given its implication in centriole biology, PLK2 was reported to endogenously localize at the centrosomes throughout the cell cycle. The expression of PLK2 varies widely across tissues and, given its importance in mammary gland development, was shown to be particularly high in mammary tissues (Villegas et al., 2014). On the other hand, PLK3 is more steadily expressed throughout the cell cycle and its function mostly relates to stress response pathways involving p53 during DNA damage and spindle disruption (Donohue et al., 1995; Xie et al., 2001). PLK4, derived from PLK1 (Carvalho-Santos et al., 2010) and sharing with PLK2 a role in centriole duplication, is characterized as a master regulator of MTOC formation and centrosome amplification (Habedanck et al., 2005). The last member of the PLK family, PLK5, has a slightly different structure than other members of its group in that it completely lacks a kinase domain in humans. Opposite to its other orthologs, the expression of PLK5 was shown to be very low throughout cell division and high in quiescent cells. PLK5 expression is highest in brain tissues and plays a core function in the nervous system, including neuron differentiation (de Cárcer et al., 2011a). For more information regarding the PLK family, its family members and its evolution across species,

we direct readers towards reviews covering these topics (Archambault and Glover, 2009; de Cárcer et al., 2011b).

MTOCs are crucial scaffolding structures used by PLKs to reach specific substrates and promote cell division (Figure 1.R1). In *S. cerevisiae*, Cdc5 decorates the nuclear surface of duplicating SPBs from late S phase to early anaphase and is also located in the nucleus. In late anaphase, Cdc5 enriches specifically on the cytoplasmic side of the parental SPB segregated to the daughter bud as well as on the bud neck (Botchkarev and Haber, 2017). Once the cell cycle is completed, Cdc5 is degraded by the anaphase-promoting complex (APC) throughout the G1 phase of the next cell cycle (Visintin et al., 2008).

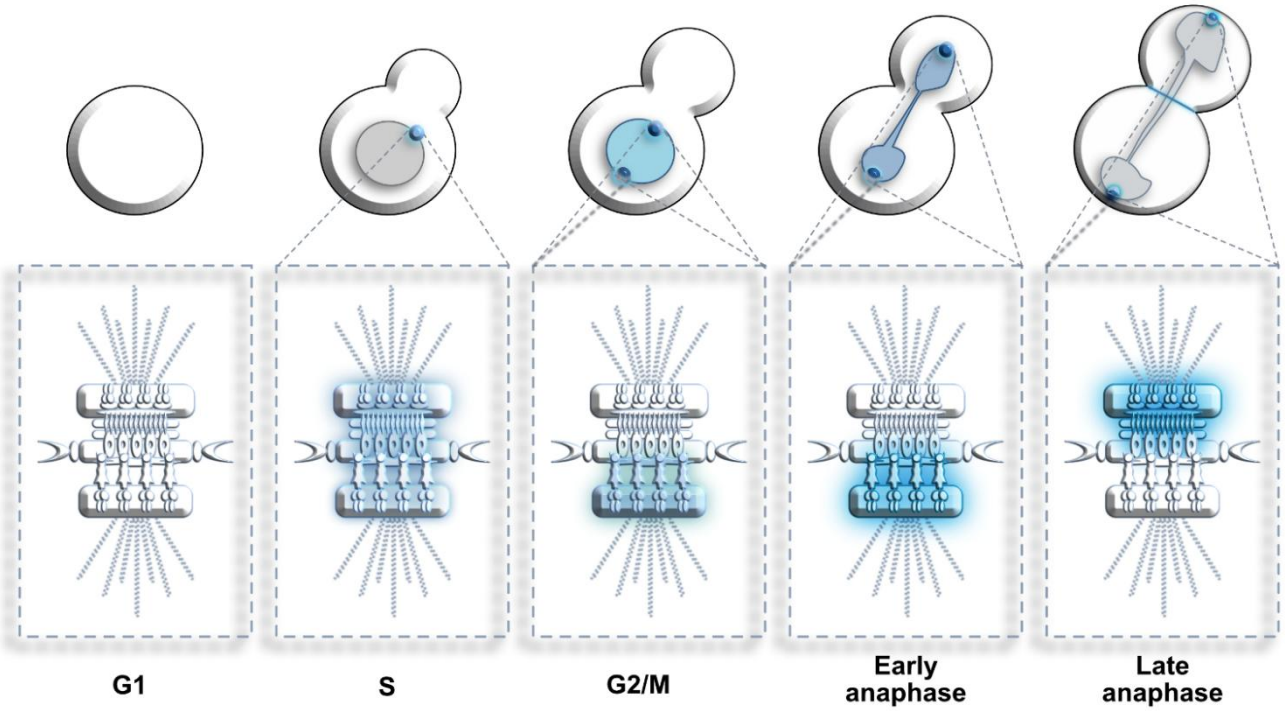

Figure 1.R1. Dynamic localization of Cdc5/Polo kinase at SPBs. G1: Cdc5 is absent from cells. S: Cdc5 enriches at the non-duplicated SPB. G2 to metaphase: Cdc5 decorates the nucleus and the nuclear surface of both SPBs. Early anaphase: Cdc5 concentration at the nuclear surface of both SPBs increases. Late anaphase: Cdc5 relocates from the inner to the outer surface of both SPBs (and bud neck) where it stimulates mitotic exit. Blue color represents enrichment of Cdc5. Color intensity represents Cdc5 concentration levels.

In fission yeast, the polo-related kinase Plo1 shows equally important roles in cell cycle progression and displays high levels of functional overlap with budding yeast Cdc5 and human Plk1 (Lee et al., 2005).

Amongst its key roles, Plo1 is required for mitotic entry, formation of the mitotic spindle, establishment of the actin ring prior to cytokinesis as well as septation activation preceding mitotic completion (Ohkura et al., 1995). Similar to Cdc5 and Plk1, Plo1 requires the SPBs as a docking platform and transiently enriches on the structure in a spatiotemporal manner throughout the cell cycle. Similar to the enrichment of Cdc5 at the SPBs, which is low in S phase but high in G2/M (Simpson-Lavy and Brandeis, 2011), the enrichment of Plo1 on the SPBs is high during mitosis but absent in interphase. Additionally, Plo1 activity at the SPBs is highly reliant on the activity of the kinase Cdc2 (Mulvihill et al., 1999). Upon Cdc2 activation, Plo1 enriches at the SPBs and remains until spindle breakdown whilst keeping steady expression levels throughout the cell cycle (Lee et al., 2005).

The enrichment of Plo1 at the SPBs plays a pivotal role in the commitment to cell division and mitotic progression. The process of mitotic commitment is tightly regulated by M-phase promoting factor (MPF) (Ohi and Gould, 1999), composed of the regulatory subunit cyclin B and the catalytic subunit Cdc2. Following its recruitment to the SPBs in G2 phase (Alfa et al., 1989; Decottignies et al., 2001; Grallert et al., 2013), MPF activity can promote mitotic entry at any point during the cell cycle. Consequently, its activity must remain strictly restrained to the instant where cell division is timely and suitable. The kinase Wee1, via the phosphorylation of Cdc2, is responsible for such inhibitory effect on MPF activity (Russell and Nurse, 1987). Once all conditions for mitotic progression have been fulfilled, the phosphatase Cdc25 removes the inhibitory phosphorylation on Cdc2 and thus promotes cell division. Once MPF is activated, the complex creates a positive feedback loop that further promotes mitotic commitment through increased Cdc25 activity and Wee1 inhibition. Downstream of this feedback loop instigated by MPF, Plo1 interacts with the SPB component Cut12 in a way that supports entry into mitosis. The NIMA-related kinase Fin1, along with Plo1, was also reported to contribute to this positive feedback loop (Grallert and Hagan, 2002).

Apart from its involvement in mitotic entry, Plo1 is equally important throughout cell division. Plo1 shows two mechanistically distinct activity peaks during mitotic progression: First during prophase, where

the formation of the actin ring occurs; second in late mitosis, corresponding to septum formation (Tanaka et al., 2001). Indeed, Plo1 was reported to localize to the medial ring structures as soon as they arise, a subcellular zone that correlates with its key function in the setting of partition sites (Bähler et al., 1998). Akin to Cdc5 in budding yeast, fission yeast Plo1 relies on the APC for its disassociation from the SPBs upon mitotic completion (Mulvihill et al., 1999). Overall, the enrichment of Plo1 at the SPBs is reflective of its implication in the spatial organization of mitotic processes and represents an essential step in the regulation of mitotic entry and cell cycle progression (Lee et al., 2005; Grallert et al., 2013).

In human cells, the Aurora-A kinase, in complex with its co-factor Bora, phosphorylates Plk1 on a conserved residue located in the T-loop of its kinase domain (T210). This G2 phase phosphorylation event uniquely occurs at the centrosomes (Bruinsma et al., 2015). Throughout the cell cycle, Plk1 localization and activity varies greatly. In late G2/early prophase, Plk1 preferentially enriches at the centrosomes to promote mitotic entry and then becomes enriched at the kinetochores to support microtubule-kinetochore connections in prometaphase, with lower Plk1 levels remaining at the centrosomes to instruct spindle assembly.

1.3.2 Roles of Cdc5 in key cell cycle processes

Cdc5 plays an extensive role in cell cycle regulation, orchestrating the activity of hundreds of substrates through a wide range of cell cycle events (Botchkarev and Haber 2018; Archambault and Glover 2008; 2009). Under normal cycling conditions, the essential and non-redundant functions of Cdc5 predominantly occur in the later stages of mitosis, as evidenced by the large-budded telophase arrest and abnormal nuclear morphology observed upon Cdc5 inactivation in dividing cells (Charles et al. 1998; Hartwell et al. 1973; Jaspersen et al. 1998). However, its contributions to cell cycle events throughout all stages of mitosis remain critical for ensuring the fidelity and appropriate timing of cell division. This section outlines the well-

established functions of Cdc5 during the budding yeast cell cycle, highlighting parallels and distinctions with PLK1 function in human cells.

1.3.2.1 Mitotic entry

Cdc5 facilitates timely mitotic entry via two distinct mechanisms. First, Cdc5 behaves as a negative regulator of the Swe1 (human WEE1) kinase, which role is to prevent mitotic Clb-Cdc28 interactions prior to the timely transition from S to G2/M phase (Nakashima et al. 2008; Sakchaisri et al. 2004; Booher et al. 1993; Hu et al. 2008). Upon the coupled Hsl1/Hsl7-mediated recruitment of Cdc5 and Swe1 at the bud neck, Cdc5 promotes mitotic entry by phosphorylating Swe1 and initiating its degradation, consequently relieving mitotic Clb-Cdc28 complexes from a Swe1-induced inhibitory phosphorylation. In parallel, Cdc5 activity within the nucleus contributes to enhanced mitotic cyclin (Clb2) expression via sustained phosphorylation of the key transcription factors Mcm1, Fkh2 and Ndd1 (Darieva et al. 2006). Consistent with this, the nuclear exclusion of Cdc5 at the G2/M transition correlates with delays in mitotic onset (Nakashima et al. 2008; Botchkarev et al. 2014).

In human cells, PLK1 plays roles analogous –but not identical– to those of yeast Cdc5 to drive mitotic entry (Pintard and Archambault 2018; Colicino and Hehnly 2018). By 1) phosphorylating WEE1 to promote its degradation, 2) activating CDC25 phosphatases and, in the broader G2/M control network, 3) counteracting the CDK1 inhibitory kinase MYT1, PLK1 promotes full cyclin B-CDK1 activity and commitment to mitosis (Gheghiani et al. 2017; Joukov and De Nicolo 2018; Ghelli Luserna di Rorà et al. 2020; Sur and Agrawal 2016). While Cdc5 does support mitotic entry in yeast, its essential roles occur later in cell division; by contrast, PLK1 function is critical for G2/M commitment in human cells (Pintard and Archambault 2018). Unlike Cdc5, which directly boosts *CLB2* transcription via the Mcm1-Fkh2-Ndd1 module, PLK1 in humans regulates G2/M transcription mainly by phosphorylating FOXM1 –within the MMB-FOXM1 network– leading to its activation (Moore et al. 2023; Liao et al. 2018).

1.3.2.2 MTOC duplication and activity

The dynamic and reversible phosphorylation of SPB components and associated factors is crucial for modulating spindle assembly and function during cell division. Across eukaryotes, PLKs are known to regulate MTOC duplication, maturation and activity (Llamazares et al. 1991; Sunkel and Glover 1988; Tsou et al. 2009; Lane and Nigg 1996). In budding yeast, Cdc5 collaborates with Cdc28 to control the timing of SPB duplication. This regulatory mechanism involves inhibitory phosphorylation of the half-bridge component Sfi1, a conserved factor essential for SPB duplication events, to avoid aberrant SPB overduplication during mitosis (Elserafy et al. 2014). Cdc5 also phosphorylates several SPB-associated proteins, including the core SPB component Nud1, the kinetochore protein Slk19, and the MT-associated factor Stu2, to ensure proper spindle function, elongation and activity (Park et al. 2008). Mutations in Cdc5, such as the temperature-sensitive (*ts) cdc5-11* allele, were shown to result in reduced kinase activity and abnormal spindle morphology, shorter spindle length, and delayed MT regrowth (Park et al. 2008).

Human PLK1 is similarly deployed to regulate multiple aspects of MTOC behavior (Joukov and De Nicolo 2018; Colicino and Hehnly 2018). PLK1 is a key regulator required for efficient centrosome maturation, and is dynamically enriched at centrosomes/MTOCs where it phosphorylates core pericentriolar material (PCM) components –including pericentrin and CEP215/CDK5RAP2– to drive PCM self-assembly and expansion (Fry, Sampson, et al. 2017; Colicino and Hehnly 2018; Lee and Rhee 2011). During centrosome separation, PLK1 and Aurora A act within the mitotic kinase network to activate NEK2, which phosphorylates linker proteins –including C-NAP1/CEP250 and rootletin/CROCC– to dissolve the centrosome linker and support bipolar spindle formation (Smith et al. 2011; Fry, Bayliss, et al. 2017; Joukov and De Nicolo 2018). By contrast, centriole biogenesis and duplication is instead orchestrated by PLK4 in humans; PLK1 roles are mainly in duplication, maturation, and disjunction, rather than procentriole assembly (Nigg and Holland 2018; Ryniawec and Rogers 2022). Overall, yeast Cdc5 and human PLK1 functionally converge at MTOCs to prepare spindle poles for mitosis. For a detailed overview of SPB and

centrosome duplication, see Section *1.7.1: Function and structural organization of eukaryotic MTOCs: An overview*.

1.3.2.3 Formation of mitotic nuclear flares

The absence of nuclear envelope (NE) breakdown is a defining feature of closed mitosis in budding yeast. To accommodate the dynamic changes in NE morphology during chromosome segregation, Cdc5 regulates phospholipid synthesis to support NE expansion (Walters et al. 2014). The resulting membrane excess is partitioned into nuclear flares –protrusions of the NE– that compartmentalize surplus growth as chromosomes migrate to opposite poles (Walters et al. 2014). The function of Cdc5 in supporting this membrane supply becomes particularly critical during mitotic arrest, when the NE continues to elongate despite the temporary halt in chromosome segregation (Witkin et al. 2012). By sustaining phospholipid synthesis and enabling flare formation, Cdc5 facilitates spatial sequestration of excess NE material around the nucleolus, thereby preserving intranuclear organization (Walters et al. 2014; Witkin et al. 2012). This mitotic role of Cdc5 in regulating nuclear shape is independent from its other cellular functions, such as rDNA condensation (St-Pierre et al. 2009). Notably, the *cdc5-nf* mutant is selectively defective in phospholipid-dependent flare formation but retains other essential mitotic functions, underscoring a dedicated role for Cdc5 in NE remodeling (Walters et al. 2014).

Because mammalian cells undergo open mitosis, PLK1 facilitates NE breakdown (NEBD), a process that contrasts with the NE expansion observed in closed mitosis (Dey and Baum 2021). PLK1 contributes to NE dismantling by promoting nuclear pore complex (NPC) disassembly: CDK1-dependent priming phosphorylation of interconnecting nucleoporins, such as NUP53, enables PLK1 recruitment which, together with CDK1, hyperphosphorylates NUP53 along with the gatekeeper NUP98 to weaken NUP53-NDC1 interactions and destabilize NPC architecture, increasing NE permeability (Linder et al. 2017; Kalous and Aleshkina 2023; Kutay et al. 2021).

1.3.2.4 Chromosome condensation

The accurate partition of sister chromatids to opposite poles of the cell during mitosis is contingent on the effective reorganization of amorphous chromatin into highly condensed and rigid chromosomes that can withstand spindle-pulling forces and safely undergo extensive intracellular movements (Hudson et al. 2003; Ono et al. 2003; Gerlich et al. 2006). Chromosomal reorganization events are promoted by a highly conserved pentameric ATPase complex termed condensin, which binds DNA molecules and associates with chromatin regions to extrude DNA loops and consequently induce localized compaction (Hirano 2005). A key functional feature of condensin during loop extrusion is its ATP-dependent supercoiling activity, which converts relaxed DNA into positively supercoiled strands that stabilize DNA loops and impart structural rigidity (Kimura and Hirano 1997; Kimura et al. 2001). Cdc28-primed, Cdc5-mediated phosphorylation of the non-SMC condensin subunits Brn1, Ycg1, and Ycs4 is essential for hyperactivating condensin supercoiling activity, particularly during late mitotic events (St-Pierre et al. 2009; Sutani et al. 1999; Kimura et al. 2001; 1998). As Cdc28 activity declines in late mitosis, the anaphase-specific hyperactivation of condensin by Cdc5 becomes especially critical for keeping chromosomes tightly compacted until mitosis is complete, thereby safeguarding genome stability.

Unlike budding yeast, which possesses a single condensin complex, human cells contain two – condensin I and condensin II (van Ruiten and Rowland 2018; Skibbens 2019)– and, because vertebrate mitosis is open, their sequential access to chromosomes introduces additional regulatory layers in chromosome condensation (Shah et al. 2024; Dey and Baum 2021; Paul et al. 2019; Paulson et al. 2021). PLK1 contributes to early prophase condensation by acting on condensin II: CDK1-driven phosphorylation of CAP-D3 promotes PLK1 recruitment on condensin and subsequent hyperphosphorylation of condensin II, including a key site on the kleisin subunit CAP-H2, leading to prophase DNA compaction through condensin II accumulation on chromatin (Abe et al. 2011; Kagami et al. 2017; Dekker and Dekker 2022; Archambault et al. 2022). In conjunction with CDK1 and Aurora B, mitotic phosphorylation of condensin subunits

promotes the loading and activation of both complexes –stimulating DNA supercoiling and loop-extrusion activity– with condensin II acting inside the intact nucleus in prophase, and condensin I accessing chromosomes only after NE breakdown (Paulson et al. 2021; Tane et al. 2022; Archambault et al. 2022).

1.3.2.5 Sister chromatid segregation

Cdc5-mediated phosphorylation is essential for the timely separation of sister chromatids during mitosis. Before the metaphase-to-anaphase transition, sister chromatids remain tethered at their centromeres by cohesin, a multiprotein complex composed of Scc1/Mcd1/Rad21, Scc3, Smc1 and Smc3 (Gruber et al. 2003), and Cdc5 accumulates at centromeric chromatin in this window (Mishra et al. 2016). Cohesin establishes a robust ring-like structure that encircles sister chromatids, ensuring they remain paired until specific cell signals trigger their separation. During anaphase, the cohesin complex is dismantled as Separase (Esp1) cleaves Scc1/Mcd1/Rad21, allowing sister chromatids to segregate to opposite poles.

Because timely Scc1/Mcd1/Rad21 cleavage is critical for preserving genome stability, several regulatory mechanisms are in place to safeguard this process. Here, the function of Cdc5 is pivotal: a *cdc5-99* mutant, unable to remove cohesin from centromeric chromatin, shows severe chromosome segregation defects and fails to exit mitosis (Mishra et al. 2016). Prior to the metaphase-to-anaphase transition, Scc1/Mcd1/Rad21 remains unphosphorylated via the sustained activity of Cdc55, the regulatory subunit B of the protein phosphatase 2A (PP2A)(Yaakov et al. 2012), which directly counteracts Cdc5-dependent priming phosphorylation on Scc1 (Alexandru et al. 2001; Yaakov et al. 2012). Separase is concomitantly kept inactive through the inhibitory function of Pds1 securin. Once all mitotic spindle MTs have captured sister chromatid kinetochores, silencing of the spindle assembly checkpoint (SAC) allows Cdc20 to activate the APC/C, which then targets Pds1 securin for degradation (Visintin et al. 1997; Cohen-Fix et al. 1996). Separase, now active, counteracts the inhibitory function of $PP2A^{Cdc55}$ towards Scc1/Mcd1/Rad21 (Yaakov et al. 2012). This enables centromere-bound Cdc5 to phosphorylate Scc1/Mcd1/Rad21 at ten distinct

residues, consequently initiating Separase-mediated Scc1/Mcd1/Rad21 cleavage and the subsequent segregation of sister chromatids (Mishra et al. 2016; Alexandru et al. 2001; Hornig and Uhlmann 2004).

Human cells, as opposed to yeast cells, deploy a prophase pathway in which PLK1 –together with CDK1 and Aurora B– promotes WAPL-dependent release of cohesin from chromosome arms (Kalous and Aleshkina 2023; Hindriksen et al. 2017). By contrast, budding yeast executes the major cohesin removal at anaphase, following SAC satisfaction, when APC/C^{Cdc20} activation triggers separase-driven cohesin cleavage (Konecna et al. 2023). During prophase/prometaphase, PLK1 directly phosphorylates the SA2/STAG2 cohesin subunits and, in conjunction with CDK1/Aurora B-dependent phosphorylation of sororin, drives WAPL-mediated cohesin release from chromosome arms (Hauf et al. 2005; Kalous and Aleshkina 2023; Prusén Mota et al. 2024). This pathway, coordinated with Aurora B and WAPL, individualizes sister chromatids while centromeric cohesin is protected by the SGO1-PP2A complex until anaphase (García-Nieto et al. 2023; Ueki et al. 2021). PLK1 further facilitates APC/C activation by promoting the destruction of the APC/C inhibitor EMI1 which, together with APC/C phosphorylation and SAC silencing, allows for APC/C^{Cdc20}-dependent anaphase onset (Reimann et al. 2001; Hansen et al. 2004; Qiao et al. 2016; Kernan et al. 2018; Lara-Gonzalez et al. 2017).

1.3.2.6 Mitotic exit: FEAR and MEN

During anaphase, Cdc5 coordinates two complementary but temporally distinct mechanisms that drive mitotic exit: the Cdc Fourteen Early Anaphase Release (FEAR) pathway and the mitotic exit network (MEN). These act in a sequential and tightly regulated manner, with both converging on the common goal of releasing the Cdc14 phosphatase from the nucleolus. There, Cdc14 is sequestered and kept inactive by the Regulator of Nucleolar Silencing and Telophase (RENT) complex subunit Net1 until the appropriate time for mitotic exit (Shou et al. 1999; Visintin et al. 1999). The FEAR pathway provides the initial pulse of Cdc14 activity, whereas the MEN sustains this release until mitotic exit is complete.

First, in early anaphase, the FEAR network triggers the partial release of Cdc14 from the nucleolus into the nucleoplasm to coordinate essential mitotic processes such as spindle elongation, nucleolar segregation, and sequential MEN activation (Roccuzzo et al. 2015; D'Amours et al. 2004). Key members of the FEAR include Separase, Separase-interacting kinetochore protein Slk19, nucleolar protein Spo12, $PP2A^{Cdc55}$ phosphatase, as well as Cdc5 (Tomson et al. 2009; Queralt et al. 2006; Stegmeier et al. 2002). In the FEAR, Cdc5 promotes the proteolytic degradation of Swe1, an inhibitory kinase that dampens Cdc28 mitotic activity, thereby enhancing Cdc28 activity and Cdc28-mediated Net1 phosphorylation (Liang et al. 2009). At the onset of anaphase, Separase-driven $PP2A^{Cdc55}$ inhibition facilitates Cdc5-dependent phosphorylation of Net1. This modification reduces Net1 affinity towards Cdc14, leading to the nucleolar release of Cdc14 occurring in synchrony with sister chromatid separation (Shou et al. 2002; Queralt et al. 2006). Cdc5 physically interacts with other members of the FEAR pathway, including Separase and Slk19, in a cell cycle-dependent manner (Rahal and Amon 2008). Interestingly, the RSC^{Rsc2} ATP-dependent chromatin-remodeling complex was shown to regulate the FEAR function of Cdc5 through physical PBD-mediated interactions, promoting Net1 phosphorylation and partial Cdc14 nucleolar release (Rossio et al. 2010). To prepare for mitotic exit, Cdc5 undergoes regulated translocation from the nucleus to the cytoplasm, a process contingent on Cdc14 activity (Botchkarev et al. 2014).

Then, in late anaphase, the MEN orchestrates the complete and final release of nucleolar Cdc14 in a tightly coordinated spatiotemporal manner. Ultimately, Cdc5 functions at multiple junctures within the MEN to promote mitotic exit. Its dual role in both the FEAR and the MEN pathways establishes Cdc5 as a fundamental temporal regulator that synchronizes nuclear migration with mitotic exit, ensuring the timely and coordinated release of Cdc14 from the nucleolus. The Ras-like GTPase Tem1, anchored at the SPB via the scaffold protein Nud1, functions as a spatial sensor that safeguards MEN activation (Gruneberg et al. 2000). To ensure timely exit from mitosis, the Bfa1-Bub2 GTPase-activating protein (GAP) complex maintains Tem1 in an inactive GDP-bound state (Geymonat et al. 2002; Pereira et al. 2000). When the

mitotic spindle aligns correctly along the mother-daughter axis, Cdc5 phosphorylates Nud1-bound Bfa1 on the daughter-facing SPB, thereby circumventing Bfa1-Bub2 inhibitory function towards Tem1 (Geymonat et al. 2002; Gruneberg et al. 2000; Pereira et al. 2000). To become active, Tem1 must spatially encounter the GEF Lte1. In S phase, Lte1 is asymmetrically anchored to the daughter bud cortex. In medium and large-budded cells, Lte1 is released from its cortical anchors and disperses throughout the daughter bud cytoplasm (Seshan et al. 2002). Upon SPB migration within the daughter bud, Tem1 spatially overlaps with cytoplasmic Lte1 and becomes active (Bardin et al. 2000). Tem1 orchestrates the recruitment of Cdc15 kinase to the SPB via the Nud1 scaffold, and the ensuing Tem1-Cdc15 interaction promotes Cdc15 catalytic activation (Asakawa et al. 2001). Active Cdc15 directly phosphorylates Nud1, consequently generating SPB phospho-docking sites to facilitate Dbf2-Mob1 complex anchoring on the structure (Rock et al. 2013). While GTP-bound Tem1 functions as a key spatial regulator of the MEN by ensuring timely Cdc15 recruitment to SPBs, Cdc5 can concurrently facilitate Cdc15 recruitment in a Tem1-independent manner (Rock and Amon 2011). In fact, ectopic Cdc5 expression alone was shown to be sufficient to trigger Cdc14 nucleolar release in a way that supports mitotic exit (Shou et al. 2002; Visintin et al. 2003; Rodriguez-Rodriguez et al. 2016). Both Cdc15 and Cdc5 contribute to Dbf2-Mob1 kinase complex activation (Mah et al. 2001; Lee, Frenz, et al. 2001). In its active form, the Dbf2-Mob1 complex translocates to the nucleus and exerts an inhibitory phosphorylation event on Cdc14 nuclear localization signal (NLS), facilitating Cdc14 accumulation in the cytoplasm and thereby triggering the deactivation of key mitotic regulators to drive mitotic exit (Lee, Frenz, et al. 2001; Mohl et al. 2009; Stoepel et al. 2005).

In contrast to budding yeast, human cells lack a dominant Cdc14-based mitotic exit circuit (Partscht and Schiebel 2023) and instead initiate exit with a rapid drop in CDK1-Cyclin B activity triggered by APC/C^{Cdc20}-mediated ubiquitination and proteolysis of cyclin B1 (and securin), commencing in anaphase and sustained by APC/C^{Cdh1} in late mitosis and G1 to clear mitotic regulators (Greil et al. 2022; Cirillo et al. 2024). Consequently, mammalian mitotic exit is primarily governed by CDK1 inactivation coupled to the PP1-

initiated reactivation of PP2A-B55, together with other CDK-opposing phosphatases –PP1 and PP2A– rather than a FEAR/MEN-type, Cdc14-centered hierarchy that orchestrates exit in budding yeast (Holder et al. 2019).

1.3.2.7 Cytokinesis

Upon mitotic exit, mother and daughter cells initiate cytokinesis to stage their physical separation. In budding yeast, cytoskeletal dynamics at the division site are primarily driven by the Rho1 GTPase, a key regulator of contractile actin ring (CAR) assembly (Tolliday et al. 2002). To promote CAR assembly, Cdc5 phosphorylates the Rho1 GEFs Tus1 and Rom2 in a way that supports Rho1 recruitment at the bud neck (Yoshida et al. 2006). Concurrently, Cdc5 regulates the activity of Cdc42, a small GTPase critical for coordinating actin dynamics and septin organization (Adams et al. 1990; Caviston et al. 2003). Through a direct PBD-mediated interaction with the GAPs Bem2 and Bem3, Cdc5 inhibits Cdc42 activity in late mitosis to support septum formation (Atkins et al. 2013). Vesicle tethering and membrane deposition at the cleavage site are also regulated through Cdc5 kinase activity. Cdc5-mediated phosphorylation of Sec4, a Rab GTPase essential for plasma membrane trafficking, disrupts its interaction with exocyst component Sec15 to temporarily halt vesicle fusion and plasma membrane deposition at the site of division (Lepore et al. 2016). In the final stages of cytokinesis, Cdc5 phosphorylates the F-BAR tether protein Hof1, a regulator of actin cytoskeleton organization, to regulate dynamics of CAR constriction. This modification primes Hof1 for a second phosphorylation event driven by the MEN kinase complex Dbf2-Mob1, which supports Hof1 relocalization from the septin scaffold to the CAR in a way that ultimately promotes membrane ingression and cytokinesis (Meitinger et al. 2011).

Given that human cytokinesis occurs after nuclear envelope breakdown (i.e., open mitosis) (Dey and Baum 2021), cleavage furrow positioning is directed primarily by signals from the spindle midzone, with other inputs from astral MTs, which often sharpen the equatorial zone by inhibiting RhoA at the poles

(Basant and Glotzer 2018; Verma et al. 2019). PLK1 regulates cleavage furrow formation via central spindle signaling, wherein it phosphorylates the centralspindlin subunit HsCYK-4 (also termed RACGAP1/MgcRacGAP) to recruit the RhoGEF ECT2 and ignite RhoA activation at the equatorial cortex (Adriaans et al. 2019; Wolfe et al. 2009; Burkard et al. 2009; Petronczki et al. 2007), yielding assembly and ingression of the contractile furrow (Yüce et al. 2005; Wagner and Glotzer 2016). Therefore, each strategy tailors to the structural differences between budding yeast and humans while converging on the common goal of cell separation.

1.3.3 Implications of combinatorial substrate recognition in cell cycle modulation

Most Cdc5 interactions occur through canonical PBD-mediated docking, whereby Cdc28-primed, pre-phosphorylated substrates bind the conserved phosphopeptide-binding groove located at the interface of both Polo-boxes of Cdc5 (Elia, Cantley, et al. 2003; Elia, Rellos, et al. 2003). However, structural and biochemical studies have identified a second hydrophobic pocket on the surface opposite to the canonical Ser/Thr-binding groove, capable of recognizing distinct, non-phosphorylated protein sequences (Miller et al. 2009; Chen and Weinreich 2010). This second aromatic pocket enables the simultaneous, non-competitive binding of additional interactors to Cdc5 PBD, expanding both the range of partner proteins and the regulatory outcomes of Cdc5 activity throughout the cell cycle (Almawi et al. 2020). Through its non-canonical hydrophobic pocket, Cdc5 PBD engages the exonuclease Exo1 during meiotic crossover formation and binds Dbf4 –the regulatory subunit of the Cdc7-Dbf4 (DDK) kinase complex– under spindle position checkpoint (SPoC) activation, contributing to crossover regulation and transient inhibition of Cdc5-dependent MEN initiation, respectively (Sanchez et al. 2020; Miller et al. 2009). This combinatorial mode of substrate binding confers Cdc5 with switch-like versatility, enabling precise signal integration and dynamic tuning of its catalytic activity to preserve genome stability. As Cdc5 functions at the crossroads of cell cycle progression and checkpoint feedback, the dual binding capacity of Cdc5 PBD may provide a unique

advantage in modulating cell cycle transitions under both normal conditions and in response to genotoxic stress.

1.4 Stress responses: Critical cell cycle modulators

The inherent complexity and unidirectional nature of the eukaryotic cell cycle make the process of cell division particularly susceptible to both endogenous and exogenous stressors that can compromise its fidelity. Endogenous damage, often triggered by replication errors or oxidative stress, can take the form of DNA mismatches, spontaneous base deamination and abasic sites, DNA methylation, or global replication fork collapse; genotoxic stress may also stem from exogenous sources that include ultraviolet and ionizing radiations, alkylating agents, or other genotoxic or oxidative chemicals (Chatterjee and Walker 2017; Gupta and Lutz 1999). To ensure high-fidelity genome replication and subsequent cell division, cells have evolved a complex network of surveillance pathways termed cell cycle checkpoints (Hartwell et al. 1994). Together with core mitotic regulators and stress response pathways, these checkpoints constitute a robust control system that meticulously synchronizes phase transitions and ensures that all requirements are satisfied before the cell irreversibly commits to the next stage of cell division.

Cell cycle checkpoints represent the critical cycling nodes where a range of stress responses are activated and modulated according to the nature of the threat. To fully grasp the functional significance of these checkpoints, it is essential to survey the specific stress responses they activate and modulate. This section first explores the diverse signaling pathways at the heart of these checkpoint responses, with an emphasis on how they are calibrated to accommodate different stages of mitosis. Where relevant, contributions of Cdc5 are highlighted, together with brief comparisons to the corresponding pathway in human cells.

1.4.1 The heat shock response (HSR)

The budding yeast heat shock response (HSR) is an evolutionarily conserved cytoprotective transcriptional program initiated in response to thermal up-shifts to safeguard proteostasis and sustain cell viability (Singh et al. 2024). The HSR transiently halts cell cycle progression and activates a range of protective systems that together prevent protein misfolding and stabilize cell wall function until temperature stress subsides (Mühlhofer et al. 2019). The heat shock protein Hsf1 (human HSF1), a central trimeric transcription factor operating at the core of the HSR, binds DNA upon heat stress to induce the transcriptional activation of critical heat shock protein (HSP) genes (Sorger and Pelham 1988). The main molecular pillars of this response –Hsp70, Msn2/4, Hsp104, Hsp12, and trehalose– operate at different levels to fine-tune HSR execution (Masser et al. 2020; Dea and Pincus 2024). Hsp70 binds to Hsf1 and, in doing so, modulates transcriptional activation to swiftly curb the response when required (Baler et al. 1992; Peffer et al. 2019; Masser et al. 2020). At the transcriptional level, the active stress regulators Msn2/4 widen the heat transcriptome –in complement to Hsf1 activity– by incorporating signals related to nutrient, osmotic and oxidative stressors, which effectively protects against compound stresses (Parrou et al. 1997; Sadeh et al. 2011). Following the Hsp70-facilitated triage of proteins for proteasomal degradation or refolding, the Hsp104 disaggregase resolubilizes clumped proteins in a way that supports cellular detoxification (Sanchez and Lindquist 1990; Lum et al. 2004). The hydrophilin Hsp12, in conjunction with trehalose, operates at the membrane level to improve desiccation resistance and support barrier integrity (Sales et al. 2000; Kim et al. 2018; De Virgilio et al. 1994; Tapia and Koshland 2014).

In most cases, the HSR becomes activated at the Start (G1) point and blocks entry into the cell cycle by lowering Cln1/2 cyclin mRNA levels, and physically trapping both Cln3 mRNA and Cdc28 within heat-induced stress granules (Rowley et al. 1993; Yahya et al. 2021). As heat stress dissipates, a critical chaperone-driven feedback loop induces stress granule disassembly, consequently releasing active Cdc28 and promoting cell division only when cycling conditions are optimal once more (Yahya et al. 2021). Recovery

from a G1-phase heat shock was also shown to involve Cln3 along with the S phase cyclin Clb5 (Li and Cai 1999). HSR activation is tied to cell cycle checkpoints, and, consequently, its response can be modulated as cells progress through cell division. Whereas the G1 HSR brings a clear halt to the cell cycle, its impact in S phase cells differs slightly. If replisomes are threatened by heat-induced misfolding, the openness of chromatin in actively replicating cells allows for a faster and more efficient Hsf1-mediated HSR which, coupled with the DDR, simulates a replication stress response (RSR) (Venturi et al. 2000; Duch et al. 2018). At the G2/M transition, akin to the S phase response, the HSR is rapidly activated (Venturi et al. 2000). Importantly, prior to anaphase onset, the heat-induced hyper-condensation of the metaphase rDNA array requires the combined action of Cdc5 and condensin to resolve compacted loops, without which cells could not safely complete chromosome segregation (Matos-Perdomo and Machín 2018).

In humans, while primarily mediated by HSF1, the HSR is more complex in that it integrates p53 signaling (Logan et al. 2009; Isermann et al. 2021) and implicates additional heat shock factors (such as HSF2) (Ostling et al. 2007; Gomez-Pastor et al. 2018). It is also wired into mitotic control via PLK1, which phosphorylates HSF1 in early mitosis to regulate its stability and mitotic activity (Lee et al. 2008). Notably, mammalian HSF1 can directly influence certain cell cycle regulators –as seen in p53-null cells, where HSF1 inhibition causes delayed cyclin B1 degradation (Wang et al. 2004)– and, via crosstalk with the p53-p21 axis, links the HSR to CDK1 restraint (Charrier-Savournin et al. 2004).

1.4.2 The osmotic stress response

Fluctuations in external osmolarity can disrupt the trans-membrane gradient and, in doing so, alter cell volume and threaten cell homeostasis. Under osmotic stress, the High Osmolarity Glycerol (HOG) pathway stimulates the translocation of the stress-activated protein kinase (SAPK) Hog1 to the nucleus (Ferrigno et al. 1998). This event modulates the expression of key regulators of cell metabolism and stress-response genes that can counteract osmotic imbalance (de Nadal and Posas 2022). Importantly, Hog1 modulates cell

cycle progression under osmotic imbalance by transiently quenching cyclin expression and stabilizing Sic1, effectively leading to a temporary cycling arrest at the G1/S transition (González-Novo et al. 2015; Adrover et al. 2011; Escoté et al. 2004). It is worthy of note that the function of the HOG pathway is not exclusively tied to the G1/S transition. Beyond these early cell cycle roles, Hog1 also interfaces with key mitotic regulators –including Cdc5– to influence mitotic exit under osmotic stress. First, during S phase, osmotic imbalance can activate Hog1, which transiently inhibits DNA replication through the phosphorylation of Mrc1, a key member of the replication fork (Yaakov et al. 2009; Duch et al. 2013; 2018). In G2 phase, Hog1 can promote Swe1 accumulation, thereby delaying mitotic entry and providing cells with additional time to effectively respond to hyperosmotic stress (Alexander et al. 2001; Clotet et al. 2006). Finally, during metaphase, osmostress can trigger Hog1 activation, leading to Net1 phosphorylation. This modification enhances Net1 affinity toward Cdc14 and shields Net1 from Cdc5-driven hyperphosphorylation, consequently delaying mitotic exit (Jiménez et al. 2020; Tognetti et al. 2020).

Rather than operating through Hog1, which they lack, human cells instead deploy the p38 MAPK pathway upon osmotic stress: under hyperosmotic conditions during S phase, p38 phosphorylates Claspin –the functional homolog of yeast Mrc1– to protect S phase integrity and limit DNA damage (Ulsamer et al. 2022).

1.4.3 The oxidative stress response

Reactive oxygen species (ROS) –such as hydroxyl radicals, hydrogen peroxide, and superoxide anions– are inevitable and highly reactive by-products of aerobic respiration that induce DNA lesions and compromise genome stability at high concentrations (Poetsch 2020). Under physiological conditions, ROS accumulation is effectively prevented through a range of enzymatic antioxidant defences including catalases, peroxidases and superoxide dismutases that constitutively curb oxidative damage (Herrero et al. 2008). When this first line of defense is unable to preserve homeostasis, cells facing harmful levels of oxidative stress trigger the

oxidative stress response. This adaptive transcriptional program is orchestrated by the bZIP transcription factor Yap1, and operates to support cellular redox balance upon subsequent insults (Kuge and Jones 1994; Delaunay et al. 2000). Alongside the general stress regulators Msn2/4 and Hsf1 –and with modulatory input from Skn7–, Yap1 upregulates genes encoding thioredoxins (TRX1/2), glutathione biosynthetic enzymes (GSH1/2), catalases (CTT1), peroxiredoxins (TSA1) and other key antioxidant effectors providing improved stress tolerance (Gasch et al. 2000; Lee et al. 1999).

During the G1 phase, the oxidative stress response relies on enzymes of the base-excision repair (BER) pathway to remove oxidized bases without triggering the full Rad53-mediated checkpoint response. This process occurs in parallel to the transcription factor-driven expression of key stress response genes, enabling cells to build resilience against subsequent insults without imposing a full cell cycle arrest (Leroy et al. 2001). Given the direct impact of ROS on DNA integrity, S phase cells exposed to oxidative stress trigger the full Mec1-mediated DDR, resulting in Rad53 activation and Dun1-mediated downstream signaling. This response slows replication by inhibiting late origin firing and stabilizing stalled forks, and boosts dNTP pools in favor of DNA repair (Iyer and Rhind 2017; Leroy et al. 2001; Zhao and Rothstein 2002). At the G2/M transition, the BER-mediated repair of oxidative DNA lesions occurs silently (i.e., without checkpoint activation), akin to the stress response occurring in G1, keeping the DDR inactive unless DNA repair is incomplete or unsuccessful (Leroy et al. 2001).

In response to oxidative stress, mammalian cells utilise the nuclear factor erythroid 2-related factor 2 (NRF2) –via KEAP1-CUL3-driven sensing, which stabilizes and localizes NRF2 to the nucleus– to induce cytoprotective gene expression upon ROS accumulation (Itoh et al. 1999; Wakabayashi et al. 2004). By blocking G1 CDKs, NRF2-dependent upregulation of key CDK inhibitors –such as $p15^{INK4b}$, $p21^{Cip1}$, and $p27^{Kip1}$– can prevent entry into S phase, thereby safeguarding genome stability (Polyak et al. 1994; Xiong et al. 1993; J. Li et al. 2011; Jana et al. 2018). In parallel, substantial ROS can activate ATM –along with the p53

program– yielding arrest (via p21) or apoptosis (via BAX) when damage is irreparable – a p53-dependent axis that budding yeast lacks (Guo et al. 2010; el-Deiry et al. 1993; Miyashita and Reed 1995).

1.4.4 The nutrient stress response

In the face of nutrient scarcity, cell growth is modulated through a range of nutrient-stress programs designed to restrain cell cycle progression and stimulate the expression of crucial starvation-related survival genes. To effectively address nutrient scarcity, this response modulates three pillars of cell growth and biogenesis. First is the TORC1-PKA axis, which closely regulates nitrogen/amino acid/glucose dynamics to promote cell growth under normal conditions (Foltman and Sanchez-Diaz 2023). Second is the Snf1/AMP-activated protein-kinase (AMPK) pathway, which senses limited fermentable carbon sources and modulates the exploitation of alternative carbon sources when needed (Hedbacker and Carlson 2008; Coccetti et al. 2018). Third is the famine switch Rim15 which, under normal conditions, remains inactive via TORC1/PKA inhibitory effect. Upon energy shortage, Rim15 is essential to drive cells into a quiescent-like state (Bontron et al. 2013).

Nutrient stress impacts cell cycle control at multiple nodes – with Cdc5 acting as the TORC1-sensitive effector that delays mitotic commitment upon limited nutrients (Nakashima et al. 2008). First, in G1, the compounded effect of TORC1 inactivation and Rim15-mediated $PP2A^{Cdc55}$ inhibition results in limited Cln3 cyclin expression and a robust stabilization of the Cdc28 inhibitor Sic1 (Moreno-Torres et al. 2015; Sarkar et al. 2014). The resulting decrease in Cdc28 activity effectively impedes cell cycle entry and, in parallel with other key programs, drives cells into a quiescent-like state (Miles et al. 2021). Akin to the HSR, nutrient scarcity stimulates Rim15-mediated Msn2/4 induction to promote the expression of key stress-related genes required to support cell survival (Lee et al. 2013). In S phase cells, nutrient deprivation generates a marginally different response. Rather than entirely blocking cell cycle progression, the S phase nutrient stress response concomitantly exploits Snf1 to maintain Clb5 transcription and sustain origin firing in a way

that limits replication fork collapse while amino-acid pools are replenished (Nicastro et al. 2015). As cells approach mitosis, TORC1 inactivation leads to Cdc5 mislocalization, which consequently delays mitotic entry until the nutrient supply is replenished (Nakashima et al. 2008).

Across eukaryotes, nutrient starvation activates AMPK and inhibits TORC1/mTORC1, permitting a reversible G1 brake designed to conserve energy (Barbet et al. 1996; Gwinn et al. 2008; Inoki et al. 2003). In mammals, a second quiescence reinforcement layer is driven by the retinoblastoma (RB) family-E2F (DREAM) repressor program, which permits the silencing of key cell cycle genes as another safeguard (Uxa et al. 2019; Litovchick et al. 2007). By elevating AMPK and concomitantly suppressing mTORC1 –suppression that, under amino-acid starvation, is reinforced by PLK1 binding the mTOR-RAPTOR core to dampen mTORC1 and promote autophagy (Ruf et al. 2017)– prolonged nutrient scarcity reduces cyclin synthesis and maintains RB in a hypophosphorylated, growth-suppressive state (Averous et al. 2008; Decker et al. 2003; Fingar et al. 2004; Chellappan et al. 1991).

1.4.5 The DNA damage response (DDR)

To preserve genome integrity, cells confronted with genotoxic stress can activate the DNA damage response (DDR) –a dynamic signaling circuit that spans the entire cell cycle and reconfigures at each stage to meet distinct challenges– with critical control exerted over Cdc5 through cell cycle checkpoints. Although genotoxic stress can originate from many sources –including oxidative radicals, polymerase errors, ionizing or UV radiation, DNA-protein or inter-strand crosslinks, R-loop accumulation, telomere uncapping, and other forms of replication stress (Tubbs and Nussenzweig 2017; Skoneczna et al. 2015)– these insults ultimately converge on two types of perceived DNA damage signals: single-strand breaks (SSBs), or the more deleterious double-strand breaks (DSBs) (Caldecott 2014; Vítor et al. 2020). While SSBs and DSBs are structurally distinct and differ in early recognition steps, both feed into shared DDR modules (Figure 1.3.). By altering local chromatin structure and evicting nucleosomes flanking a DSB, the RSC chromatin-

remodeling complex establishes an accessible signal initiation site directly at the lesion (Liang et al. 2007). MRX (Mre11-Rad50-Xrs2; human MRN: MRE11-RAD50-NBS1) complex-bound DNA ends at the unprocessed break site serve as a platform for the recruitment and activation of the DDR apical kinase Tel1, the yeast ortholog of ATM. This event triggers Tel1-dependent phosphorylation of histone H2A (γH2A) and facilitates the recruitment of the Rad9 (human 53BP1) mediator to the damage site. Processing of DSB ends by Dna2-Sgs1 and Exo1 (human EXO1) generates RPA-coated single-stranded DNA (ssDNA), which provides a docking site for the Ddc2 (human ATRIP)-bound apical kinase Mec1, the yeast ortholog of ATR (Mantiero et al. 2007; Zou and Elledge 2003). This portion of DSB processing, essential for DDR signaling, shares the same activation signal that initiates SSB recognition. The simultaneous recruitment of the 9-1-1 complex – comprising Ddc1, Mec3, and Rad17 (human RAD9A-RAD1-HUS1)– along with the checkpoint activator Dpb11 (human TOPBP1) to ssDNA-dsDNA junctions primes the damage site for subsequent Mec1/Tel1-dependent phosphorylation of histone H2A (γH2A; γH2AX in mammals) and Ddc1. Shortly after, the SWR1 remodeler exchanges H2A for H2A.Z, a non-phosphorylatable variant that transiently accumulates near DNA damage and contributes to the relocation of persistent lesions to the nuclear periphery, establishing a reversible chromatin ‘’timer’’ that can later be overturned by the Ino80 chromatin remodeling complex to facilitate adaptation (Papamichos-Chronakis et al. 2006; Horigome et al. 2014; Kalocsay et al. 2009). Together with Rad9 phosphorylation, these modifications create binding platforms for the docking of downstream DDR effectors (Schwartz et al. 2002). Active, oligomerized Rad9 then recruits Rad53 (human CHK2) –the central effector kinase of the DDR– for a crucial Mec1-mediated phosphorylation event required for its activation (Sanchez et al. 1996). Rad9 functions as a bona fide Mec1 adaptor for Rad53 activation, and its oligomerization sustains DDR checkpoint signaling (Sanchez et al. 1996; Sweeney et al. 2005; Usui et al. 2009; Pizzul et al. 2022). (Baldo et al. 2012; Zhou et al. 2010)

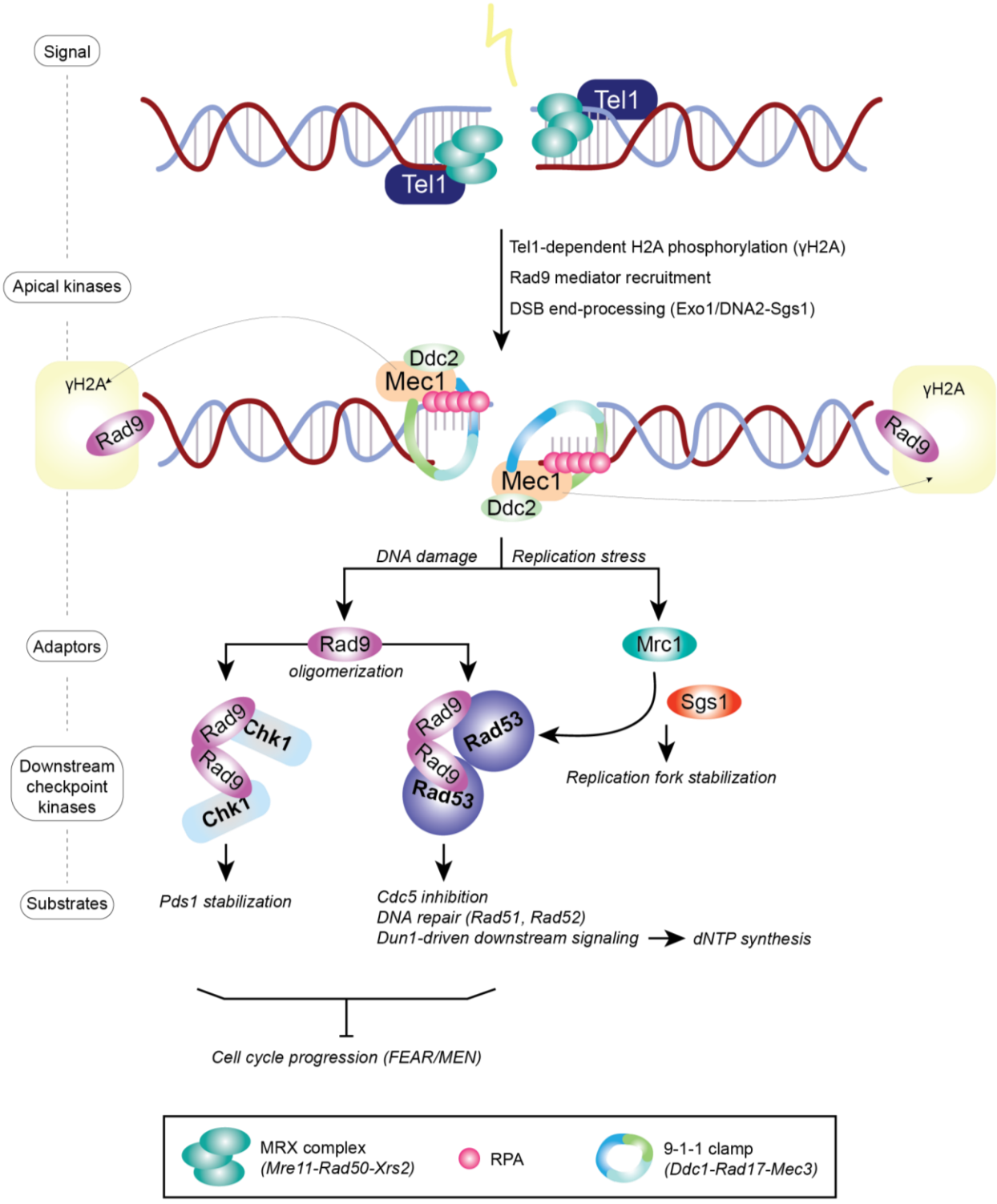

Figure 1.3. Schematic overview of the DNA damage response (DDR) signaling cascade in the budding yeast *Saccharomyces cerevisiae*. Major sensors, adaptor proteins, and effector kinases activated following DNA lesions are depicted. Key downstream outcomes include checkpoint kinase regulation, widespread phosphorylation events leading to cell cycle arrest, recruitment and activation of DNA repair machinery, and induction of stress-responsive transcriptional programs. Conceptual design inspired by Baldo et al. 2012 and Zhou et al. 2010.

In G1 phase, these DDR effectors work to stabilize Sic1, inhibit G1/S cyclin activity, and enforce a transient cell cycle arrest that will effectively prevent the replication of damaged templates (Siede et al. 1994; Pizzul et al. 2022). When DNA lesions arise during S phase, the DDR operates through the intra-S DNA damage checkpoint by sensing RPA-coated ssDNA at stalled forks and activating a signaling cascade that safeguards genome integrity and chromosome stability (MacDougall et al. 2007; Zou and Elledge 2003; Hustedt et al. 2013). This response is mainly transduced through the DNA damage sensor kinase Mec1, the canonical damage mediator Rad9, and the effector kinase Rad53 (Vialard 1998). Activated Rad53 inhibits DDK to prevent late origin firing, stabilizes stalled replication forks, and –in parallel with Chk1– contributes to preventing chromosome segregation (Paulovich and Hartwell 1995; Santocanale and Diffley 1998; Szyjka et al. 2008; Tercero and Diffley 2001; Ogi et al. 2008) (See section *1.5.3: The intra-S DNA damage checkpoint* for more details). In response to replication stress, the Mec1/Rad53-driven DDR is transduced through the DNA replication checkpoint (DRC). In this context, the fork-associated mediator Mrc1 (Katou et al. 2003; Osborn and Elledge 2003; Alcasabas et al. 2001), and the Sgs1 helicase (Bjergbaek et al. 2005; Hegnauer et al. 2012) act to stabilize stalled forks and prevent late origin firing through Rad53-mediated DDK inhibition, until replication stress is resolved (Pardo et al. 2017) (See section *1.5.2: The DNA replication checkpoint (DRC)* for more details). By organizing these crucial programs, the DDR ensures that DNA replication occurs safely and stalls mitotic entry until cycling conditions are optimal once more (Paulovich and Hartwell 1995; Tercero and Diffley 2001). At the G2/M transition, Pds1 is phosphorylated by the downstream checkpoint kinase Chk1 to inhibit separase, transiently preventing anaphase entry (Wang et al. 2001; Sanchez et al. 1999; Agarwal and Cohen-Fix 2002). At this stage, Rad53-dependent phosphorylation dampens Cdc5 activity, thereby preserving APC/C-Cdh1 in its active form –which curtails spindle elongation (T. Zhang et al. 2009)– and blocking Cdc5-mediated phosphorylation of Bfa1, thus keeping the MEN inactive and preventing mitotic exit (Valerio-Santiago et al. 2013). Crucially, Rad53 and Chk1 act in concert to block both the FEAR and the MEN, thereby sustaining the DNA damage-induced G2/M arrest until lesions are resolved (Liang

and Wang 2007). By dynamically rewiring its sensor-mediator-effector axis across different stages of the cell cycle, the DDR effectively preserves genome integrity while still permitting productive cell division.

While the human checkpoint response shares the core ATM/ATR cascade with yeast, the distinguishing addition is a p53-mediated branch that broadens checkpoint outcomes beyond transient arrest (Abuetabh et al. 2022; Zhang et al. 2024; Liu et al. 2024). Following DNA damage, p53 transactivates $p21^{Cip1}$ –enforcing cell cycle arrest– and, in permanently damaged cells, triggers apoptosis or senescence (el-Deiry et al. 1993; Nakano and Vousden 2001; Xue et al. 2007). Importantly, p53 couples genome surveillance with broader cellular physiology notably by 1) promoting DNA repair via induction of p53R2, a ribonucleotide-reductase subunit (Tanaka et al. 2000); 2) rewiring metabolism via TIGAR, a glycolysis-regulating target (Bensaad et al. 2006); 3) triggering non-apoptotic death programs, including ferroptosis (Jiang et al. 2015). The effects of p53 are especially consequential in multicellular organisms, where it couples genome surveillance to organismal homeostasis – enforcing repair, senescence, or apoptosis to restrain tumorigenesis and preserve tissue function (Kastenhuber and Lowe 2017; Levine 2020).

1.4.5.1 Canonical DNA repair mechanisms

Yeast and humans share a core repertoire of six evolutionarily conserved DNA repair pathways that together mitigate DNA lesions arising from endogenous metabolism and exogenous genotoxins (Ramotar and Masson 1996; Chalissery et al. 2017). Collectively, these six canonical pathways –direct damage reversal, base-excision repair (BER), nucleotide-excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ)– span the full spectrum of DNA lesions. By virtue of their overlapping specificities, these responses provide the necessary functional redundancy required to prevent deleterious genome instability (Prorok et al. 2021; Bauer et al. 2015; Boiteux and Jinks-Robertson 2013).

1.4.5.1.1 Direct damage reversal

In budding yeast, the pathway of direct damage reversal relies on two incision-free enzymes, the Phr1 photolyase and the Mgt1 O^6-alkylguanine-DNA alkyltransferase, to respectively resolve UV-induced cyclobutane pyrimidine dimers (CPDs) and O^6-alkylguanine lesions (Sancar 1985; Xiao and Samson 1992). This mechanism enables rapid, direct restoration of modified bases without backbone cleavage, making it a safe, metabolically efficient first-response repair branch (Prorok et al. 2021). Following UV or MMS exposure, Rad53-dependent DDR signaling phosphorylates the zinc-finger repressor Rph1, leading to increased Phr1 photolyase expression (Kim et al. 2002). Photo-excitation within Phr1 reduced flavin cofactor (FADH-) triggers rapid electron transfer that cleaves the CPD, yielding two restored pyrimidines (Prytkova et al. 2007). Alternatively, in response to spontaneous or chemical-driven alkylation, the Mgt1 O^6-alkylguanine-DNA alkyltransferase selectively transfers an O^6-alkyl adduct onto its catalytic cysteine residue, restoring the altered guanine (Tubbs et al. 2007). Unlike Phr1, Mgt1 is constitutively expressed and is not induced by DNA damage signaling (Joo et al. 1995). Together, these two direct-reversal enzymes prevent replication-fork stalling and restore DNA integrity, safeguarding the genome with high fidelity and minimal energy expenditure (Prorok et al. 2021; Quinet et al. 2016).

Unlike budding yeast, human cells –and other placental mammals– lack DNA photolyases and instead rely on NER to remove UV-induced CPDs (Li et al. 1993; Vechtomova et al. 2021). However, human cells do possess an O^6-methylguanine-DNA methyltransferase (MGMT), enabling rapid repair of O^6-alkylguanine lesions (Pegg et al. 1982; Grafstrom et al. 1984; Fang 2024). Through a stoichiometric ‘’suicide’’ transfer reaction, MGMT restores altered guanines by transferring the alkyl group from the damaged base onto its own active-site cysteine, irreversibly inactivating the enzyme (Pegg et al. 1982). Importantly, high MGMT expression (typically with an unmethylated promoter) commonly drives resistance to alkylating chemotherapies –including temozolomide (TMZ)– especially in glioblastoma, underscoring the relevance of

MGMT status in clinical decision-making (Hegi et al. 2005; Stupp et al. 2005; Yu et al. 2020; Oldrini et al. 2020; Fang 2024).

1.4.5.1.2 Base excision repair (BER)

Highly accurate by design, the BER is a five-step pathway specialized in the removal of non-bulky lesions that frequently result from oxidative stress, spontaneous hydrolysis, or deamination. First, the BER deploys a lesion-specific monofunctional or bifunctional DNA N-glycosylase (*i.e.*, Ung1/Mag1 or Ogg1/Ntg1/Ntg2, respectively) that excises the damaged base, giving rise to a transient apyrimidinic/apurinic (AP) site (Krokan et al. 1997; Hindi et al. 2021). Then, a central AP endonuclease –Apn1/Apn2– nicks the phosphodiester backbone while a phosphodiesterase evicts the leftover deoxyribose phosphate residue (Johnson and Demple 1988; Ramotar et al. 1991). This facilitates DNA polymerase ε (Pol2)-mediated gap filling (Wang et al. 1993), followed by Cdc9-catalyzed sealing of the repaired backbone (Wu et al. 1999).

In human cells, the BER pathway operates in a similar manner and deploys APE1 as its central AP endonuclease (Mol et al. 2000). While DNA polymerase δ and/or Pol ε (Pol3/Pol2) primarily mediates gap filling in budding yeast, DNA polymerase β (Pol-β) is used in human BER (Sobol et al. 1996; Bennett et al. 1997), rapidly followed by DNA ligase III-XRCC1 (short-patch) or DNA ligase I (long-patch) to seal the nick and complete BER (Kubota et al. 1996; Cappelli et al. 1997; Pascucci et al. 1999; Matsumoto et al. 1999).

The importance of BER in the repair of DNA lesions is substantial, and tumor-associated *POLB* variants are repeatedly linked to genomic instability and cellular transformation – particularly in colorectal and gastric cancers (Wang et al. 1992; Lang et al. 2004; 2007; Nemec et al. 2012; Rozacky et al. 2015; Murphy et al. 2012). In fact, a large colorectal cancer study found that approximately 40% of tumors harbor mutations in the coding sequence of *POLB*, highlighting the crucial role of Pol-β in the continual repair of thousands of DNA lesions through BER (Donigan et al. 2012).

1.4.5.1.3 Nucleotide-excision repair (NER)

Dedicated to larger, helix-distorting adducts, the NER pathway mainly excises UV-induced photoproducts and other bulky DNA lesions by removing a short oligonucleotide fragment encompassing the damage (Prakash and Prakash 2000). By virtue of duplex DNA and the unidirectionality of transcription, the NER system typically detects bulky lesions via two distinct mechanisms. First is the global genome NER pathway (GG-NER), which surveys all DNA, including nontranscribing strands, across the genome. This global-genome NER branch depends on the Rad7-Rad16 complex which, with the DNA-binding factor Abf1, remodels local chromatin to promote the recognition of helix-distorting DNA lesions by the downstream core NER machinery (Verhage et al. 1994; Reed et al. 1999). Second is the transcription-coupled NER (TC-NER), which rapidly locates lesions occupying the transcribed strand by sensing lesion-blocked RNA polymerase II (RNAPII) (You et al. 1998). During TC-NER, the SWI/SNF2-family ATPase Rad26 facilitates clearance of lesion-stalled RNAPII while Def1 can mediate its degradation, thereby allowing access to the core NER machinery at the bulky lesion (van Gool et al. 1994; You et al. 1998; Tijsterman and Brouwer 1999; Woudstra et al. 2002; Reid and Svejstrup 2004). While the recognition steps required for GG-NER and TC-NER activation differ, both converge on a common NER core pathway. This pathway relies on the sequential recruitment of key enzymes, starting with the docking of the Rad4-Rad23 complex, followed by TFIIH and Rad14, at the site of damage (Guzder et al. 1996; Nemzow et al. 2015). To facilitate subsequent excision events, Rad14 promotes TFIIH-driven, ATP-dependent DNA unwinding, resulting in an RPA-stabilized ssDNA bubble (Guzder et al. 1995). The exposed fragment is cleaved on the 5’ side by the Rad1-Rad10 heterodimeric endonuclease, and on the 3’ side by the Rad2 endonuclease (Bardwell et al. 1994; Tomkinson et al. 1993; Harrington and Lieber 1994). The resulting gap, generated by the excision of a 25-30 nucleotide fragment, is filled by the replicative DNA polymerases δ (Pol3) or ε (Pol2), after which Cdc9 seals the nick (Wu et al. 1999; 2001; Shivji et al. 1995).

The process of NER is highly conserved from yeast to humans, though with notable species-specific differences (Guzder et al. 1995). Akin to yeast, both the GG-NER and the TC-NER sub-pathways detect lesions –GG-NER by scanning the genome, and TC-NER by sensing stalled RNA polymerase II– converging on the same core NER machinery (Volker et al. 2001; Moreno et al. 2023). During GG-NER, the XPC-RAD23B-CETN2 heterotrimer cooperates with the $CRL4^{DDB2}$ UV-DDB ubiquitin ligase –composed of DDB2, DDB1, CUL4, and RBX1– to bind and process UV photoproducts (Araki et al. 2001; Nishi et al. 2005; Wakasugi et al. 2002; Sugasawa et al. 2005). Budding yeast lacks a functional DDB2 homolog, and thereby diverges from the human GG-NER machinery in that respect (Sakai et al. 2020). In TC-NER, CSB –together with CSA and UVSSA– recognizes the stalled RNA polymerase II and recruits TFIIH to promote repair (van der Weegen et al. 2020; Schwertman et al. 2012; Duan et al. 2021; Moreno et al. 2023). The core NER machinery, responsible for the processing of lesions recognized by both GG-NER and TC-NER, operates via the TFIIH helicase/translocase activities (XPB and XPD) to assess the lesion with help from the verification scaffold XPA and RPA (Drapkin et al. 1994; Li et al. 1995; Moreno et al. 2023). Dual incision –enabled by the XPF-ERCC1 endonuclease heterodimer on the 5’ side and the XPG endonuclease on the 3’ side– releases a fragment of approximately 30 nucleotides (Sijbers et al. 1996; O’Donovan et al. 1994; Hu et al. 2013), yielding a gap filled by DNA polymerases δ/ε (Shivji et al. 1995; Aboussekhra et al. 1995) and preferentially sealed by DNA ligase I in proliferating cells, or DNA ligase III in non-dividing cells (Mortusewicz et al. 2006; Paul-Konietzko et al. 2015).

Hereditary defects in the human NER pathway can yield serious autosomal-recessive disorders and developmental abnormalities that span a characteristic spectrum (Spivak and Hanawalt 2015; Hozhabrpour et al. 2024). Xeroderma pigmentosum (XP) presents with severe UV sensitivity and markedly increased risks of early-onset cutaneous and ocular cancers –often due to defects in GG-NER factors (i.e., XPC, DDB2)– with approximately 25% of affected individuals exhibiting progressive neurologic manifestations including cognitive impairment, acquired microcephaly, and ataxia (Kraemer et al. 2022; Leung et al. 2022; Bradford

et al. 2011; Yurchenko et al. 2023). While XP is cancer-prone because of impaired GG-NER, other syndromes –such as Cockayne syndrome (CS) and UV-sensitive syndrome (UV^{S}S)– are characterized primarily by impaired TC-NER and are typically not associated with increased cancer risk (Reid-Bayliss et al. 2016). In CS, patients mainly present growth failure, neurodevelopmental degeneration, and photosensitivity due to failure to resume transcription after UV-induced, transcription-blocking lesions; UV^{S}S patients exhibit milder cutaneous photosensitivity with little to no neurodegeneration, reflecting a different TC-NER defect (Laugel et al. 2010; Calmels et al. 2018). Mechanistically, CS results from biallelic mutations in CSB (*ERCC6*) or CSA (*ERCC8*), whereas UV^{S}S is caused by mutations in UVSSA (*UVSSA*) (Schwertman et al. 2012; Nakazawa et al. 2012; Gonzalo-Hansen et al. 2024; Kordon et al. 2022). Trichothiodystrophy (TTD) –caused by mutations in TFIIH subunits XPB (*ERCC3*) or XPD (*ERCC2*), among others– manifests as brittle nails/hair, photosensitivity, and intellectual impairment (Theil et al. 2023; Ferri et al. 2025; Lanzafame et al. 2025). Other NER-related disorders include De Sanctis-Cacchione (DSC, a severe XP variant) syndrome, cerebro oculo-facio-skeletal (COFS) syndrome, and XPF-ERCC1 progeroid syndrome (XFEPS), which share outcomes such as microcephaly, growth failure, and varying degrees of neurodevelopmental impairment (Spivak and Hanawalt 2015). The wide range of consequences associated with defective NER underscores the importance of this process in organismal homeostasis and its broad physiological reach.

1.4.5.1.4 Mismatch repair (MMR)

The highly conserved eukaryotic DNA mismatch repair (MMR) pathway is essential for correcting replication errors that evade the proofreading activity of high-fidelity DNA polymerases (Kunkel and Erie 2015; Huang and Li 2020). To correct base-base mismatches or short insertion/deletion loops (IDLs) –a process conserved in budding yeast and human– the MutSα complex, comprising the Msh2 (human MSH2) and Msh6 (human MSH6) subunits, first recognizes the mispair-containing DNA region and converts into an ATP-bound sliding-clamp state on the DNA duplex (Alani 1996; Kunkel and Erie 2015; Kadyrova et al. 2023). For larger loops,

the Msh2-Msh3 MutSβ complex, operating in a similar manner to MutSα, is instead deployed (Palombo et al. 1996). In this ATP-bound conformation, MutSα/MutSβ interacts with and recruits the Mlh1-Pms1 (human MLH1-PMS2) MutLα endonuclease complex at the site of the recognized mismatch, allowing for the introduction of a PCNA/RFC-stimulated MutLα-driven strand-specific nick (Kadyrov et al. 2007; Kunkel and Erie 2015; Witte et al. 2023). This nick serves as an entry point for Exo1-mediated excision, which leaves a single-stranded gap spanning the length of the now removed erroneous sequence (Tishkoff et al. 1997; Tran et al. 1999). Akin to other types of DNA repair pathways, DNA polymerase δ (Pol3) fills the gap that is then sealed by Cdc9 in yeast and DNA ligase I in human cells, restoring fidelity (Reyes et al. 2021; Sallmyr et al. 2020; Huang and Li 2020).

Perhaps one of the best representations of the clinical importance of MMR in human diseases is Lynch syndrome –historically termed hereditary non-polyposis colorectal cancer (HNPCC)– an autosomal-dominant cancer predisposition syndrome stemming from germline pathogenic variants in *MLH1, MSH2, MSH6,* or *PMS2* (Peltomäki et al. 2023; Dominguez-Valentin et al. 2023; Bhattacharya et al. 2025). Patients with Lynch syndrome have inherited one defective MMR allele and, upon inactivation of the second allele, develop MMR-deficient tumors bearing microsatellite instability (MSI) – i.e., length alterations at short tandem repeats from uncorrected replication slippage (Yamamoto et al. 2024; Baretti and Le 2018). Individuals with Lynch syndrome present substantially elevated lifetime risks of developing MSI-high colorectal and endometrial cancers –that vary by the affected gene– along with elevated risks of other malignancies including ovarian cancer, upper tract urothelial, small bowel, and gastric cancers (Peltomäki et al. 2023; Dominguez-Valentin et al. 2023; Eikenboom et al. 2024; Dueñas et al. 2020; Bhattacharya et al. 2025). By driving a high mutation burden, loss of MMR highlights the essential role this pathway fulfills in the maintenance of genome stability and its central importance in clinical cancer genetics (Kunkel and Erie 2015; Baretti and Le 2018; Yamamoto et al. 2024).

1.4.5.1.5 Homologous recombination (HR)

DSBs caused by ionizing radiations and mutagenic enzymes or chemicals are profoundly disruptive to cellular homeostasis and therefore must be repaired swiftly and accurately to preserve genome stability (Ciccia and Elledge 2010). During homologous recombination (HR), an intact DNA strand showing homology to the DSB ends (*i.e.*, the duplicated sister chromatid, or a homologous chromosome) behaves as a template to precisely repair the broken dsDNA. This process begins when the MRX complex and the Sae2 endonuclease engage the broken DNA to induce a 5'-3' nick necessary for its subsequent resection, a process supported by Cdc5 activity (Mimitou and Symington 2009; Donnianni et al. 2010). Exo1, in partnership with the Dna2-Sgs1 endonuclease/helicase complex, carries out long-range resection as duplexed DNA becomes unwound (Zhu et al. 2008; Mimitou and Symington 2008). The resulting ssDNA overhangs, rapidly coated with RPA, behave as docking platforms that recruit key recombination mediators, including Rad52 (Gasior et al. 1998). RPA-bound Rad52 performs the crucial task of replacing RPA molecules with polymerized Rad51, resulting in the formation of an ATP-generated presynaptic Rad51 nucleoprotein filament essential for homology search and strand invasion (Deveryshetty et al. 2023). The D-loop intermediate formed as Rad51 filament locates and invades a homologous duplex primes the 3' invading strand for DNA synthesis, a process canonically performed by the DNA polymerase δ (Pol3) (Li et al. 2009). Throughout this process, the Srs2 helicase plays a pivotal role in regulating the timing and outcome of Rad51 filament formation and subsequent disassembly (Krejci et al. 2003; Ira et al. 2003).

Upon completion of strand extension, the joint molecule can be processed by four distinct mechanistic routes, yielding either crossover (CO) or non-crossover (NCO) outcomes. During synthesis-dependent strand annealing (SDSA), the Sgs1-Top3-Rmi1 (STR) complex unwinds and dismantles the formed heteroduplex to allow for the annealing of the extended 3' tail back to its complementary end, located downstream of the removed break, yielding an NCO product (Allers and Lichten 2001). In the case of double Holliday junction (dJH) formation, the second 3' overhand is captured before the D-loop is dismantled, making both ends of

the break pair with the donor duplex and generating two separate heteroduplex regions (Bzymek et al. 2010). To dissolve double Holliday junctions, the STR complex partners with the Mlh1-Mlh3 MutLγ complex and Exo1 to bring both junctions closer to each other and decatenate the crossed DNA strands, resulting in the exclusive production of NCO products (Ira et al. 2003; Zakharyevich et al. 2010). When dissolution is incomplete, Holliday junction resolution can rely on structure-selective nucleases that include Mus81-Mms4, notably activated in a Cdc5-dependent manner (Gallo-Fernández et al. 2012), Yen1, or Slx1-Slx4. These enzymes cleave each junction to generate either CO or NCO products, depending on the orientation of the inflicted cut, leading to HJ resolution (Zakharyevich et al. 2012; Jessop and Lichten 2008). When only one end of the DSB is available (*i.e.,* at a chromosome end, or a collapsed replication fork) the extended D-loop can morph into a migrating replication fork through an HR pathway termed break-induced replication (BIR). In that situation, DNA polymerase δ (Pol3) synthesizes DNA by copying the donor chromosome continuously until its end (Lydeard et al. 2007). Ultimately, this spectrum of HR resolution pathways allows cells to maximise genome stability by favoring NCO outcomes whenever possible, while still permitting COs when essential (Johnson and Jasin 2000).

While the logic of HR is highly conserved from yeast to humans, key differences must be stated. First, rather than relying on MRX-Sae2 to engage broken DNA, human cells deploy MRN with CtIP, and CDK-dependent control restricts productive end-resection to S/G2 (Huertas and Jackson 2009). In addition, the 53BP1-RIF1-Shieldin axis opposes resection outside S/G2 – a mammalian module that budding yeast lacks (Bunting et al. 2010; Noordermeer et al. 2018; Sartori et al. 2007). In humans, the recombination mediator required for RAD51 filament loading hinges on the BRCA1-PALB2-BRCA2 cascade, making *BRCA* genes central to HR-driven DNA repair (F. Zhang et al. 2009; Jensen et al. 2010; Sullivan and Bernstein 2018). Cell cycle kinases also modulate RAD51 function, including PLK1, which directly phosphorylates RAD51 to promote its recruitment and support DNA damage-induced HR (Yata et al. 2012). To regulate RAD51 filament formation and disassembly, human cells exploit FBH1, RECQ5, and the PCNA-associated inhibitor

PARI as anti-recombinases (Simandlova et al. 2013; Hu et al. 2007; Moldovan et al. 2012). Joint molecules are preferentially channeled to NCOs in both species; humans specifically exploit the BLM-TOP3A-RMI1/2 dissolvasome and, when dissolution is insufficient, structure-selective nucleases (MUS81-EME1, SLX1-SLX4, GEN1) yielding either COs or NCOs according to cleavage geometry (Xue et al. 2013; Wyatt et al. 2013; Rass et al. 2010). Finally, while budding yeast uses BIR when only one DSB end is available, human cells commonly deploy POLD3-dependent mitotic DNA synthesis (MiDAS) at common fragile sites (CFS) and break-induced telomere synthesis in alternative lengthening of telomere (ALT) cancers – BIR-like solutions adapted to mammalian genome maintenance (Bhowmick et al. 2016; Minocherhomji et al. 2015; Dilley et al. 2016).

Serious hereditary cancer syndromes can arise from germline defects in the HR pathway – most notably loss-of-function variants in *BRCA1* or *BRCA2*, which underlie hereditary breast and ovarian cancer (HBOC) and confer substantially increased risks of breast, ovarian, prostate, and pancreatic cancers (Li et al. 2022; Kuchenbaecker et al. 2017). These elevated cancer risks reflect the central role of BRCA1/2 in genome maintenance, as biallelic inactivation of core HR genes drives accumulation of unrepaired DSBs and gross chromosomal rearrangements resulting from defective repair fidelity (Lord and Ashworth 2016). Other genetic disorders –including Fanconi anemia (FA)– can stem from biallelic mutations in *BRCA2* (FANCD1) or other genes at the BRCA/HR interface (i.e., *PALB2* (FANCN), *BRIP1* (FANCJ), *FANCC*, *FANCE*), establishing the clinical continuum between HR failure and human disease (Howlett et al. 2002; Reid et al. 2007; Levitus et al. 2005; Ceccaldi et al. 2016; Mehta and Ebens 2021). Fanconi anemia is characterized by bone marrow failure, congenital abnormalities, and markedly increased cancer predisposition, arising from defects in HR-mediated DNA interstrand crosslink (ICL) repair pathway (Ceccaldi et al. 2016; Nalepa and Clapp 2018; Niraj et al. 2019). From a therapeutic perspective, the characteristic vulnerability of HR-deficient cancer cells has been exploited via poly(ADP-ribose) polymerase (PARP) inhibition, a canonical synthetic-lethal strategy first proven in BRCA-deficient models and translated to patients (Bryant et al. 2005; Farmer et al. 2005; Fong et al. 2009; Lord and Ashworth 2017).

1.4.5.1.6 Non-homologous end-joining (NHEJ)

In haploid or G0/G1-stage cells, non-homologous end joining (NHEJ) is the primary pathway deployed to rejoin free DNA ends arising from a chromosomal DSB (Emerson and Bertuch 2016; Ghosh and Raghavan 2021b). The NHEJ pathway directly ligates broken ends back together with minimal processing, preventing erratic chromosomal rearrangements or prolonged checkpoint-mediated cell cycle arrest (Emerson and Bertuch 2016). The pathway begins by the recruitment of the Yku70-Yku80 telomeric Ku complex onto both DNA termini, where bound heterodimers protect ends from excessive resection and dock key downstream effectors at the site of damage (Boulton and Jackson 1996; Clerici et al. 2008). Simultaneously, the MRX complex tethers onto both DNA ends to maintain them in close proximity, facilitating synapsis and preventing excessive end mobility (Chen et al. 2001; Tisi et al. 2020). If DNA ends present gaps, incompatible overhangs, or damaged bases, Pol4- and MRX-driven end processing steps can restore blunt ends to favor effective ligation (Moore and Haber 1996; Daley et al. 2005). Compatible ends are then assembled and ligated through the recruitment of the DNA ligase IV homolog Dnl4 which, bound to its cofactor Lif1, firmly remains at the break site via the DNA end-binding protein Nej1 and sustained tethering to the MRX complex (Wilson et al. 1997; Palmbos et al. 2008; Herrmann et al. 1998; Deshpande and Wilson 2007). Dnl4 then catalyzes phosphodiester bond formation between aligned DNA ends, thereby sealing the break (Wilson et al. 1997).

Relative to budding yeast, mammalian cells deploy additional mediators throughout the NHEJ pathway. First, rather than relying on the KU heterodimer alone, human cells form the KU-DNA-PKcs holoenzyme: KU70-KU80 recruits DNA-PKcs to assemble DNA-PK, which gates end-processing and organizes the transition from long-range to short-range synapsis (Chen et al. 2021; Stinson and Loparo 2021). The Artemis nuclease –activated by DNA-PKcs– processes hairpins/overhangs, while Pol μ and Pol λ align and extend broken or incompatible ends to promote compatibility (Poinsignon et al. 2004; Watanabe and Lieber 2022; Yosaatmadja et al. 2021; Cerqueira et al. 2023; Ghosh and Raghavan 2021a; Zhao et al. 2020; Capp et

al. 2006). Ligation is then catalyzed by the XRCC4-LIG4 complex, which is scaffolded and stabilized within the synaptic assembly via APLF and supported by the KU-binding tethers XLF and PAXX (Bragança et al. 2023; Stinson and Loparo 2021; Deshpande and Wilson 2007; Seif-El-Dahan et al. 2023). During mitosis –when classical NHEJ and HR are attenuated– PLK1 phosphorylates DNA polymerase θ (Polθ/POLQ), activating Polθ-mediated end joining to repair mitotic DSBs and help preserve genome stability (Brambati et al. 2023; Gelot et al. 2023).

The core function of NHEJ in DSB repair makes germline mutations in NHEJ components especially threatening for cell homeostasis and can lead to severe human diseases manifesting as immunodeficiency and developmental failures (Slatter and Gennery 2020; Fournier et al. 2022). For example, DNA ligase IV deficiency (LIG4 syndrome) stems from variants in *LIG4* and is characterized by microcephaly, growth retardation, distinctive facial features, bone marrow dysfunction/pancytopenia, and severe combined immunodeficiency (SCID) (Altmann and Gennery 2016; Staines Boone et al. 2019). The immunodeficiency arises because NHEJ is required during V(D)J recombination in developing lymphocytes; when DSB intermediates cannot be rejoined, functional B and T cells fail to develop (Stinson and Loparo 2021; Christie et al. 2022). Beyond immunodeficiency, patients with LIG4 syndrome show marked radiosensitivity to DNA-damaging agents –such as ionizing radiation– reflecting the ubiquitous role of ligase IV in DSB repair across cell types (Altmann and Gennery 2016; Staines Boone et al. 2019). Another NHEJ-related disorder is Artemis deficiency, caused by mutations in *DCLRE1C* encoding Artemis – an endonuclease that, in conjunction with DNA-PKCs, opens RAG-generated hairpins during V(D)J recombination (Ma et al. 2002; Ghadimi et al. 2023). Artemis-deficient individuals typically present with radiosensitive SCID and marked cellular radiosensitivity from impaired DSB repair (Cowan et al. 2022). In contrast to LIG4 syndrome, microcephaly and growth retardation are not consistent features of Artemis deficiency, underscoring its immunologic –rather than syndromic– phenotype (Volk et al. 2015; Ghadimi et al. 2023). Clinically, DNA-PK inhibitors –such as

peposertib and AZD7648– are being tested in early-phase trials to augment radiotherapy/chemotherapy efficiency by blocking the NHEJ pathway in tumors (Samuels et al. 2024; Kagawa et al. 2024; Yap et al. 2025).

1.5 Cell cycle checkpoints: Guardians of genome stability

Checkpoints are remarkably conserved from yeast to humans, underscoring the fundamental role these pathways play in cell cycle regulation and stress response. In budding yeast, six sequential checkpoints safeguard discrete cell cycle transitions: The G1/S (Start) checkpoint, the DNA replication checkpoint (DRC), the intra-S DNA damage checkpoint, the morphogenesis checkpoint in late S phase/early G2, the G2/M checkpoint, the spindle assembly checkpoint (SAC) in metaphase, the spindle position checkpoint (SPoC) in anaphase, and the NoCut checkpoint at the onset of cytokinesis (Figure 1.4.). The following sub-sections present a brief overview of every budding yeast checkpoint and their relevance in the preservation of genomic integrity.

1.5.1 The G1/S (Start) checkpoint

The G1/S or Start checkpoint, also termed restriction point in metazoans, constitutes a pivotal regulatory node at which cells integrate signals related to nutrient availability, growth conditions, and cell size before irreversibly committing to a new cycle of cell division (Adler et al. 2022; Pennycook and Barr 2020). In early G1, active Cln3-Cdc28 complexes phosphorylate and inactivate the transcriptional repressor Whi5, leading to the expression of key SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) transcription factors (Palumbo et al. 2016; Nasmyth and Dirick 1991; Koch et al. 1993). This prompts the transcriptional activation of several genes involved in the irreversible transition from G1 to S phase, including G1 cyclins (*CLN1* and *CLN2)* and S cyclins (*CLB5* and *CLB6*), establishing a strong feedback loop that further reinforces the commitment to cell division (Rubin et al. 2020; Adler et al. 2022). Active Cln1/2-Cdc28 then initiates multi-site phosphorylation on the Cdc28 inhibitor Sic1. This primes Sic1 for a subsequent phosphorylation event carried by Clb5/6-Cdc28,

culminating in rapid Sic1 destruction. This negative feedback loop triggers a robust surge in active S phase Clb5/6-Cdc28, ensuring a single and irreversible commitment to S phase entry (Yang et al. 2013).

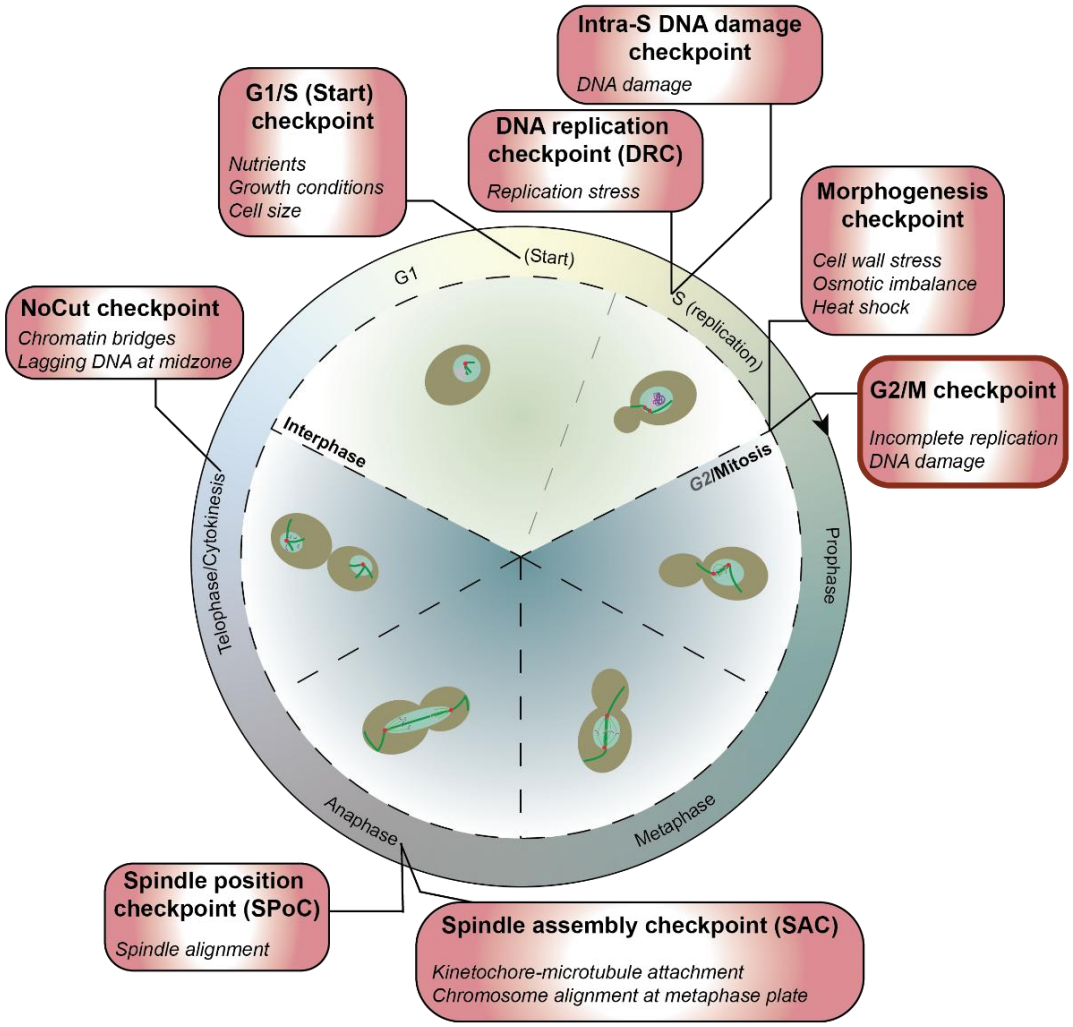

Figure 1.4. Overview of the major cell cycle checkpoints in *Saccharomyces cerevisiae.* These include the G1/S (Start) checkpoint, the DNA replication checkpoint (DRC), the intra-S DNA damage checkpoint, the morphogenesis checkpoint, the G2/M checkpoint, the spindle assembly checkpoint (SAC), the spindle position checkpoint (SPoC), and the NoCut checkpoint.

Under suboptimal cycling conditions or significant cellular stress –such as nutrient deprivation, osmotic imbalance, oxidative strain, heat shock, or DNA damage – cells can activate a range of signaling pathways designed to safeguard genomic integrity (Gasch and Werner-Washburne 2002). These pathways, while mechanistically distinct and tailored to particular forms of cell stress, collectively operate to delay cell cycle progression. In G1, their downstream effects converge on a common set of outcomes: inhibition of

cyclin-Cdc28 activity, stabilization of the key Cdc28 inhibitor Sic1, and global repression of the G1/S transcriptional program (Duch et al. 2012). This tailored response therefore ensures that cells do not initiate DNA replication under unfavorable cycling conditions, which could otherwise jeopardize genomic integrity.

1.5.2 The DNA replication checkpoint (DRC)

During S phase, cells prepare for division by launching the delicate process of genome duplication (Bell and Labib 2016). In G1 phase, cells proactively establish the fundamental framework for DNA replication through the formation of pre-replication complexes (pre-RCs), defined as multiprotein assemblies competent for the licensing of replication origins (Diffley et al. 1994). Upon entry into S phase, the concurrent rise in Cdc28 and Cdc7-Dbf4 kinase (DDK) activity prompts the transformation of licensed origins into functional replication forks, notably via activation of the replicative Cdc45-MCM-GINS (CMG) helicase complex (Sheu and Stillman 2010; Bousset and Diffley 1998). This modification allows for the loading of DNA polymerases α (Pol1), δ (Pol3), and ε (Pol2) onto newly unwound DNA strands, thereby initiating bidirectional DNA synthesis (Bell and Labib 2016). Importantly, cells exploit a range of regulatory mechanisms to ensure that the genome is replicated once and only once per cell cycle. A key protective measure is the temporal separation between helicase loading in G1 phase and its activation in S phase, which is closely regulated by oscillating levels of Cdc28 activity. Between G1 and S phase, Cdc28 activity rises swiftly from low to high and remains elevated until it drops during mitotic exit. This effectively blocks relicensing of replication origins by preventing a potential return to a pre-replicative state (Dahmann et al. 1995).

The DNA replication checkpoint (DRC), which operates through the DDR, specifically addresses stalled or stressed replication forks to protect genome stability (Pardo et al. 2017; Saldanha et al. 2023). Upon replication insults –such as nucleotide depletion, DNA secondary structures, or polymerase-helicase uncoupling– stretches of ssDNA accumulate and are rapidly coated by RPA (Zou and Elledge 2003). DDR signaling initiates when RPA-coated ssDNA recruits Mec1-Ddc2 at stalled forks, and becomes rapidly

amplified at adjacent ssDNA-dsDNA junctions via 9-1-1 clamp loading (Zou and Elledge 2003; Navadgi-Patil and Burgers 2009). Unlike the canonical DDR –which can respond to a single DNA lesion– the replication checkpoint exhibits a threshold behavior for Rad53 activation in a way that avoids spurious responses to transient RPA-coated ssDNA naturally found at moving forks (Shimada et al. 2002). Downstream, this checkpoint operates to stabilize the replisome –in part by Mrc1-dependent control of CMG/Pol ε dynamics– blocks late-origin firing via Rad53-dependent phosphorylation of Sld3 and Dbf4, and raises dNTP pools by stimulating the Dun1-RNR/Sml1 (and Crt1) axis (McClure and Diffley 2021; Katou et al. 2003; Osborn and Elledge 2003; Santocanale and Diffley 1998; Zegerman and Diffley 2010; Lopez-Mosqueda et al. 2010; Zhao and Rothstein 2002; Huang et al. 1998). Together, these mechanisms preserve fork integrity until replication stress resolves, effectively safeguarding genome stability.

1.5.3 The intra-S DNA damage checkpoint

Upon detection of lesions during S phase (i.e., MMS adducts, UV lesions, resected DSBs), the intra-S DNA damage checkpoint activates the Mec1-Rad9-Rad53 branch of DDR signaling (Vialard 1998). While both the DRC and the intra-S DNA damage checkpoint converge on Mec1/Rad53 DDR signaling, they differ in their inputs and mediators (Pardo et al. 2017). In contrast to the fork-derived DRC, in which Mrc1 is the primary mediator, the intra-S checkpoint engages the DNA damage-specific mediator Rad9 to delay cell cycle progression and promote DNA repair (Pardo et al. 2017; Sweeney et al. 2005). This DNA damage checkpoint-driven delay is enforced by Chk1-dependent stabilization of Pds1 securin and by regulation of MT-associated proteins, collectively preventing premature anaphase onset and spindle elongation (Palou et al. 2015; Agarwal et al. 2003; Wang et al. 2001; Krishnan et al. 2004). In parallel, Mec1/Rad53 signaling suppresses late origin firing –via Rad53 phosphorylation of Sld3 and Dbf4– and stabilizes stalled forks, akin to the DRC, thereby coordinating DNA repair with replication restart (Zegerman and Diffley 2010; Lopez-Mosqueda et al. 2010; Tercero and Diffley 2001; Szyjka et al. 2008).

1.5.4 The morphogenesis checkpoint

In budding yeast, cell proliferation relies on the timely and spatially organized emergence of a polarized bud. In late G1, bud formation is initiated at a single site on the mother cell cortex through the localized action of the GTPase Cdc42, which activity depends on the GEF Cdc24 (Ziman et al. 1993; Sloat et al. 1981; Zheng et al. 1994; Shimada et al. 2000). This process establishes the site of future bud formation and promotes the recruitment of key polarity regulators, including the scaffold protein Bem1, to support polarized growth (Peterson et al. 1994). Actin-mediated vesicle trafficking and localized cell wall remodeling drive the expansion of the developing daughter bud, thereby spatially restricting growth and temporally coordinating this process with cell cycle progression (Adams and Pringle 1984; Pruyne and Bretscher 2000). To ensure that bud emergence occurs only once per cell cycle, the G1 phase Cln-Cdc28 complex promotes bud formation, while Clb-Cdc28 complexes active during later stages of mitosis prevent rebudding (Lew and Reed 1993).

In response to cell wall stress, osmotic imbalance, or heat shock, cells can detect disrupted actin organization and activate the morphogenesis checkpoint (McMillan et al. 1998). This leads to the robust stabilization and accumulation of the Cdc28 inhibitor Swe1 which, under normal cycling conditions, would be targeted for degradation upon its enrichment at the mother bud neck (McNulty and Lew 2005; Sia et al. 1998). Disruptions in septin organization prevent proper localization of the Nim1-related kinase Hsl1 and the methyltransferase Hsl7 to the bud neck, compromising their ability to recruit Swe1 and promote its phosphorylation-dependent degradation (Longtine et al. 2000; Crutchley et al. 2009; King et al. 2012; McMillan et al. 1998). Active Swe1 inhibits Cdc28 function through tyrosine phosphorylation at a key conserved residue (Y19), effectively preventing the formation of active Clb-Cdc28 complexes required for mitotic entry and consequently pausing cell cycle progression (Lew and Reed 1995).

Importantly, once bud formation and cell polarity are reestablished, Cdc5 plays a pivotal role in silencing the morphogenesis checkpoint by promoting Swe1 hyperphosphorylation at the bud neck, which

sends Swe1 for ubiquitin-mediated degradation and effectively promotes mitotic entry (Bartholomew et al. 2001; Sakchaisri et al. 2004; Asano et al. 2005).

1.5.5 The G2/M checkpoint

The G2/M checkpoint operates at the interface of irreversible chromosome segregation and, therefore, serves as the final gatekeeper of genome integrity before the onset of mitosis. As such, its role as a regulatory barrier is essential in ensuring the detection and repair of residual DNA damage or incomplete replication that may propagate into subsequent cell divisions. Under normal conditions, the G2/M transition operates as an all-or-nothing bistable mitotic switch initiated by a rapid accumulation of mitotic cyclins (Clb3-4/Clb1-2) (Murray et al. 1989; Surana et al. 1991). At this stage, the persistent inhibitory phosphorylation imposed by Swe1 on Cdc28 leads to the accumulation of predominantly inactive Clb-Cdc28 complexes (Booher et al. 1993). As Cdc5 accumulates in late S phase, Swe1 localizes to the bud neck in a Hsl1/Hsl7-dependent manner and undergoes a Cdc5-mediated phosphorylation event that targets it for ubiquitin-mediated degradation (Sakchaisri et al. 2004; Asano et al. 2005). The concurrent rise in Mih1 phosphatase activity generates a positive feedback loop that further activates Clb-Cdc28 complexes, which, in turn, reinforces Mih1 activity (Kumagai and Dunphy 1992; Pal et al. 2008). As this feedback loop progressively overrides Swe1-mediated inhibition of Cdc28, the resulting surge in Cdc28 activity establishes a robust and irreversible commitment to mitosis (Solomon et al. 1990; Pomerening et al. 2003; Ferrell 2008).

The G2/M checkpoint, akin to the G1 and intra-S DNA damage checkpoints, can integrate stress signals related to damaged DNA and activate the DDR as a critical protective response. While the DDR primarily addresses oxidative stress in G1 and DNA damage/replication errors in S phase, the G2/M checkpoint DDR is specifically responsive to DNA lesions caused by ionizing radiations, chemical mutagens, aberrant DNA metabolism, or spontaneous chemical modifications at the DNA level (Paulovich et al. 1997). Under DNA damage conditions, Cdc28 inhibition occurs through the sustained signaling of the DDR sensor

kinase Mec1 and its downstream checkpoint kinase Rad53, which redundantly modulates Cdc28 activity alongside Swe1 (Palou et al. 2015). Importantly, the Mec1-mediated DNA damage response simultaneously promotes Chk1-dependent stabilization of Pds1 securin and Rad53-dependent inhibition of Cdc5 activity, together establishing a robust checkpoint mechanism that prevents chromosome segregation under damaging cycling conditions (Palou et al. 2015; O'Connell et al. 1997; Sanchez et al. 1999; Hu et al. 2001).

1.5.6 The spindle assembly checkpoint (SAC)

During mitosis, the spindle assembly checkpoint (SAC) ensures the accurate segregation of replicated chromosomes to daughter cells. This checkpoint assesses whether chromosomes have achieved amphitelic kinetochore-MT attachments –where sister chromatids are bi-oriented and each kinetochore is attached to MTs from opposing spindle poles– and whether sister chromatids are properly aligned at the metaphase plate (Musacchio 2015). Under optimal conditions, the transition into anaphase is contingent on the release of the APC/C co-activator Cdc20 from SAC inhibition –specifically, from inhibitory binding by Mad2 and the Mad3-Bub3 module– thereby enabling full APC/C^{Cdc20} activity (Hwang et al. 1998; Fang et al. 1998; Lim et al. 1998). This event triggers the degradation of key mitotic pillars, including securin and B-type cyclins, leading to separase activation, cohesin cleavage, and a concomitant reduction in Cdc28 activity that irreversibly commits cells to chromosome segregation and mitotic exit (Cohen-Fix et al. 1996).

Barring bipolar attachment of well-aligned chromosomes to the mitotic spindle, the SAC prevents entry into anaphase via a set of conserved proteins together forming the mitotic checkpoint complex (MCC) (Musacchio 2015; Liu and Zhang 2016). This diffusible complex, of which the main players include Mad1, Mad2, Bub1, Bub3, Mad3/BubR1 and the kinase Mps1, assembles at unattached kinetochores upon detection of abnormal chromosome tension or positioning (Hoyt et al. 1991; Weiss and Winey 1996). Specifically, the Ndc80-mediated binding of Mps1 to kinetochores creates phosphorylation-induced docking sites on the scaffold protein Spc105, allowing for the recruitment of other MCC members (Ciferri et al. 2007;

London et al. 2012). Then, kinetochore-anchored Mad1 catalyzes the conversion of open Mad2 (O-Mad2) to its closed (C-Mad2) conformation, allowing for the direct binding of C-Mad2 to Cdc20 (Lara-Gonzalez, Kim, et al. 2021; De Antoni et al. 2005; Luo and Yu 2008; Lara-Gonzalez, Pines, et al. 2021). This leads to the accumulation of complexed C-Mad2-Cdc20 which, in partnership with Mad3 and Bub3, operate as the active MCC complex (Fang et al. 1998; Sczaniecka et al. 2008). The MCC competes against free Cdc20 for APC/C binding and, in doing so, prevents APC/C activation (Chao et al. 2012; Li et al. 1997). The resulting stabilization of securin and B-type cyclins, pending the resolution of chromosome-related tension and alignment issues, halts cell cycle progression in metaphase to safeguard genome stability (Yamamoto et al. 1996; Clute and Pines 1999). Importantly, Cdc5 hyperphosphorylates Mad3 during SAC activation, further supporting MCC assembly and activity (Rancati et al. 2005). RSC^{Rsc2}-bound Cdc5 was also reported to support SAC adaptation, likely through its function in the FEAR and the MEN (Rossio et al. 2010).

1.5.7 The spindle position checkpoint (SPoC)

Due to the intrinsically asymmetric nature of budding yeast cell division, the precise orientation of the mitotic spindle along the mother-daughter axis is essential for accurate DNA segregation. The spindle position checkpoint (SPoC), functionally linked to the MEN and its downstream effectors, passively ensures that cells do not exit mitosis in the absence of proper spindle alignment (Caydasi et al. 2017; Wang et al. 2000, 200)(*See Section 1.3.2.6 for an overview of the MEN*). In case of spindle misorientation, the Bfa1/Bub2 GAP complex maintains the small GTPase Tem1, the upstream MEN activator, in a GDP-bound state to restrain MEN activation (Bloecher et al. 2000). This regulation is mediated by the kinase Kin4 which, under normal conditions, asymmetrically localizes to the mother SPB in mid-anaphase. MEN activation occurs only when the daughter-directed SPB enters the bud, allowing Tem1 to encounter its GEF Lte1, which initiates the mitotic exit cascade (Bardin et al. 2000). In response to spindle misalignment, Kin4 is recruited to both SPBs via Spc72, where it sustains Bfa1/Bub2 activity by opposing Cdc5-mediated inhibitory phosphorylation,

keeping Tem1 transiently inactive (Pereira and Schiebel 2005; D'Aquino et al. 2005; Maekawa et al. 2007). In parallel, by engaging Cdc5 PBD through a non-canonical interface, Dbf4 simultaneously inhibits Cdc5 ability to activate the MEN (Miller et al. 2009; Chen and Weinreich 2010). Together, these interactions ensure that mitotic exit is delayed until proper spindle alignment is achieved.

1.5.8 The NoCut checkpoint

Late DNA segregation errors –such as anaphase bridges and lagging chromosomes– leave chromatin in the division plane at abscission and, if unaddressed, risk chromosome breakage and compromised genome stability (Petsalaki and Zachos 2021). The budding yeast NoCut checkpoint counters this risk via an Ipl1/Aurora B kinase-dependent surveillance pathway that monitors chromosome clearance at the division site and delays membrane abscission upon detection of residual chromatin (Norden et al. 2006). Mechanistically, the NoCut checkpoint relies on localization of the chromosomal passenger complex (CPC: Ipl1/Aurora B with its regulator Sli15/INCENP and associated subunits) to the spindle midzone, which –when chromatin persists at the bud neck– triggers recruitment and retention of the anillin-like proteins Boi1 and Boi2 at the division site to delay abscission (Mendoza et al. 2009). The SH3 domain of Boi2 is specifically required to inhibit abscission upon NoCut checkpoint activation, making Boi1 dispensable for this function (Masgrau et al. 2017). Once DNA retracts from the neck, the reversible abscission delay imposed by the NoCut checkpoint is lifted, permitting membrane severing and completion of cytokinesis. In humans, cytokinesis failure can result in tetraploidization through cleavage furrow regression and, when chromatin bridges break, trigger breakage-fusion-bridge (BFB) cycles or chromothripsis – catastrophic processes that fuel cancer progression and pose a major threat to genomic stability (Lens and Medema 2019; Petsalaki and Zachos 2021; Guérin and Marcand 2022). This checkpoint shares conceptual similarities with the Aurora B-mediated abscission checkpoint described in human cells, suggesting a conserved role for cytokinesis surveillance across species (Steigemann et al. 2009).

1.6 DNA damage adaptation: Bridging genome surveillance and cell proliferation

The unifying principle at the interface of stress response circuits and cell cycle checkpoints is the inevitable trade-off between short-term proliferation and long-term fitness. This trade-off becomes especially consequential in the context of DNA damage signaling, where chronic or irreparable lesions can render checkpoint arrest counterproductive by drastically constraining clonal survival (Sadeghi et al. 2022). At this crossroads, cells may engage the adaptation response to DNA damage, a regulated override of the DNA damage checkpoint that permits cell cycle re-entry despite moderate levels of persistent DNA lesions (Sandell and Zakian 1993; Toczyski et al. 1997). This pathway is best characterized in the budding yeast *Saccharomyces cerevisiae* where Cdc5, in coordination with other key effectors, attenuates Mec1/Tel1-dependent checkpoint signaling within hours of arrest following either an irreparable DSB, SSBs, or partial telomeric resection (Sandell and Zakian 1993; Toczyski et al. 1997; Lee et al. 1998; Vidanes et al. 2010). An analogous PLK1-driven circuit operates in human cells, where it is frequently modulated by tumors to override damage-induced arrest and resist genotoxic therapy (Syljuåsen et al. 2006; Rödel et al. 2010; Hagege et al. 2021). This section provides an in-depth exploration of the current knowledge surrounding DNA damage adaptation, with a specific focus on the budding yeast model, the molecular logic underpinning its checkpoint override program, and the broader implications of DNA damage adaptation for cancer progression.

1.6.1 Beyond recovery: Apoptosis and the path to DNA damage adaptation

Upon damage detection, cells encountering broken DNA or exposed telomeric regions activate the reversible Mec1/Tel1-dependent G2/M DNA damage checkpoint to halt cell division and support DNA repair and genome stability (Weinert and Hartwell 1988; Garvik et al. 1995; Usui et al. 2001). Recovery –the process by which DDR signaling is deactivated as cells resume mitosis with a restored genome– relies on the efficient execution of DNA repair pathways, and the subsequent downregulation of checkpoint effectors

(Vaze et al. 2002; Yam et al. 2022)(*see section 1.4.5.1 - Canonical DNA repair mechanisms*). These pathways ensure that checkpoint activation remains transient and appropriately scaled to the extent of the genomic damage. In budding yeast, Cdc5 contributes to a range of DNA repair mechanisms via phosphorylation-dependent modifications. This applies to the process of homologous recombination, where Cdc5 phosphorylates the Mms4 subunit of the Mus81-Mms4 Holliday junction resolvase to amplify its activity and enhance processing of recombination intermediates (Gallo-Fernández et al. 2012). In a similar manner, Cdc5 facilitates DSB processing via Sae2 endonuclease phosphorylation, stimulating end resection at chromosomal break sites (Donnianni et al. 2010). However, Cdc5 activity is dispensable during active recovery from DNA damage, as a *cdc5-ad* (L251W) mutant remains proficient for checkpoint recovery and cell-cycle re-entry while showing specific defects in the process of DNA damage adaptation (Toczyski et al. 1997). While these mechanisms are widely conserved from yeast to humans, PLK1 plays a broader role in DDR regulation than its yeast homolog Cdc5, contributing to both DNA repair and checkpoint recovery (Li and Hao 2024). To first support the DNA repair machinery, PLK1 can phosphorylate the DNA repair mediator BRCA1 –promoting foci formation at damage sites– and targets RAD51, enhancing filament formation and overall homologous recombination efficiency (Chabalier-Taste et al. 2016; Yata et al. 2012). PLK1 role in DNA repair also extends to the phosphorylation of CtIP, a key cofactor in DNA end resection during DSB processing, as well as DNA polymerase θ, facilitating end-joining at mitotic DSBs (Wang et al. 2018; Gelot et al. 2023). Following efficient repair, PLK1 targets several effector proteins to terminate the DDR and promote checkpoint recovery. Notably, the checkpoint mediator Claspin –which is essential for ATR-dependent CHK1 (Chk1) activation– is rapidly targeted for degradation in a PLK1-dependent manner during checkpoint reversal (Mailand et al. 2006). Other substrates include the CDK1 inhibitor WEE1 (Swe1), as well as the checkpoint mediators 53BP1 (Rad9) and CHK2 (Rad53) (van Vugt et al. 2004; 2010; Peng 2013).

When the burden of DNA lesions overwhelms repair capacity, cells switch to an apoptosis-like death program (Burhans et al. 2003). In budding yeast, this protective response translates into ROS accumulation,

enhanced chromatin condensation, DNA fragmentation, and metacaspase Yca1 activation (Burhans et al. 2003; Madeo et al. 2002). A similar cascade is triggered in mammalian cells bearing severe levels of DNA damage, where the tumor suppressor p53 transcriptionally induces the BH3-only protein PUMA –activating caspase proteinases and other apoptogenic factors– to eradicate extensively damaged cells (Yu et al. 2003; Aubrey et al. 2018; H. Wang et al. 2023; Hao et al. 2022). While apoptosis represents a fail-safe mechanism to purge cells with irreparable DNA lesions, surrendering immediate survival for long-term genomic preservation ultimately amounts to programmed self-destruction that contradicts the cell's innate need to proliferate. An evolutionary bias toward clonal survival drives cells with persistent DNA damage to deploy all available strategies for lineage defence. DNA damage adaptation lies in this narrow corridor –between checkpoint recovery and apoptosis– reflecting the cell's intrinsic drive to propagate. Although passing unresolved DNA lesions to offspring seems counter-intuitive given its obvious costs, such strategy can still confer significant advantages for overall fitness (Kaye et al. 2004; Galgoczy and Toczyski 2001). This double-edged tactic may allow a subset of progeny to retain enough fitness to survive and proliferate while simultaneously extending the time window during which lingering DNA lesions can be repaired (Serrano and D'Amours 2014).

1.6.2 Thresholds and molecular circuitry of DNA damage adaptation

In budding yeast, DNA damage detection activates a G2/M DDR-dependent checkpoint that results in a transient signaling cascade-driven cell cycle arrest. Within 8-15 hours –a timing that varies with the type of lesion– cells bearing moderate levels of persistent DNA damage can override this checkpoint and re-enter mitosis, while original damage persists (Sandell and Zakian 1993; Toczyski et al. 1997; Pellicioli et al. 2001; Dotiwala et al. 2007). The process of DNA damage adaptation is highly dependent on both the quantity and the severity of DNA lesions and there is, in budding yeast, a well-defined damage threshold above which adaptation becomes inaccessible. While a single irreparable DSB is generally well-tolerated (Lee et al. 1998),

two persistent DSBs enforce an extended –and often permanent– arrest maintained by the SAC (Zhou et al. 2024). Depending on context, cells unable to repair or adapt to persistent DNA damage execute regulated cell death (RCD) (Carmona-Gutierrez et al. 2018), encompassing apoptosis-like RCD –which often involves the metacaspase Yca1 (Madeo et al. 2002)– or caspase-independent RCD (Wysocki and Kron 2004). The relationship between damage extent and adaptation strongly correlates with a detection system where ssDNA abundance at sites of damage directly dictates DDR activation threshold (Lee et al. 1998). It is understood that cumulative DNA damage is sensed via detection of RPA- or Rad52-bound 3' ssDNA overhangs, such that a *rfa1-t11* RPA mutant or a *rad52Δ* mutant can tolerate multiple breaks and adapt as a result of reduced checkpoint signal intensity (Zou and Elledge 2003; Lee et al. 1998; 2003; Pellicioli et al. 2001). Supporting this model, forcibly tethering DNA damage sensors and mediator proteins to one or more chromosomal loci –even in the absence of lesions– suffices to trigger a full G2/M checkpoint response (Bonilla et al. 2008). Multiple minor lesions –including exposed telomeric regions– can therefore equate to a single DSB in terms of checkpoint activation, making cumulative perceived DNA damage the signaling output that really matters. In line with this, mutant *yku70Δ* cells bearing exposed telomeres undergo extensive resection and, as a consequence, fail to adapt following a G2/M checkpoint-mediated cell cycle arrest (Lee et al. 1998). Consistent with the principle that perceived checkpoint signal intensity establishes the threshold for adaptation, deletion/inactivation of resection factors –such as Mre11 and Rad50– or ssDNA-binding proteins –such as RPA– restores adaptation in *yku70Δ* mutants (Lee et al. 1998). In human cells, the upstream signaling logic is conserved, but the fate decision differs: p53-proficient cells are far less prone to checkpoint adaptation and instead mount a robust G2/M arrest (van den Berg et al. 2018). By contrast, p53-deficient cancer cell lines can undergo checkpoint recovery/slippage and enter mitosis with unrepaired lesions, often yielding mitotic catastrophe (Amornwichet et al. 2014; Lukin et al. 2015). PLK1-high cancers often favor survival over mitotic death notably by accelerating checkpoint shut-off –as seen during mitotic override/slippage– and enhancing DNA repair, a pattern associated with resistance to

genotoxic therapies across multiple tumor types (Yata et al. 2012; Chiappa et al. 2022)(See section *1.6.3*. *DNA damage adaptation: Physiological consequences and implications in cancer* for more details).

A seminal genetic screen first identified Cdc5 as a central regulator of DNA damage adaptation (Toczyski et al. 1997). Subsequent functional studies confirmed that Cdc5 operates in a dose-dependent, rate-limiting manner to drive checkpoint escape (Toczyski et al. 1997; Rawal et al. 2016; Vidanes et al. 2010; Donnianni et al. 2010). Following the induction of a single DSB, cells carrying the adaptation-defective *cdc5-ad* allele sustain a G2/M arrest indefinitely, while remaining fully proficient for mitotic progression (Toczyski et al. 1997; Rawal et al. 2016). This striking phenotype underscores the functional dissociation between Cdc5 canonical mitotic roles and its capacity to override a DNA damage-induced cell cycle arrest, highlighting how its contribution to adaptation is mechanistically distinct from its other cellular responsibilities. As such, the hypomorphic catalytic mutant *cdc5-T238A* impairs DNA damage adaptation from reduced kinase activity while permitting normal chromosome segregation, further illustrating how Cdc5 specific role in adaptation is both functionally and catalytically distinct (Rawal et al. 2016). The observation that two adaptation-defective alleles, *cdc5-ad* and *cdc5-16*, complement each other to restore DNA damage adaptation is consistent with distinct, domain-specific Cdc5 functions acting non-redundantly to enable adaptation (Coutelier et al. 2023). Consistent with this, overexpression of *CDC5* partially suppresses adaptation defects associated with *cdc5* alleles and considerably enhances adaptation kinetics (Donnianni et al. 2010). Adaptation to the G2/M DNA damage checkpoint therefore proceeds through a coordinated, Cdc5-driven cascade that progressively silences checkpoint signaling and reinstates cell cycle progression.

To promote DNA damage adaptation, both the apical DDR kinase Mec1 and its binding partner Ddc2 undergo dynamic modifications that support Mec1 deactivation and dissociation from sites of damage, dampening checkpoint signal intensity. Autophosphorylation of Mec1 on Ser1964 curbs its kinase activity and licenses checkpoint override, while phosphorylation of its co-activator Ddc2 on Ser174/Ser182 weakens Mec1-Ddc2 retention at RPA-coated lesions, adding another layer of negative feedback to facilitate DNA

damage adaptation (Memisoglu et al. 2019). Under replication stress, the Slx4 scaffold competes with the Rad9 adaptor from both the Dpb11 sensor complex and γH2A-marked nucleosomes, thereby dampening Rad53 activation while Mec1 signaling remains intact (Ohouo et al. 2013). Shortly after Mec1 autophosphorylation, PP2C/PP4 phosphatases (Ptc2/Ptc3, Pph3) gradually dephosphorylate Rad53 along with other substrates to dismantle the checkpoint (Memisoglu et al. 2019). At persistent DSBs, the Ino80 complex also counteracts SWR1 function by evicting the H2A.Z histone variant, supporting the decline of Rad53 activity and aiding adaptation (Papamichos-Chronakis et al. 2006).

A central function of Cdc5 during DNA damage adaptation is the antagonization of Rad53 activity, which remains hyperphosphorylated throughout the DDR to sustain checkpoint function. Under normal cycling conditions, Cdc5 directly phosphorylates Rad53 on cell cycle-regulated sites (*i.e.,* Ser789/791), both *in vivo* and *in vitro*, modulating its activity (Schleker et al. 2010). Early in the cell cycle, this Cdc5-dependent phosphorylation event is proposed to reinforce Rad53 activity upon DDR activation by lowering Rad53 activation threshold, strengthening its effect during the G2/M checkpoint (Schleker et al. 2010). During a prolonged G2/M arrest, Cdc5 conversely blocks Rad53 hyperphosphorylation to drive DNA damage adaptation (Vidanes et al. 2010). As Cdc5 is dynamically regulated upon checkpoint activation in a Rad53-dependent manner and blocks Rad53 auto-phosphorylation during checkpoint silencing, a feedback loop involving Cdc5 and Rad53 must partially regulate not only checkpoint intensity, but also the timeline of adaptation (Sanchez et al. 1999; Toczyski et al. 1997; Pellicioli et al. 2001; Lopez-Mosqueda et al. 2010; Vidanes et al. 2010). In fact, the adaptation defect of a *cdc5-ad* mutant impeded for Rad53 inactivation can be rescued by overexpressing the PP2C-like phosphatase Ptc2, suggesting a priming function for Cdc5 in the Ptc2/Ptc3-mediated inactivation of Rad53 during adaptation (Leroy et al. 2003). As such, a double *ptc2Δ ptc3Δ* phosphatase mutant is defective for DNA damage adaptation and remains in a permanent cell cycle arrest with high levels of Rad53 phosphorylation (Leroy et al. 2003). The productive docking of Ptc2/Ptc3 onto Rad53 is reliant on Casein Kinase II (CKII) activity, which directly phosphorylates Ptc2 *in vivo* (Guillemain

et al. 2007). Therefore, Cdc5 primes the checkpoint for Rad53 deactivation, while CKII licenses Ptc2/Ptc3 activity through phospho-dependent substrate docking, ensuring timely Rad53 dephosphorylation and promoting checkpoint silencing. Strikingly, the prolonged G2/M arrest seen in the adaptation-defective mutants *cdc5-ad* and *ckb2-ad* is eliminated in a *rad9Δ* background, indicating that DNA damage adaptation operates downstream of the Rad9-mediated checkpoint signaling cascade (Toczyski et al. 1997). Another critical DDR effector, Chk1, is likewise dephosphorylated during checkpoint adaptation, thereby contributing to the dismantling of the DNA damage-induced arrest (Pellicioli et al. 2001).

As core DDR effectors are deactivated, mitotic pathways resume. During checkpoint adaptation, the Cdc28 inhibitor Swe1 is rapidly hyper-phosphorylated and targeted for proteolysis by a multi-kinase cascade that includes the bud neck kinases Hsl1/Hsl7, Gin4, Cla4, Cdc5, and Clb2-Cdc28 itself (Lee et al. 2005; Asano et al. 2005). Swe1-driven phosphorylation of Cdc28 on Tyr19 is then indirectly reversed in a two-step process: the $PP2A^{Cdc55}$ complex, recruited by Zds1/2, dephosphorylates and activates the Mih1 phosphatase, which then removes the Tyr19 phosphate and restores full Cdc28 G2/M activity (Russell et al. 1989; Wicky et al. 2011; Yang et al. 2000; Lin and Arndt 1995). As Rad53 and Chk1 are rendered inactive, both the FEAR and the MEN regain control and drive mitotic exit by promoting Cdc14 nucleolar release (Liang and Wang 2007; Valerio-Santiago et al. 2013; Wang et al. 2000). Together, these mechanisms enable checkpoint adaptation and promote cell cycle resumption despite unresolved DNA damage.

While the DNA repair machinery itself does not directly drive the adaptation response to persistent DNA damage (Ratsima et al. 2016), the damage-associated signals that persist at repair intermediates can critically shape the timing and efficiency of adaptation. Notably, by remodeling nucleoprotein filaments at sites of DNA damage, the Swi2/Snf2-like DNA translocase Tid1/Rdh54 modulates the persistence and amplitude of checkpoint signaling in a Mec1- and Rad53-dependent manner, such that an ATPase-dead *tid1-K318R* mutant fails to adapt to a single irreparable DSB (Lee, Pellicioli, et al. 2001; Ferrari et al. 2013). The Sae2 endonuclease, a key substrate for both Mec1/Tel1 and Cdc5, regulates checkpoint signaling in a similar

manner (Donnianni et al. 2010; Baroni et al. 2004). Accordingly, the adaptation defect of a *sae2Δ* mutant – unable to displace MRX from persistent DSBs– can be fully rescued by *rad9Δ* or *tel1Δ*, highlighting Sae2 central role as a Mec1/Tel1-regulated rheostat that modulates DNA damage signaling during the adaptation response to a DSB (Clerici et al. 2006; Donnianni et al. 2010). Beyond Rdh54 and Sae2, DNA damage adaptation hinges on several other end-processing and DNA repair factors that include the Swi2/Snf2 homolog Fun30, Ku70/Ku80, Rad51/Rad52, as well as the Srs2 helicase (Eapen et al. 2012; Lee et al. 1998; 2000; 2003; Vaze et al. 2002). Therefore, while the assembly of DNA damage sensors and mediators at lesion sites is essential for initiating the DNA damage response, their timely disassembly is equally critical in dampening damage signals, adjusting stimulus sensitivity, and enabling checkpoint adaptation.

Beyond the combined effects of 1) DDR shut-down, 2) reactivation of mitotic processes via restored Cdc28 activity, and 3) dynamic clearance of DNA damage sensors at sites of persistent lesions, other Cdc5-dependent processes positively contribute to DNA damage adaptation. While the MEN in itself is not sufficient to drive adaptation, the molecular cascade facilitating mitotic exit does partially support checkpoint override. During a G2/M DNA damage checkpoint-mediated cell cycle arrest, the MEN is actively inhibited to prevent mitotic exit by engaging the Bfa1-Bub2 GAP complex, which maintains the Tem1 GTPase in an inactive state (Wang et al. 2000; Liang and Wang 2007). Thus, cells undergoing checkpoint adaptation must also overcome MEN inhibition imposed by the DNA damage checkpoint, a process facilitated by the Cdc5-dependent phosphorylation and inactivation of the MEN inhibitor Bfa1 (Hu et al. 2001; Valerio-Santiago et al. 2013). Accordingly, a *cdc5-16* adaptation-deficient PBD mutant becomes proficient for G2/M DNA damage adaptation upon *BFA1* deletion (*bfa1Δ*) (Ratsima et al. 2016). Yet, *CDC5* overexpression can trigger full checkpoint override in phosphorylation-deficient *bfa1-4a* and *bfa1-11a* mutants, suggesting that Cdc5 deploys at least one other mechanism separate from Bfa1-dependent MEN activation to promote DNA damage adaptation (Ratsima et al. 2016). Thus, although Cdc5-mediated MEN activation does facilitate adaptation, the adaptation pathway engages additional, non-canonical drivers beyond Bfa1-dependent

mitotic exit to redundantly ensure timely cell cycle re-entry in the presence of DNA damage. Another pivotal effector of the DDR is the RSC chromatin-remodeling complex, which intervenes at multiple levels of the cascade to enhance nuclease access, stabilize DNA repair intermediates, and –notably through the FEAR– partners with Cdc5 to drive adaptation (Liang et al. 2007; Chambers et al. 2012; Shim et al. 2005; Oum et al. 2011; Chai et al. 2005; Rossio et al. 2010; Ratsima et al. 2016). RSC exists in two partially redundant isoforms, the Rsc1- and Rsc2-types (Chambers et al. 2012). While both can promote DNA repair, RSC^{Rsc1} specializes in the initial chromatin remodeling events occurring at DSB sites upon checkpoint activation, whereas RSC^{Rsc2} primarily enables Cdc5-dependent checkpoint adaptation downstream of the DDR (Shim et al. 2005; Chambers et al. 2012; Ratsima et al. 2016). Under normal cycling conditions, RSC^{Rsc2} and Cdc5 physiologically associate through a non-canonical phosphorylation-independent PBD binding event and together contribute to FEAR-mediated Net1 phosphorylation, resulting in the early release of nucleolar Cdc14 (Rossio et al. 2010). Upon exposure to genotoxic stress, the RSC^{Rsc2}-Cdc5 interaction is markedly enhanced, pointing to a particular requirement for RSC^{Rsc2} activity under damaging cycling conditions (Ratsima et al. 2016). While deletion of either RSC subunit (*rsc1Δ* or *rsc2Δ*) compromises checkpoint adaptation following telomere uncapping in a *cdc13-1* background, *rsc2Δ* mutants show a near-complete block in DNA damage adaptation (Ratsima et al. 2016). Crucially, an overexpression of *CDC5* –which restores adaptation in a *cdc5-16* PBD mutant– fails to rescue the defects associated with *rsc2* deletion, placing RSC^{Rsc2} as a key co-factor that cooperates with –but cannot be bypassed by– Cdc5 during DNA damage adaptation (Ratsima et al. 2016). Since Rad53 dephosphorylation kinetics are unaffected in *rsc2Δ* cells relative to *RSC2* controls, the role of RSC^{Rsc2} during checkpoint silencing likely takes place within a branch of the DDR unrelated to Rad53 inactivation (Ratsima et al. 2016). Beyond its role in the DDR, RSC^{Rsc2} has also been implicated in SAC adaptation, underscoring a broader requirement for the RSC complex in relicensing cell cycle progression under checkpoint-activating conditions (Rossio et al. 2010).

At the molecular level, the adaptation response to persistent DNA damage constitutes a multifaceted, Cdc5-dependent process that integrates diverse cellular signals to collectively attenuate DDR signaling, reactivate mitotic effectors, and recalibrate the perceived DNA damage burden below a threshold permissive for cell cycle re-entry. From a physiological standpoint, adapting to a checkpoint-mediated cell cycle block –even at the expense of genome stability– represents an undeniably advantageous opportunity in terms of survival fitness for unicellular organisms otherwise destined for cell death. In higher eukaryotes, the process of DNA damage adaptation can severely compromise organismal homeostasis and may yield malignant transformation. Compared to their yeast counterparts, human cells must overcome a broader array of safeguarding mechanisms –specifically designed to prevent genome instability– to undergo checkpoint adaptation. The following section explores the adaptation response to DNA damage in humans, its molecular peculiarities, and the physiological consequences of its dysregulation in the context of cancer development.

1.6.3 DNA damage adaptation: Physiological consequences and implications in cancer

In vertebrate cells, checkpoint adaptation unfolds when the primary DNA damage signal is progressively dampened as mitotic regulators become active once more (Syljuåsen et al. 2006; Kubara et al. 2012). The PP2C phosphatase WIP1/PPM1D directly dephosphorylates key ATM/ATR substrates that include CHK2, p53, γ-H2AX and ATM itself, reducing checkpoint strength while allowing for low Cyclin B-CDK1 G2 activity (Fujimoto et al. 2006; Oliva-Trastoy et al. 2007; Lu et al. 2005; Cha et al. 2010; Shreeram et al. 2006; Müllers et al. 2017). Residual CDK1 phosphorylates RAD9, leads to PLK1 recruitment and RAD9 hyperphosphorylation, which promotes a loosening of the 9-1-1 clamp that further dampens ATR-CHK1 signaling (Wakida et al. 2017). Simultaneously, PLK1 primes the checkpoint adaptor Claspin for degradation by the SCF-βTrCP complex, causing rapid CHK1 dephosphorylation and collapse of the ATR-CHK1 signaling axis (Mamely et al. 2006; Mailand et al. 2006). PLK1 also phosphorylates the inhibitory kinase WEE1,

targeting it for the same SCF-βTrCP ligase which, together with WIP1-driven p53 attenuation, frees CDC25B phosphatase and unleashes a Cyclin B-CDK1 positive feedback loop that commit cells to mitosis (Watanabe et al. 2004; Lindqvist et al. 2009; de Gooijer et al. 2017; van Vugt et al. 2004; Lobjois et al. 2009).

Because checkpoint adaptation reflects a dynamic balance between pro-mitotic CDK1/PLK1 feedbacks and ATM/ATR-CHK1/CHK2-p53 checkpoint safeguards, the same upstream damage-response network can resolve into distinct single-cell fates; accordingly, human cells bearing persistent DNA lesions may undergo apoptosis, bona fide G2 checkpoint silencing with continued cycling (i.e., adaptation), or mitotic slippage (i.e., exit from mitosis without division) (Syljuåsen et al. 2006; Haschka et al. 2018; Sinha et al. 2019; Liang et al. 2014). During mitotic catastrophe, premature or faulty mitotic entry in the face of damage culminates in apoptosis or necrosis (Vakifahmetoglu et al. 2008). In checkpoint silencing with continued cycling, cells enter and complete mitosis with persistent lesions, frequently displaying γ-H2AX-positive micronuclei and accumulating chromosomal rearrangements – hallmarks of genomic instability after adaptation (Syljuåsen et al. 2006; Lewis and Golsteyn 2016; Kalsbeek and Golsteyn 2017). Mitotic slippage arises when DNA damage-driven ATM/ATR-CHK signaling restrains the pro-mitotic branch (i.e., Cyclin B/CDK1-PLK1-Greatwall (Gwl/MASTL)-pENSA/ARPP19), a pathway that normally inhibits the PP2A-B55 phosphatase via the ENSA/ARPP19 regulatory proteins (Lacroix et al. 2022; Castro and Lorca 2018; Fatima et al. 2020). Foundational work defined the Gwl/MASTL-ENSA/ARPP19-PP2A-B55 switch and its conservation across species in great detail (Archambault et al. 2007; Larouche et al. 2021; Wang et al. 2014; Williams et al. 2014; Gharbi-Ayachi et al. 2010; Mochida et al. 2010) and, in human cells, the balance between DDR-driven repression of MASTL and reactivation of pro-mitotic factors during mitotic DNA damage is a key determinant of fate (Kim et al. 2019; Wong et al. 2016). ATM/ATR-driven inhibition of MASTL leaves PP2A-B55 active, triggering widespread dephosphorylation of mitotic substrates and thereby launching the exit program (i.e., chromosome decondensation, nuclear envelope re-formation, spindle breakdown) without division (i.e., chromosome segregation, cytokinesis) (Kim et al. 2019). Mitotic slippage

therefore yields undivided tetraploid interphase cells which, in p53-proficient backgrounds, are often eliminated by arrest or apoptosis to limit dissemination of damaged karyotypes (Andreassen et al. 2001; Orth et al. 2012).

When p53 gatekeeper activity is lost or altered, damaged cells pursue cycling and rapidly diversify their genomes as they accumulate chromosomal instability (CIN) (Hashimoto and Todo 2013). Genetic or epigenetic erosion of the p53 axis can occur through p53 inactivation, overexpression of p53 negative regulators –such as MDM2 or WIP1/PPM1D–, or transcriptional rewiring (Hashimoto and Todo 2013; Oliner et al. 2016; Bulavin et al. 2002; Kleiblova et al. 2013; McKenzie et al. 2010; Jung et al. 2021). In combination with loss-of-function mutations in high fidelity repair genes –such as BRCA1–and DDR/checkpoint signaling irregularities, these aberrations mount a powerful system that drives the rapid dissemination of damaged cells experiencing large micronuclei, chromothripsis, and inflammatory signaling (Cao et al. 2006; Yadav et al. 2025; Lewis and Golsteyn 2016). In the context of cancer, damaged cells harboring impaired p53-ATM surveillance and heightened CDK1-PLK1 activity can co-opt DNA damage adaptation as a mechanism to override cell cycle arrest despite genomic instability. This adaptation process facilitates continued proliferation in the presence of DNA lesions and, in doing so, directly contributes to clonal selection, tumour heterogeneity, enhanced resistance to genotoxic therapies, and malignant transformation (Gheghiani and Fu 2023; Syljuåsen 2007; Kalsbeek and Golsteyn 2017).

1.6.4 Contribution of SPBs/centrosomes to DNA damage adaptation

In both budding yeast and human cells, SPBs and centrosomes respectively serve as critical spatial signaling platforms where Cdc5/PLK1 must localize and accumulate to effectively initiate the adaptation response to persistent DNA damage. This spatial enrichment is essential to promote cell cycle re-entry by modulating the activity of key regulators of cell cycle progression, including MEN inhibitor Bfa1 and, indirectly, the APC complex activator Cdh1 (Rawal et al. 2016; T. Zhang et al. 2009; Valerio-Santiago et al. 2013). The critical

importance of this docking interaction is underscored by the behavior of *cdc5-16* and *cdc5-T238A* mutants, which exhibit defective localization to SPBs and are consequently unable to reinitiate cell division following exposure to irreparable DNA damage (Ratsima et al. 2016; Rawal et al. 2016). The fact that the adaptation defect of the *cdc5-16* PBD mutant –unable to localize at SPBs– can be fully rescued by artificially tethering the kinase to SPBs underscores the central role of this organelle. This rescue, observed in *cdc5-16-bbp1* and even in a PBD deletion mutant such as the *cdc5-ΔPBD-CNM67* chimera, provides compelling evidence that SPBs act as the principal organizing hub for checkpoint adaptation and cell cycle re-entry (Ratsima et al. 2016). The SPB, by acting as a nexus for multiple intertwined signaling pathways, functions as far more than a mere structural scaffold. It orchestrates the spatial enrichment of essential kinases, phosphatases, cell cycle regulators, and remodeling factors within a confined microenvironment, thereby enabling the efficient deactivation of checkpoint effectors and the seamless resumption of cell cycle progression. Accordingly, Cdc5 enrichment at SPBs is tightly regulated in a cell cycle-dependent manner and its sequential, surface-specific docking to both faces of the organelle is essential for coordinated progression from S phase through anaphase (Botchkarev et al. 2017). In response to DNA damage, the regulated spatial enrichment of Cdc5 at SPBs modulates cell proliferation by facilitating cell cycle machinery reprogramming and thereby accommodates adaptation to genotoxic stress. Although the precise extent to which the centrosome contributes to DNA damage adaptation in human cells remains unclear, its role as a facilitator of PLK1-dependent cell cycle progression has also been documented, particularly in the context of cancer cell behavior (Xie et al. 2020).

The following section offers a detailed exploration of how SPBs and centrosomes, beyond their canonical role as MTOCs, function as versatile signaling platforms across diverse cell proliferation pathways –including DNA damage adaptation– in both yeast and human cells.

1.7 The spindle pole body (SPB): A signaling hub for cell division and DNA damage adaptation

*Section 1.7 has been reproduced verbatim from my previously published review article: [Langlois-Lemay L, D'Amours D. Moonlighting at the Poles: Non-Canonical Functions of Centrosomes. Front Cell Dev Biol. 2022 Jul 14;10:930355. doi: 10.3389/fcell.2022.930355. PMID: 35912107; PMCID: PMC9329689.]

Abstract

Centrosomes are best known as the microtubule organizing centers (MTOCs) of eukaryotic cells. In addition to their classic role in chromosome segregation, centrosomes play diverse roles unrelated to their MTOC activity during cell proliferation and quiescence. Metazoan centrosomes and their functional doppelgängers from lower eukaryotes, the spindle pole bodies (SPBs), act as important structural platforms that orchestrate signaling events essential for cell cycle progression, cellular responses to DNA damage, sensory reception and cell homeostasis. Here, we provide a critical overview of the unconventional and often overlooked roles of centrosomes/SPBs in the life cycle of eukaryotic cells.

Introduction

Ever since the centrosome was first discovered in the late 1800s, intense research efforts have been devoted to understanding its roles and life cycle in eukaryotic organisms. In their classic roles as microtubule-organizing centers (MTOCs), centrosomes and SPBs are classified amongst the most primitive organelles but gained complex ancillary functions throughout evolution (Bornens and Azimzadeh, 2007; Nabais et al., 2020). Increasingly, centrosomes are now recognized as important determinants of cell differentiation, self-renewal and aging processes in multicellular organisms.

Visualized for the very first time through electron microscopy, SPBs were described as “small knobs” found at either ends of a long straight fiber during mitosis (Robinow and Marak, 1966). Subsequent studies uncovered that SPBs and centrosomes are morphologically distinct; SPBs are tri-layer structures closely

embedded in the nuclear membrane whereas centrosomes are surrounded by pericentriolar material. However, both function as MTOCs. Interestingly, a third class of eukaryotic organelle, the nucleus-associated bodies (NABs), is typically responsible for MTOC-related functions in amoebozoans (Gräf et al., 2015; Gräf, 2018; Ito and Bettencourt-Dias, 2018).

Beyond MTOC activities, centrosomes/SPBs also promote cell signaling events induced by diverse stimulatory and stress signals. Here, we will review the non-canonical roles of MTOCs in cell homeostasis, with a specific focus on how the structural organization and subcellular position of centrosomes/SPBs play a central role in the modulation of cellular processes.

1.7.1 Function and structural organization of eukaryotic MTOCs: An overview

1.7.1.1 Centrosomes as MTOCs

Characterized as a protein-dense scaffolding structure responsible for the nucleation of *α*- and *β*-tubulin, centrosomes arrange and anchor microtubules that form the bipolar spindle in mitosis (reviewed in Wu and Akhmanova, 2017; Gomes Pereira et al., 2021) (Figure 1.R2). The main microtubule nucleator is the γ-tubulin ring complex (γ-TuRC), a highly conserved complex responsible for the capping of microtubule minus ends (Oakley et al., 1990; Zheng et al., 1995). Formed of several proteins including γ-tubulin and actin (Liu et al., 2020; Wieczorek et al., 2020), this complex is located in the pericentriolar material (PCM) and was shown to rely on pericentriolar proteins such as CDK5RAP2 to attach to centrosomes (Fong et al., 2008). The γ-TuRC complex, operating as an organizational template for the nucleation of microtubules, forms the cytoplasmic microtubule array in interphase as well as the mitotic spindle during mitosis and was shown to regulate nucleation dynamics via conformational changes (Consolati et al., 2020). From interphase to mitosis, the function of centrosomes as MTOCs is highly dynamic and supports the ongoing division of proliferating cells (Mazia, 1987). Both the size and function of centrosomes as MTOCs may fluctuate according to the state of a given cell, or even its cell type (Decker et al., 2011). To behave in such a dynamic

manner, MTOCs rely on centrosomal components and associated proteins that enrich at the centrosomes to stabilize or release microtubule organization and involve a large array of components that can even selectively enrich to one centriole over the other throughout the cell cycle (Andersen et al., 2003; Jakobsen et al., 2011). Combined together, all these factors allow for a personalized MTOC function specifically catered to cell conditions at a given time to accurately support cell cycle progression through microtubule nucleation.

Aside from its classic function as MTOC, the centrosome also plays crucial roles in cell polarity, shape and migration. When Van Beneden first discovered the centriole in 1883 (Van Beneden, 1883), he hypothesized that the polarity of a cell could be conferred by the orientation of both its nucleus and centrosome (Luxton and Gundersen, 2011). The nuclear-centrosomal (NC) axis exists in the majority of metazoan differentiated cell types, as well as in some unicellular organisms including yeast (Nelson, 2003). The polarity of a cell defined by the orientation of its centrosome is an important feature at the core of many biological processes. Research performed on normal fibroblast to study wound healing reported that both the Golgi apparatus and the centrosome (MTOC) were necessary for directional migration towards the edge of a lesion. Authors speculated that the coordinated orientation of both the MTOC and the Golgi apparatus towards the wound was required to modulate vesicular transport to the edge of the cell, thus leading to the growth of this extremity towards the wound (Kupfer et al., 1982). Akin to this, the centrosome was also reported to play a crucial role in directional mesenchymal cell migration. In a study published in 2017, Zhang and others used micropatterned one-dimensional adhesive strips to study cell polarity in mesenchymal cells and reported that the centrosome was involved in directional cell migration. Specifically, the centrosome was proposed to dynamically localize at the rear of mesenchymal cells to organize the microtubule network and distribute signals related to protrusive activity as a way to establish tail formation during directional migration (Zhang and Wang, 2017).

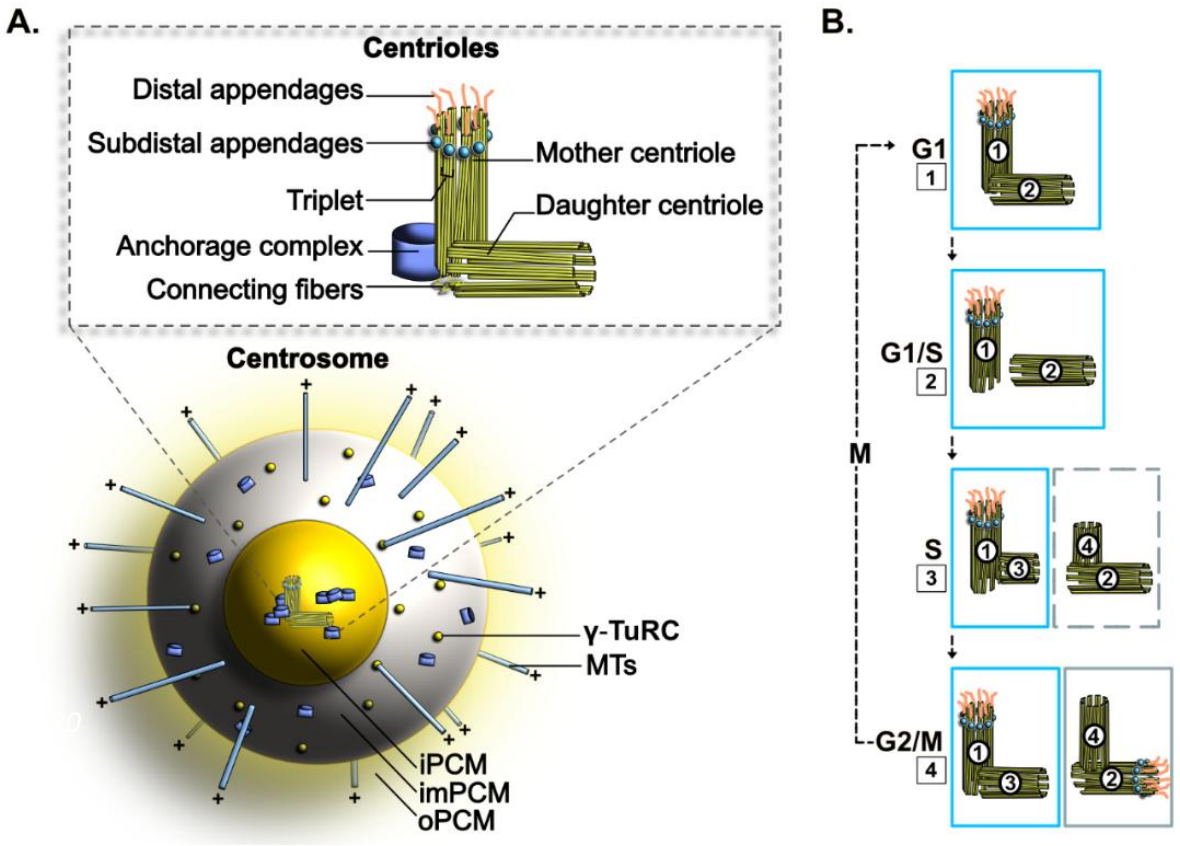

Figure 1.R2. (A) Schematic representation of the centrosome. PCM, Pericentriolar material; iPCM, Inner PCM; imPCM, Intermediate PCM; oPCM, Outer PCM **(B)** Centrosome duplication cycle. The duplication of centrosomes is termed semi-conservative, as each older centriole will generate a new centriole. **1–2**. At the G1/S transition, the two centrioles separate. **3**. In S phase, PCM forms around each parting centriole. **4.** Daughter centrioles expand orthogonally and reach opposite poles. See text for more details.

Asymmetric cell division, a process equally reliant on cell polarity for its occurrence, can also depend on the orientation of centrosomes to effectively reach completion (as reviewed in Chen and Yamashita, 2021). Asymmetric cell division is a common process routinely observed from yeast to humans. In *S. cerevisiae*, aging determinants are partitioned asymmetrically, resulting in a young daughter bud expanding from an older parental yeast. This process directly impacts the replicative lifespan of both parental and daughter cells, which represents the finite number of divisions a cell can undertake before reaching senescence (Longo et al., 2012). Spindle orientation and other factors established by the cell polarity machinery can

guide this asymmetric process, which results in the transfer of new components such as mitochondria, endoplasmic reticulum (ER), vacuoles and rejuvenating factors to the daughter cell whilst a number of older components remain in the parental cell (Higuchi-Sanabria et al., 2014). Moreover, SPBs themselves undergo asymmetric inheritance. The older parental SPB migrates towards the new daughter bud, whilst the daughter SPB remains in the parental yeast cell (see section "MTOC duplication cycle" for more details). The asymmetric SPB segregation was shown to be regulated by the spindle positioning protein Kar9 as well as the SPB component Nud1, via its role in the mitotic exit network (MEN) (Hotz et al., 2012a). Along the same lines, asymmetric division is also a feature broadly reported in stem cells, in which the cell type of resulting cells –one self-renewed stem cell and one differentiating cell –differs. In *Drosophila* male germ lines, adult stem cells (GSCs) were shown to asymmetrically divide by relying on the inheritance pattern of mother and daughter centrosomes through directional orientation of the mitotic spindle (Yamashita et al., 2003). Using specific labeling techniques, Yamashita and others later observed that the mother centrosome preferentially remains affixed to the GSCs, whilst the daughter centrosome migrates to the differentiating cell (Yamashita et al., 2007). The authors hypothesized that a high number of astral microtubules may be responsible for the anchorage of the mother centrosome to the GSC, thereby keeping them in close proximity during asymmetric cell division. In accordance with this, the predetermined anchoring of the mother centrosome was suggested to act as an orientation mechanism for the mitotic spindle as a way to ensure the success of asymmetric stem cell division and highlights the core role that centrosomes can play in asymmetric stem cell division. Yet, the non-random segregation of mother and daughter centrosomes is not always a prerequisite for spindle alignment and subsequent asymmetric division. After each of the asymmetric divisions undergone by the germline lineage of the nematode *C. elegans*, centrosome rotation occurs as a way to re-align the spindle to the anterior-posterior (AP) axis. This rotation requires that one of the centrosomes, called the leading centrosome and chosen randomly, travels near the anterior border of the cell (Hyman and White, 1987; O'Connell, 2000). This example demonstrates that the non-random

segregation of centrosomes during asymmetric division is a common occurrence in some species and does not represent an essential feature of spindle alignment for asymmetric cell division in all biological systems.

Another important function for centrosomes as MTOCs can also be observed in neuronal development. A decisive part of neuronal differentiation lies in axon specification, a process through which one of the neurites matures into a functional axon. This is of high importance for the fate of a neuron, as this process permanently defines its polarization and connectivity. In the current literature, the contribution of centrosomes to this specific stage of neuronal development has met some controversy (as reviewed in Meka et al., 2020). Several reports describe a key role for the centrosome in axonal outgrowth and specification (Rivas and Hatten, 1995; Schaar and McConnell, 2005; Tsai and Gleeson, 2005; Higginbotham and Gleeson, 2007; Kuijpers and Hoogenraad, 2011), whilst other studies seem to contradict such statement and rule out a potential requirement for centrosome function throughout this neuronal process (Esch et al., 1999; Andersen and Bi, 2000; Bradke and Dotti, 2000). In cultured hipoccampal neurons, growing axons were reported to organize microtubule arrays in a centrosome-independent way once axon specification is complete. This observation is supported by the fact that during axonal elongation, centrosome ablation was shown to have no effect on axon extension or regeneration and suggests that centrosomal function may be required only in the earlier stages of neuronal development (Stiess et al., 2010). Recent studies also argue for a role for centrosomes as F-actin organization centers in developing cultured neurons (Meka et al., 2019). Disruption of centrosome function was shown to alter the content of somatic F-actin and decreased peripheral F-actin matter in neuronal growth cones, suggesting a key role for the centrosome in F-actin organization (Meka et al., 2019). During neuronal differentiation, centrosomes as MTOCs can have various other functions. The most classical and well-known function of MTOCs in neuron biology is probably cargo transport across dendrites and axons, a function performed in partnership with motor proteins (Kapitein et al., 2010). In mouse and chick neural tube cells, centrosomes were also shown to influence neuronal delamination, a process by which novel neurons detach from the neuroepithelium throughout

differentiation and morphogenesis. For delamination, the centrosome has to be retained in the newborn neuron and nucleates a wheel-like microtubule organization that supports apical abscission. In this process, the centrosome is thus of high importance in mediating microtubule activity and is involved in nervous system growth and expansion (Kasioulis et al., 2017). Another interesting function for centrosomes in neuron biology is in neuronal activity. Using fluorescent microscopy, Hu and others reported that microtubules also have the propensity to invade dendritic protrusions. This observation suggests that MTOCs, through microtubules, may have an implication in the operative exchanges between neurons. An increase in neuronal activity was notably shown to correlate with an increased number of spines occupied by microtubules, as well as with an increased contact time between microtubules and dendritic protrusions (Hu et al., 2008). However, more work is needed to establish the precise function of these microtubules in neuronal plasticity. Taken together, these examples display the various ways in which centrosomes as MTOCs can impact neuronal development and highlight the specialized –and still debated– contribution of this organelle in neuron biology.

1.7.1.2 Structural organization of MTOCs

Despite lacking a finite membrane border, the centrosome maintains its unique tri-dimensional shape via centrosome-interacting proteins, 500 of which have been identified to date (Andersen et al., 2003; Gupta et al., 2015; Gheiratmand et al., 2019). Throughout the cell cycle, its size and composition vary, allowing for diverse arrangements in microtubule organization (Devi et al., 2021; Gomes Pereira et al., 2021). Centrosomes contain centrioles, a pair of cylindrical organelles perpendicularly positioned to one another (Figure 1.R2A). Surrounding the centrioles is the pericentriolar material (PCM), a fibrous coiled-coil protein platform (Schatten, 2008) formed by the main microtubule nucleator γ-tubulin, γ-turc, actin (Liu et al., 2020; Wieczorek et al., 2020) and pericentrin proteins (Salisbury, 1995; Levy et al., 1996; Lutz et al., 2001; Martinez-Campos et al., 2004; Wu and Akhmanova, 2017). This platform allows for sustained or

transient anchoring of specific signaling proteins, such as the Nuclear Mitotic Apparatus Protein (NuMA), a key effector of the mitotic machinery. Similar to NuMA, centriolin was also reported to connect to the centrosomes during specific phases of mitosis to facilitate cell cycle progression and cytokinesis (Gromley et al., 2003). Importantly, the size of the PCM varies according to levels of γ-tubulin recruited to centrosomes in a way that supports the ongoing cell cycle state. Accordingly, the PCM is a smaller and tighter structure during interphase and becomes much larger during mitosis to support spindle formation through γ-tubulin nucleation (Robbins et al., 1968; Khodjakov and Rieder, 1999).

Aside from this core centrosomal structure comprised of centrioles and their surrounding PCM, other accessory structures including centriolar appendages and satellites positioned around the PCM further decorate centrosomes and provide this essential organelle with extra key features. The mother and daughter centrioles are different in that additional appendages can only be found on the mother centriole. Distal appendages (DAPs), existing at the distal end of mother centrioles across eukaryotic species except for *C. elegans* and *D. melanogaster* (Azimzadeh, 2014), are required for the docking of the centriole to the membrane and for the process of ciliogenesis (Tanos et al., 2013; Ye et al., 2014). Subdistal appendages (sDAPs) are found in close proximity to DAPs and are also involved in cilia formation and microtubule anchoring. In the literature, the relationship between DAPs and sDAPs remains elusive but recent evidence suggests that DAPs are important for sDAPs functionality and positioning (Chong et al., 2020). Apart from these appendages, centrosomes are also surrounded by centriolar satellites, small particles that congregate around the PCM of centrosomes (Kubo et al., 1999). These satellites are mainly composed of proteins involved in the maintenance of centrosomes, neurogenesis and ciliogenesis (reviewed in Prosser and Pelletier, 2020; Odabasi et al., 2020). Centriolar satellites can also play key roles in the transduction of several other biological cues and vary in form and function throughout the cell cycle and across cell types (Kubo and Tsukita, 2003; reviewed in Tollenaere et al., 2015).

Analogous to centrosomes, SPBs of lower eukaryotes act as key microtubule organizing centers but differ dramatically in their mechanism-of-action and structural features (Jaspersen, 2021). Across yeast species, SPBs are functionally conserved but display key architectural differences. Here, we provide a brief description of both budding yeast and fission yeast SPBs as we compare and contrast their organizational features.

In comparison to the more diffuse centrosomal organization, budding yeast *S. cerevisiae* SPBs are tightly embedded in the nuclear membrane through three highly organized interconnected disk-like structures (see Figure 1.R3A for a detailed representation of budding yeast SPB structure) (Robinow and Marak, 1966; Bullitt et al., 1997; Rüthnick and Schiebel, 2018). The outer plaque is responsible for the nucleation of cytoplasmic microtubules, whereas the inner plaque generates nuclear microtubules. The central plaque anchors the SPB into the nuclear membrane and connects to the half-bridge, an important structure for SPB duplication (Figure 1.R3A). Two tightly packed and organized disks, called intermediate layer 1 (IL1) and intermediate layer 2 (IL2), act as spacers between the outer plaque and the central plaque. In budding yeast, 17 proteins have been identified as SPB structural components (Figure 1.R3A), six of which constitute the core of the spindle pole body: Spc42, Cnm67, Nud1, Spc72, Spc29 and Spc110 (Adams and Kilmartin, 1999; Francis and Davis, 2000; Viswanath et al., 2017). Through reciprocal interactions, these SPB components are integral for creating and maintaining the core SPB structure (Jaspersen and Winey, 2004; Jaspersen, 2021). Most SPB genes are essential for viability and single point mutations in these genes often result in temperature-sensitivity or even lethality.

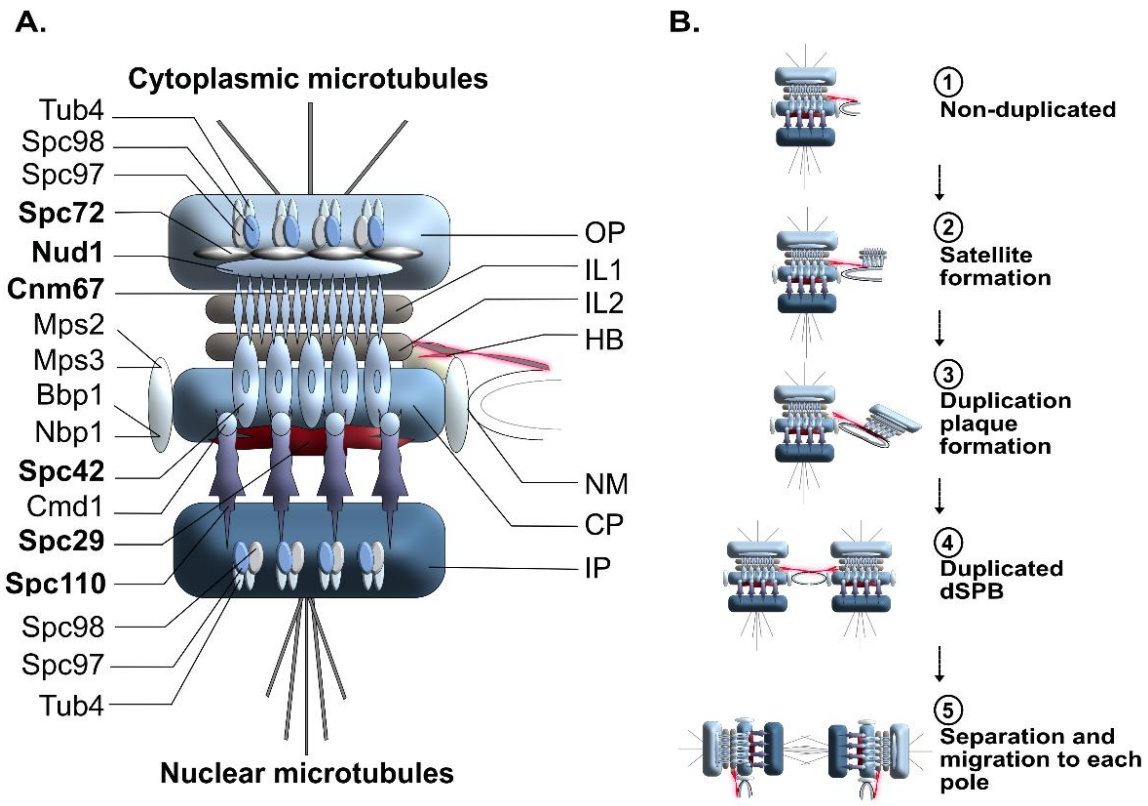

Figure 1.R3. (A) Schematic representation of budding yeast SPB organization and duplication cycle. OP, Outer plaque; IL1, Intermediate layer 1; IL2, Intermediate layer 2; HB, Half-bridge; NM, Nuclear membrane; CP, central plaque; IP; Inner plaque. Core SPB components are highlighted in bold. **(B)** SPB duplication cycle in budding yeast. The duplication process of the SPB is conservative and highly dynamic. Step 1: In early G1, the half-bridge is connected to the SPB central plaque and will act as a scaffold for SPB duplication. Step 2: The half-bridge elongates and the core of the daughter SPB (satellite) is generated on the cytoplasmic face of the half-bridge. Step 3: The duplication plaque, resulting from the elongation and growth of the satellite SPB, matures and mimics the cytoplasmic organization of a mature SPB. Step 4: The half-bridge retracts and fuses to the nuclear membrane. The daughter SPB is assembled and is embedded in the nuclear membrane next to the mother SPB. Step 5: The link between mother and daughter SPBs breaks, leading to the separation of the two organelles.

Fission yeast *S. pombe* SPBs are bipartite structures which, akin to budding yeast SPBs, are implanted in the nuclear membrane. In opposition to budding yeast SPBs, the cytoplasmic domain of fission yeast SPBs represents the bulk of its structure. The architecture of *S. pombe* SPBs also differs from that of budding yeast in that it lacks intermediate spacers and does not contain multiple separate strata except from the outer (cytoplasmic), central and inner (nuclear) layers. Despite these architectural differences,

fission yeast SPBs nucleate both cytoplasmic and nuclear microtubules as budding yeast SPBs do and encompass a half bridge required for SPB duplication (Ito and Bettencourt-Dias, 2018). Several components of *S. pombe* SPBs were classified as confirmed or probable homologues of *S. cerevisiae* SPBs, denoting a certain degree of functional and structural conservation in terms of SPB constituents across these species. These include (*S. cerevisiae/S. pombe)*: Tub4/Gtb1; Spc97/Alp4; Spc98/Alp6; Spc110/Pcp1; Spc72/Mto1; Spc42/Ppc89; Cmd1/Cam1; Cnm67/Sid4; Nud1/Cdc11; Cdc31/Cdc31; Sfi1/Sfi1; Mps3/Sad1; Mps2/Kms2. For more details on *S. pombe* SPB and its structural intricacies, we direct readers towards the study of Bestul et al. (2017).

1.7.1.3 MTOC duplication cycle

Centrosome duplication occurs once per cell cycle and is a semi-conservative process (*i.e*., the two centrioles present in each cell duplicate to generate two pairs where one template centriole is older than the newly generated copy; Figure 1.R2B) (Gomes Pereira et al., 2021). Importantly, the process of centriole assembly occurs throughout three full cell cycles. At the onset of replication, procentrioles (new centrioles) separate and by S^1 phase start their assembly (Figures 1.R2B, step 2). Both procentrioles elongate throughout S^1 phase, $G2^1$ phase and M^1 phase and grow perpendicularly from their template side (Figures 1.R2B, step 3). At the beginning of M^1 phase, additional PCM is built around each pair of centrioles as they start to separate (Figures 1.R2B, step 4). To form the mitotic spindle, procentrioles and their developing centrosome separate in early prophase of M^1. This event, mainly achieved by motor proteins, is supported by push-and-pull forces mediated by the kinesin motor Kif1 and the minus end-directed dynein motor complex. In the literature, the mechanistic intricacies of dynein function in MTOC positioning and separation remained elusive for many years (Holzbaur and Vallee, 1994; Vallee and Sheetz, 1996; Gönczy et al., 1999). Recent studies performed in one-cell *C. elegans* embryos report that different pools of dynein, localized at the cell cortex and on the nuclear surface, can influence centrosome separation. Whilst the pool of dynein located

on the nuclear surface moves centrosomes by sliding the centrosome-associated microtubules, the pool of dynein at the cell cortex pulls centrosomes through MT-mediated cortical tugging forces. In this process, dynein was shown to behave as a coupling device that transfers forces produced by polarized actomyosin cortical flows to centrosomes, thereby promoting centrosome separation (De Simone and Gönczy, 2017; Torisawa and Kimura, 2020). Along with this, the plus end-directed kinesin-related motor protein Eg5 creates outward pushing forces by tethering to plus-end antiparallel MTs (Kapitein et al., 2005). Thus, dynein and Eg5 have the ability to create opposite forces that further promotes centrosome separation (Raaijmakers et al., 2012; Agircan et al., 2014). At the end of M^1 phase, procentrioles are separated and individually assemble their PCM. This occurrence, termed centriole disengagement, signifies that the mother and daughter procentrioles are not in close association anymore. Thus, at this stage of centrosome duplication, disengaged procentrioles can be defined as daughter centrioles. From $G1^2$ to S^2 phase of the following cell cycle, daughter centrioles acquire appendages and further increase in length. Upon entry into S^2 phase of the second cell cycle, each newly formed daughter centriole begins its own cycle of centrosome duplication once more. During this process, the younger mother centriole persistently accumulates additional PCM from S^2 phase to $G2^2$, until its PCM resembles the older mother centriole PCM prior to M^2 phase. In $G2^2$ phase of the second cell cycle, the younger daughter centriole still develops and acquires distal appendages (DAPs) and subdistal appendages (sDAPs). These appendages will evolve and mature until the $G2^3$ phase of the third cell cycle, after which the corresponding round of centriole assembly is complete (Sullenberger et al., 2020).

Centrosome duplication produces two spindle poles that localize perpendicular to the plane of cell division. Achieving this precise orientation is required to support balanced chromosome segregation in mitosis (Kaseda et al., 2012; Silkworth et al., 2012; Nunes et al., 2020). Accordingly, defects in centrosome duplication can have drastic consequences for the cell. If the process of duplication fails and generates extra centrosomes, a resulting scenario may be multipolar mitosis. In multipolar mitosis, chromosomes are

segregated to more than two poles during cell division and often leads to gross aneuploidy, chromosome instability (CIN) and clonal evolution (Kwon et al., 2008; Yi et al., 2011; Yang et al., 2012; Telentschak et al., 2015; LoMastro and Holland, 2019). In some cases, clustering mechanisms allow for the formation of a functional bipolar spindle despite the presence of additional centrosomes (Kwon et al., 2008). In other cases, centrosomes may gather at the center of the cell to form a monopolar spindle, a scenario equally threatening to the maintenance of genomic integrity (Chatterjee et al., 2020). Many factors can influence the organization of the mitotic spindle following defective centrosome duplication. Overall, accurate centrosome duplication and partitioning in mitosis is decisive in the maintenance of genome stability and prevention of tumorigenesis.

Like centrosome duplication, SPB duplication is a prerequisite for effective cell division in lower eukaryotes, however, since dynamic exchanges between new and old components occur throughout duplication, SPB duplication cannot be viewed as fully conservative. In budding yeast, the half-bridge elongates in early G1 and remains connected to the central plaque and the IL2 spacer throughout the duplication process (Figures 1.R3B, step 1) (Byers and Goetsch, 1974). Once sufficiently elongated, the daughter SPB is built from satellite material (Figures 1.R3B, step 2), developing into a duplication structure formed by Cnm67, Nud1 and Spc72 through Spc42-directed self-assembly (Winey et al., 1991; Adams and Kilmartin, 1999) (Figures 1.R3B, step 3), after which the half-bridge retracts, allowing for the duplication plaque to embed itself into the membrane. At the end of G1 phase, the parental and daughter SPBs are leveled and connected through a full bridge (Figures 1.R3B, step 4), the disassembly of which allows the parental SPB to preferentially migrate into the daughter bud (Figures 1.R3B, step 5), and form a bipolar metaphase spindle (Roof et al., 1992; Jaspersen, 2021). Following the initial formation of both spindle poles, additional material is added to each SPB in a dynamic manner, hence why SPBs are considered to be dynamic: their growth process should not be viewed as exclusively conservative (Lengefeld and Barral,

2018). Instead, the continuing SPB maturation increases the ability to maintain functional integrity and has been proposed to be a mechanism for SPB repair (Jaspersen and Winey, 2004).

In fission yeast, the process of SPB duplication differs from that observed in budding yeast. The interphase SPB of *S. pombe* localizes in the cytoplasm, in close proximity to the nuclear envelope (NE), and embeds itself in the nuclear membrane only upon mitotic entry. In the literature, the timing of fission yeast SPB duplication throughout the cell cycle has been controversial for many years. Older studies state that SPB duplication occurs in G2/M (Ding et al., 1997), whereas newer studies suggest that the process instead begins in G1/S phase of the cell cycle (Uzawa et al., 2004). When describing SPB duplication and maturation, Uzawa and others separate the maturation process of *S. pombe* SPB into early and late SPB maturation. Early maturation, reported to occur upon S phase completion, represents growth of the lamellae bodies (laminated structure corresponding to the premature SPB) on the half-bridge, nuclear membrane invagination and gathering of material linking the premature SPB to the nuclear membrane. Akin to what is reported in budding yeast, the early event of SPB duplication giving rise to the lamellae bodies in fission yeast relies on the elongation of the half-bridge. The latter, without which SPB duplication could not take place, is required to support the development of the premature SPB. The newly created laminated structure, still undergoing maturation, remains linked to the mother SPB through an ellipsoid bridge (Ding et al., 1997). Late maturation, shown to take place in M phase of the cell cycle, encompasses the separation of mother and daughter SPBs, NE fenestration for SPB insertion and establishment of the mitotic spindle (Uzawa et al., 2004). While individual steps of SPB duplication differ in some respects across yeast species, the process remains broadly conserved overall.

1.7.1.4 Centrosomes and SPBs: Same, but different?

Although centrosomes are significantly larger in size than SPBs (Gräf, 2018), they share several characteristics in duplication modes and main MTOC functions (see Figure 1.R4 for centrosome/SPB homologs and orthologs). For example, Kendrin and CG-NAP are human orthologs of yeast Spc110 that localize at the PCM (Flory et al., 2000; Takahashi et al., 2002). Likewise, coiled-coil domains required to establish interactions with analogous binding partners are conserved across yeast Nud1 and human centriolin, both of which are important players in cell cycle progression, mitotic exit and cytokinesis (Gromley et al., 2003; Fraschini, 2019) (Figure 1.R4). However, microtubules nucleated by the centrosome uniquely enables motility, subcellular trafficking, and anchoring of receptors at the surface of the cell (Bettencourt-Dias, 2013), whereas yeast SPBs remain restricted to roles as MTOCs and docking stations for various signaling events.

		Localization	Shared function	Localization		
S. cerevisiae	Mob1	SPB	CoActivator/Hippo	Spindle poles	MOB1	*H. sapiens*
	Dbf2	SPB	Signaling/Hippo	Spindle poles	LATs/NDR	
	Cdc15	SPB	Signaling/Hippo	Spindle poles	MST1/2	
	Spc98	SPB O/I plaque	γ-tubulin	Centrosome	GCP3	
	Spc97	SPB O/I plaque	γ-tubulin	Centrosome	GCP2	
	Tub4	SPB O/I plaque	γ-tubulin	Centrosome	GCP1	
	Spc110	SPB central plaque	MTOC maturation	Centrosome	Pericentrin	
	Spc72	SPB outer plaque	MTOC organization	Centrosome	CDK5RAP2	
	Sfi1	SPB half bridge	MTOC duplication	Centriole	hSFI1	
	Cdc31	SPB half bridge	MTOC duplication	Centriole	Centrin	
	Nud1	SPB outer plaque	MTOC signaling	Centriole	Centriolin	
	Cmd1	SPB central plaque	MTOC structure	Nucleus/Cytoplasm	Calmodulin	

Figure 1.R4. Overview of conserved yeast and human proteins involved in MTOC structure, signaling, duplication and function. Underlined are physical constituents of centrosomes/SPBs. SPB, Spindle pole body; O/I, Outer/Inner; Hippo, Hippo pathway; Pericentrin, Kendrin/CG-NAP (Fraschini, 2019).

1.7.2 Centrosomes as signal transduction organizing centers (STOCs)

In recent years, an emerging body of evidence support non-canonical roles for centrosomes/SPBs in coordination of signal transduction events (Rincón and Monje-Casas, 2020). Indeed, in response to stimuli and cell cycle cues, kinases with functions unrelated to MTOC activity become transiently enriched at centrosomes/SPBs in a manner that is both necessary and sufficient to promote downstream signaling events. Thus, centrosomes/SPBs can modulate kinase activity in a structural capacity as signal transduction organizing centers (STOCs) (Arquint et al., 2014). This function is analogous to that of supramolecular organizing centers involved in the regulation of innate immunity and programed cell death (Kagan et al., 2014), except that centrosome-mediated events occur at much larger and structurally complex scales. In this section, we will explore the surprising relationship between several kinase families and centrosomes/SPBs and how these organelles act as powerful STOCs.

1.7.2.1 Centrosomes as STOCs: A platform to enable specialized functions of Polo-like kinases

1.7.2.1.1 PLK1 in the DNA damage response

The dynamic localization of Cdc5/Plk1 at MTOCs has major implications for signal transduction events during the cellular response to DNA damage. Upon DNA damage, cells initiate a checkpoint response that allows time for DNA repair by preventing the G2/M transition (Sandell and Zakian, 1993; Rhind and Russell, 1998; Cagney et al., 2006; Chao et al., 2017). After successful DNA damage repair, cells resume cycling through a process termed checkpoint recovery (Vaze et al., 2002). However, not all DNA lesions can be safely repaired, and the extent of damage suffered determines the fate of the damaged cells. When DNA damage is too extensive, apoptotic signals lead to programmed cell death thereby preventing the transfer of deleterious genomic errors to daughter cells. When DNA damage is less extensive, cells can resume their cell cycle through checkpoint adaptation (or bypass) despite the presence of “permanent” DNA damage (Sandell and Zakian, 1993; Toczyski et al., 1997; Lee et al., 1998; Vidanes et al., 2010; Ratsima et al., 2011).

Consequently, the process of checkpoint adaptation postpones the repair of DNA lesions to subsequent phases of the cell cycle.

The exact signaling pathway responsible for the adaptation response to persistent DNA damage is still not fully understood. In both human and yeast cells, PLK activity is required for adaptation, and Cdc5 enrichment at the SPBs is both necessary and sufficient to promote adaptation to persistent DNA damage in budding yeast cells (Ratsima et al., 2016). These observations suggest that SPBs function as docking platforms for Cdc5 to execute the adaptation response. How this is achieved is unclear, however a possible link connecting PLK, BRCA1 and centrosomes was recently proposed in human cancers (Yoshino et al., 2021). In some cases, aberrant expression of the tumor suppressor gene *BRCA1* in mammary tissues can dysregulate centrosome duplication and generate a higher centriole number *in vivo*. This reported process requires the tethering of BRCA1 to centrosomes via RACK1. This protein also acts as a scaffolding factor that promotes Aurora A and PLK1 interaction in S phase. Previous literature linked RACK1 overexpression to centriole overduplication and involved BRCA1 as a component in this process (Yoshino et al., 2019; Yoshino et al., 2020). This centriole overduplication event was shown to stem from higher levels of phosphorylated PLK1, resulting in kinase hyperactivity at centrosomes. The reported centrosome aberration phenotype in response to PLK1 hyperactivity is intriguingly reminiscent of the supernumerary SPB and polyploidy/multinucleation phenotypes observed in adaptation-defective *cdc5-16* mutants (Ratsima et al., 2011; Ratsima et al., 2016) and in cells overexpressing *CDC5* (Song et al., 2000; Bartholomew et al., 2001). However, more research is needed to assess whether there are cross-species phenotypic similarities between these two cellular processes and how this might be related to the adaptation response to unrepairable DNA damage. Despite the impact of BRCA1 aberrations reported above in centrosome amplification (Yoshino et al., 2019; Yoshino et al., 2020), other studies demonstrated that mutations in BRCA1 can induce a variety of phenotypes that do not always result in amplified centrosomes. To further evaluate the influence of BRCA1 in centrosome biology *in vivo,* Kais and others explored the effect of a

subset of mutations in the *BRCA1* locus on centrosome behavior. Remarkably, these mutations induced a range of phenotypes affecting two separate branches of centrosome biology, namely centriole pairing and centrosome number. This result suggests that BRCA1 regulates these two branches of centrosome duplication separately, and nicely underlines the separation-of-function aspect of certain BRCA1 mutations (Kais et al., 2012). Thus, some mutations in BRCA1 can affect functions unrelated to centrosome number and do not always correlate with centrosome amplification in transformed cells.

The process of DNA damage-induced centrosome amplification (DDICA) (Zou et al., 2014) represents another intriguing link connecting DNA damage responses, PLKs and MTOCs. After treatment with the DNA crosslinker mitomycin C, higher levels of BRCA1 and PLK1 were detected at centrosomes alongside increased centrosome amplification. How DDICA might enhance genomic stability and/or survival remains unclear to this day. On one hand, DDICA may promote the elimination of cells bearing extensive amounts of DNA damage through mitotic catastrophe, whilst contributing to DNA damage repair via local increase of DNA repair factors at the centrosomes (Yoshino et al., 2021). The rationale behind this is that an increased amount of DNA repair factors at the centrosomes stemming from DDICA could constitute an extra source of DNA repair proteins available for relocation from the centrosomes to nuclear sites of DNA damage, consequently supporting nuclear DNA repair as well as DDICA processing. This theory, however, remains to be proven and is a work in progress in the current literature. On the other hand, this process was suggested to be beneficial for cancer cells seeking a proliferative advantage in specific growth environments, as centrosome amplification in p53-deficient cancer cells can encourage chromosome mis-segregation (Yoshino et al., 2021), a key promoter of genomic heterogeneity. The mitotic catastrophe phenotype resulting from DDICA, observed primarily in breast cancer cells, is intriguingly evocative of the phenotype reported in budding yeast with the adaptation-defective *cdc5-16* allele. In response to DNA damage, this mutant fails to enrich at the SPB and gradually fragments its SPB, akin to DDICA (Ratsima et al., 2011). It

would be informative for future research to explore the mechanistic similarities between Cdc5-related SPB fragmentation in yeast and PLK1/BRCA1-related DDICA in breast cancer cells.

In damaged cells, the generation of extra centrosomes can also be an outcome of circumstances unrelated to PLK1 or BRCA1 expression. Dodson and others notably reported that centrosome amplification can ensue an extended G2 phase caused by DNA damage checkpoint activation, in which DNA replication is paused but centrosome duplication remains. Interestingly, the small portion of cells able to override this G2/M cell cycle arrest were shown to contain a normal number of centrosomes (Dodson et al., 2004). Other potential causes of centrosome amplification also include cytokinesis failures, as well as cell-cell fusion (reviewed in Godinho and Pellman, 2014). Overall, the relationship between centrosome amplification and DNA damage is an ongoing work in progress in the field of centrosome biology and its intricacies are yet to be fully uncovered.

1.7.2.2 Centrosomes as STOCs: PIDDosome signaling axis and the centrosome surveillance pathway

Centrosome biogenesis is a process finely coordinated with other cell cycle cues to minimize errors during centriole duplication. In some cases, this control system fails despite its global efficacy and consequently leads to aberrations in centrosome biogenesis. In the literature, centrosome aberrations sometimes are described as a common outcome of neoplastic transformation (LoMastro and Holland, 2019). However, research shows that these aberrations can in fact be at the core of neoplasia, acting as an instigator of cell transformation (Lingle et al., 2002; Pihan et al., 2003; Segat et al., 2010; Lopes et al., 2018; Burigotto et al., 2021). In recent years, a link between centrosomes and the tumor suppressor p53 was unraveled and pointed to a control system for centrosome biogenesis. This control system, termed the PIDDosome signaling axis, acts as a mitotic clock that can detect and react to centrosome aberrations and DNA damage during cell proliferation to monitor and minimize genomic instability (Tinel and Tschopp, 2004; Ando et al., 2012; Ando et al., 2017; Fava et al., 2017; Sladky et al., 2017; Tsabar et al., 2020). The PIDDosome signaling

axis is composed of the “cell-death effector caspase-2” (CASP2), the “p53-induced death domain-containing protein 1” (PIDD1) as well as the “CASP2 and RIPK1 domain containing Adaptor with Death Domain” (CRADD). In response to stress signals such as extra centrosomes or genotoxic insults, the local concentration of centrosomal PIDD1 increases and specifically enriches at the mother centriole via ANKRD26, a distal appendage protein (Burigotto et al., 2021). Processing of PIDD1 at the centrosome via auto-cleavage leads to its release in the cytoplasm, where the auto-catalytic and proximity-driven activation of CASP2 occurs (Tinel and Tschopp, 2004). Resulting CASP2 activity stimulates the cleavage of the E3 ubiquitin-ligase MDM2, a negative regulator of p53 stability, ultimately leading to the activation of the tumor suppressor p53 and upregulation of p21, a cell cycle inhibitor (Oliver et al., 2011). To limit cell proliferation, this sequence of events leads to a cell cycle arrest or cell death and thereby supports the maintenance of genomic stability (Evans et al., 2021). The increased local recruitment and resulting enrichment of centrosomal PIDD1 at the distal appendages of the mother centriole is suggested to stem from a cellular surveillance mechanism, in which an abnormally high number of mature centrioles can stimulate the activation of the PIDDosome signaling axis (Fava et al., 2017).

Similarly to the PIDDosome signaling axis in response to centrosome amplification, another pathway termed the centrosome surveillance pathway monitors and reacts to centrosome loss or prolonged mitosis (Lambrus et al., 2016; Meitinger et al., 2016; Lambrus and Holland, 2017). In response to disturbed mitosis, the scaffolding protein 53BP1 acts as a platform to recruit the protein deubiquitinase USP28 as well as p53. The resulting proximity between USP28 and p53 leads to the deubiquitination and subsequent change in p53 activity and p21 upregulation, leading to a proliferation arrest in G1 phase of the cell cycle (Fong et al., 2016; Lambrus et al., 2016; Meitinger et al., 2016). The mechanistic intricacies responsible for the activation of the centrosome surveillance pathway are not fully understood. However, variations in PLK4 expression and activity appear to be linked to centrosome loss and subsequent activation of the centrosome surveillance pathway (Wong et al., 2015). Despite both 53BP1 and USP28 proteins being known binding

partners involved in DNA damage response pathways (Zhang et al., 2006; Knobel et al., 2014; Panier and Boulton, 2014; Zimmermann and de Lange, 2014), evidence shows that the activity of the centrosome surveillance signaling pathway is independent from their canonical functions in DNA damage and uncovers a new separate line of defense against the loss of genomic integrity (Lambrus et al., 2016).

1.7.2.3 Centrosomes as STOCs: Regulation of mitotic entry by cAMP-dependent protein kinase A

Recent work has revealed that PKA activation is regulated differentially in distinct subcellular compartments, and that localized activation sites –known as signaling islands– are key in determining the profile of substrates modified by this kinase (reviewed in Omar and Scott, 2020). PKA localization and its activation kinetics at centrosomes are controlled by kinase-anchoring proteins (AKAPs). Specifically, AKAP450-controlled autophosphorylation of the PKA regulatory subunit lowers the cAMP threshold required for PKA activation at centrosomes (Figures 1.R5A,B) (Taylor et al., 1990; Di Benedetto et al., 2008; Taylor et al., 2008; Terrin et al., 2012). In parallel, cAMP-specific phosphodiesterase (PDE4D3) maintains a low cAMP concentration in the vicinity of this organelle. Combined, these two mechanisms allow for a restricted centrosomal PKA pool to maintain activity when cytosolic PKA is mostly inactive, and thereby promote cell cycle progression without inadvertently inducing gene transcription, signal transduction, or other undesired events.

At the onset of cell division, mitogenic signals trigger an increase in cAMP levels in the entire cell, including the centrosome (Vandame et al., 2014). The increase in centrosomal cAMP is believed to be partly induced by MAPK-mediated inhibition of PDE4D3, which allows the concentration of cAMP to increase (Terrin et al., 2012). However, an exogenous increase in global cellular cAMP levels is not sufficient to induce PKA-mediated cell cycle progression to promote mitosis. Instead, an increase in centrosomal cAMP levels is required; when AKAP450 is artificially relocated away from centrosomes, lack of PKA impairs mitosis and

leads to a block in G1 (Gillingham and Munro, 2000; Keryer et al., 2003). Conversely, an artificial increase of centrosomal cAMP levels induces a buildup of prophase cells (Terrin et al., 2012; Vandame et al., 2014).

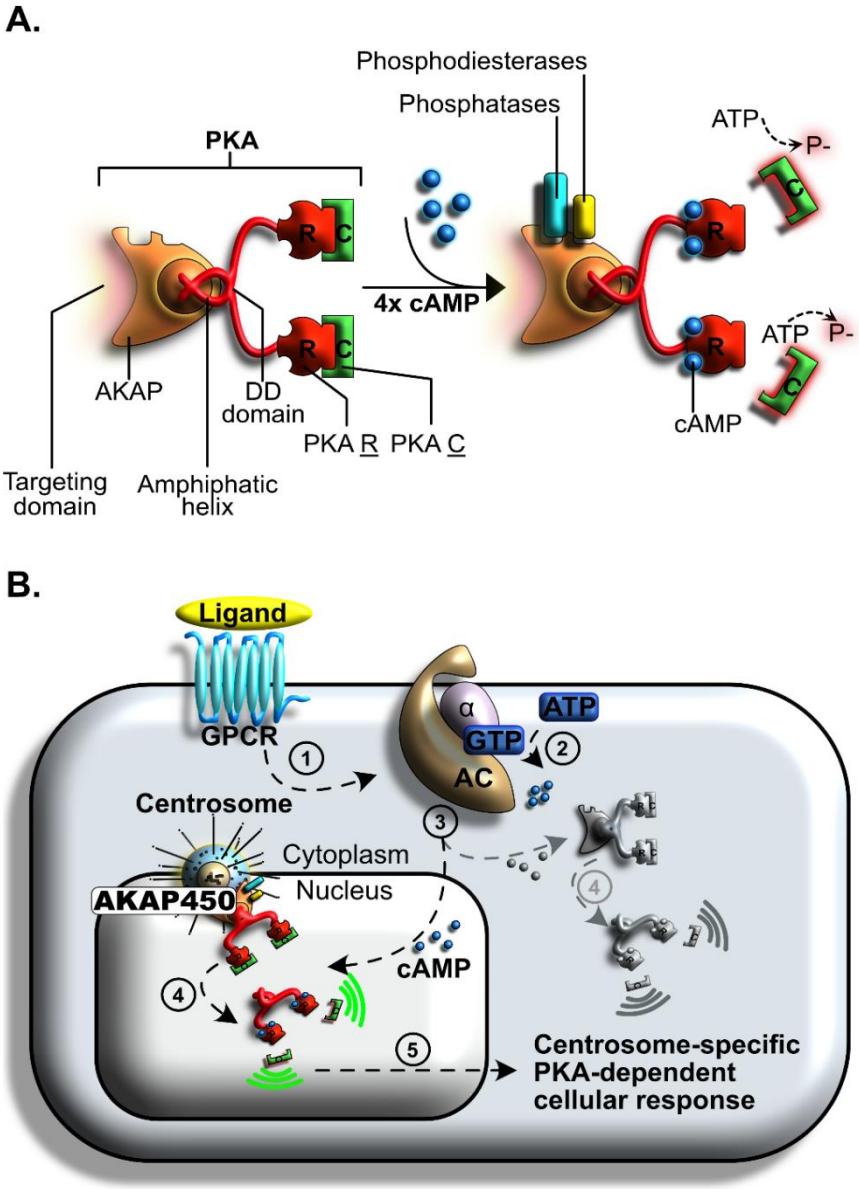

Figure 1.R5. Centrosome-specific regulation of protein kinase A (PKA) signaling. (A) PKA is a tetrameric holoenzyme composed of two regulatory subunits and two catalytic subunits. Its activity relies on cyclic AMP (cAMP) cellular levels and is involved in many regulatory processes. **(B)** Regulation of PKA following G protein-coupled receptor (GPCR) activation. A ligand binds to the GPCR (step 1), initiating the signal transduction cascade. This signal induces a GDP to GTP exchange on a heterotrimeric G complex (step 2). The Gα subunit is released and binds to adenylyl cyclase (AC), an event that induces the formation of cyclic adenosine monophosphate (cAMP) from ATP. A subpopulation of PKA anchors at the centrosomes (step 3). The resulting AKAP450 complex increases PKA affinity for cAMP. Centrosomal PKA is selectively activated by cAMP, whilst cytosolic PKA (shown in grey) remains mostly inactive (step 4). A specialized cellular response is induced by the catalytic activation of PKA at centrosomes (step 5).

Together, these studies unraveled that selective activation of centrosomal PKA is pivotal for inducing the cAMP-dependent pathway during mitosis. In this setting, centrosomes act as supramolecular docking platforms in which conditions for PKA activation differ significantly from those that prevail elsewhere in the cell.

1.7.2.4 Centrosomes as STOCs: Regulation of cell proliferation decisions by NIMA-related kinases

NIMA-related protein kinases are serine/threonine kinases involved in multiple MTOC-related processes. In metazoans, these processes include centrosome separation, during which centrosomes migrate to opposite poles of the cell, spindle assembly, and MTOC-independent regulation of mitotic checkpoints (Nigg, 2001; O'Connell et al., 2003; Moniz et al., 2011; Fry et al., 2012). In humans, seven NIMA-related kinases (Neks) have been identified, whereas lower eukaryotes typically encode a single family member.

The Nek2 isoform in humans is enriched at the centrosomes. Although Nek2 associates with centrosomes in all stages of mitosis, independently of microtubules, its activity is highest in S and G2 phases (Fry et al., 2012). Nek2 is required for centrosome integrity, as evidenced by dramatic phenotypes caused by loss or gain of function mutations. Loss of function mutations were reported to impair centrosome disjunction, a process through which the proteinaceous linker keeping the mother and daughter centrioles in close proximity normally disappears (Hinchcliffe and Sluder, 2001; Fu et al., 2015), and to elicit the formation of monopolar mitotic spindles (Faragher and Fry, 2003; O'Regan et al., 2007). On the other hand, gain of function mutations were reported to induce premature centrosome splitting where a single centrosome would separate into two distinct foci, gradual centrosome loss, and dispersal of centrosomal material (Fry et al., 1998; Fang and Zhang, 2016). Beyond its MTOC-dependent role, Nek2 promotes chromatin condensation in mouse meiotic spermatocytes (Di Agostino et al., 2004; Fry et al., 2012) and cytokinesis in *Drosophila* (Prigent et al., 2005). In fission yeast, the unique Nek2 homolog Fin1 likewise contributes to key cellular processes ranging from mitotic commitment (see section *"Centrosomes as STOCs:*

Polo-like kinases–The Polo-like kinase (PLK) family" for an overview of mitotic commitment in *S. pombe*) to spindle function, maintenance of nuclear envelope dynamics and regulation of the septum initiation network (SIN) (Krien et al., 2002; Grallert et al., 2004). Certain phenotypes observed across species upon gain or loss of function mutations in NIMA-related kinases share common themes. Fin1 overproduction in *S. pombe* was notably reported to create spindle formation defects, reminiscent of the centrosome splitting phenotype associated with Nek2 gain of function mutations in humans (Fry et al., 1998; Krien et al., 2002). Despite the lower amount of functional overlap observed in this class of protein kinases in comparison to others (such as PLKs) across species, NIMA-related kinases still share several functional features from yeast to humans and represent an important class of proteins with vital functions in cell biology.

1.7.2.4.1 Nek2-Mediated Signaling in the Wnt/Wingless Pathway

Nek2 is known to phosphorylate *ß*-catenin, a multifunctional Wnt-pathway effector implicated in a wide array of biological contexts including centrosome-related cellular processes (Kaplan et al., 2004; Bahmanyar et al., 2008; Vora et al., 2020). Throughout mitosis, Nek2-mediated *ß*-catenin phosphorylation prevents its degradation, a mechanism required to maintain high levels of centrosomal *ß*-catenin (Mbom et al., 2014) and associated with accurate centrosome disjunction. Nek2 kinase activity at the start of mitosis relies on Plk1 (Mardin et al., 2011), however, *ß*-catenin enrichment at the centrosomes is independent of its phosphorylation state (Mbom et al., 2014). Outside of the centrosome, the Nek2b isoform forms a complex with T-cell factor (TCF4) to drive *ß*-catenin-dependent cell proliferation, a mechanism associated with tumor cell invasion and metastasis (Shin et al., 2017; Zhang et al., 2017; Shen et al., 2019).

Nek2 also phosphorylates dishevelled (DVL), a scaffold protein involved in both the canonical and non-canonical branches of the Wnt pathway. In higher organisms, three genes encode DVL isoforms – *DVL1*, *DVL2* and *DVL3*. These isoforms, broadly expressed in mammalian cells, were reported to have partly overlapping functions with high levels of redundancy (Lee et al., 2008). The phosphorylation event mediated

by Nek2 on DVL isoforms is essential to promote interactions between DVL and several centrosomal linker proteins, liberating these from the centrosomes and ultimately promoting centrosomal separation. Indeed, lack of DVL impedes the dissolution of centrosomal linkers, resulting in an absence of centrosomal separation (Cervenka et al., 2016). Nek2 can also positively modulate the pool of DVL available at the centrosomes to upregulate the canonical Wnt/β-catenin pathway (Cervenka et al., 2016).

Apart from its implication in centrosome separation, the Wnt signaling pathway was also reported to play a role in cell motility. In response to exosome-transported signaling molecules named planar cell polarity (PCP) proteins, the Wnt pathway stimulates breast cancer cell (BCC) motility at the cell cortex. For this event to occur, the association of a centrosomal module is required. Specifically, DVL2 isoform was shown to mediate the assembly of this module, composed of the human centrosomal protein CEP192 and PLK4/AURKB, to promote protrusive activity in BCCs. This centrosomal module coordinates the exchange of formin DAAM1 for formin DAAM2 at the cell cortex, resulting in increased cell migration (Luo et al., 2019). This sequence of events may partly explain why aberrant expression of PLK4, AURKB and DAAM2 in breast cancer was shown to correlate with poor prognosis and increased cancer aggressiveness (http://www.cbioportal.org). Interestingly, the function of this centrosomal module was reported to be independent of centrosomes or microtubules and elegantly highlights how contextual Wnt signalling in cancer cells has the power to initiate processes such as cell migration as a means to augment metastatic potential.

In the developing *Drosophila* eye, the relationship between Nek2 and Wnt/Wingless is more direct. In a setting where the anaphase-promoting complex (APC) is inactivated, Nek2 accumulation causes hyperactivation of Wnt signaling and blocks retinal differentiation. Conversely, when Nek2 is degraded by APC, local Wnt signaling is suppressed and retinal differentiation proceeds (Martins et al., 2017). Taken together, these studies highlight how Nek2 operates in partnership with the Wnt pathway throughout the entire cell, including at the centrosomes.

1.7.2.5 Centrosomes as STOCs: Regulation of mitotic exit and/or cytokinesis by MEN and SIN kinases

The mitotic exit network (MEN) is a GTPase signaling cascade that regulates cell cycle progression in budding yeast with similarities to the Hippo signaling pathway in metazoans. MEN drives the onset of mitotic exit in late anaphase and cytokinesis primarily by inhibiting the activity of Cdk1 and reversing phosphorylation sites on Cdk1 substrates. SPBs provide spatio-temporal cues for MEN, and importantly, functions as docking platforms to initiate and amplify signaling events.

Up until anaphase, the GTPase Tem1, the main MEN initiator, is present at SPBs but is kept inactive until Cdc14 phosphatase is released from the nucleolus to create a positive feedback loop that drives the mitotic exit process (as reviewed in Manzano-López and Monje-Casas, 2020). Two spindle position checkpoint (SPOC) components, GTPase-activating proteins (GAP) Bfa1/Bub2, inhibit Tem1. In anaphase, spindle elongation allows the older SPB to progressively migrate from the mother cell into the daughter bud, at which point the Cdc5 kinase, enriched at the SPBs, phosphorylates Bfa1/Bub2 to disinhibit Tem1. Concomitantly with this, migration of the older SPB into the bud places the Lte1 guanine-exchange factor (GEF), located in the bud cortex, where it can access and convert Tem1 to its active GTP-bound form. Subsequently, the Cdc15 kinase and its downstream effector –the Dbf2-Mob1 complex– are recruited to SPBs and activated, allowing transmission of the MEN signal to the nucleolus, where it can activate Cdc14 (Renicke et al., 2017; Campbell et al., 2019).

The release of Cdc14 and its gradual accumulation outside of the nucleolus generates a robust feedback loop that promotes mitotic exit (Barberis et al., 2005; Maekawa et al., 2007). Cdc14 enriches at the SPBs via its interaction with the outer plaque component Spc72, and throughout anaphase, gradually increases on the parental/older SPB as it migrates through the daughter bud (Yoshida et al., 2002). In late telophase, once the daughter SPB is fully generated, Cdc14 accumulates on both SPBs. By acting as a docking platform for Cdc14, SPBs may act as a functionally distinct reservoir of active Cdc14 responsible for promoting effective mitotic exit (Yoshida et al., 2002).

The wealth of knowledge on the MEN and its role in mitotic exit sometimes overshadows its equally important roles in cytokinesis. In budding yeast, establishment of an actomyosin ring and septum formation between the mother and daughter bud at the beginning of anaphase are necessary processes for completing cell division and separate the two newly formed cells. Given the temporal pairing of late mitotic events and cytokinesis, many MEN components are also required for the completion of the cytokinetic process. Amongst them, SPB-bound Tem1 and the Bfa1/Bub2 complex were shown to be crucial for successful cytokinesis (Whalen et al., 2018) and the activity of the SPB-enriched Cdc5 kinase required to complete cytokinesis. In late anaphase, Bfa1 maintains Cdc5 mainly on the cytoplasmic side of the daughter SPB (Park et al., 2003). At the onset of cytokinesis, Cdc5 gradually enriches at the bud neck and promotes cell division through its kinase activity towards a specific subset of substrates. The preferential enrichment of Cdc5 at the outer side of the daughter SPB seemingly facilitates the late mitosis/cytokinesis transition by allowing for the rapid migration of Cdc5 at the bud neck (Botchkarev et al., 2017). Thus, the role played by SPBs as platforms that coordinate MEN signaling has implications beyond the area of mitotic exit, such as the regulation of key events required for the completion of cytokinesis.

The septation initiation network (SIN) in fission yeast, a GTPase signaling cascade akin to the budding yeast MEN, regulates several mitotic processes occurring in the last steps of cell division. These processes include actomyosin ring constriction (CAR), septation and cytokinesis (Feoktistova et al., 2012; Alcaide-Gavilán et al., 2014; Edreira et al., 2020). The first event leading to SIN initiation requires the activation of the Ras-like GTPase Spg1 (septum-promoting GTPase) (Schmidt et al., 1997). In metaphase, both SPBs contain uniform amounts of Spg1. The latter, ensuing its activation, recruits its effector protein kinase Cdc7 at the SPBs. Upon anaphase entry, both Spg1 and Cdc7 become inactivated on the parental SPB whilst Cdc7 concentration increases on the daughter SPB (Sohrmann et al., 1998). The resulting asymmetrical enrichment of Cdc7 on the newer SPB further induces the recruitment of Sid1 and Sid2 protein kinases on the daughter SPB, stimulating SIN activity and contributing to the transduction of septation signals from the

SPB to the division site (Guertin et al., 2000). Furthermore, Sid2 was reported to exert a positive effect on SIN activity feedback loop, thus maximizing the signaling cascade to promote septation (Feoktistova et al., 2012). The polo-like kinase Plo1, involved in several steps of mitotic progression, was also reported to positively impact SIN activity and was hypothesized to operate upstream of the aforementioned signaling cascade (Ohkura et al., 1995; Tanaka et al., 2001). Loss-of-function mutations encompassing certain *SIN* genes were reported to induce the formation of elongated multinucleated cells, resulting from the absence of cell division following several cycles of nuclear division (Nasmyth and Nurse, 1981; Balasubramanian et al., 1998). Conversely, gain-of-function mutations were linked to the establishment of numerous actomyosin contractile rings and septa in cells without divided nuclei, a consequence of mutated inhibitors of SIN (Feoktistova et al., 2012).

The function of the MEN in mitotic exit represents a late evolutionary trait. Since the regulation of mitotic exit was coupled to the mitotic exit (ME)-signaling pathway only during the development of the Saccharomycetaceae family, other yeast species such as *C. albicans* or *S. pombe* thus lack this function of the MEN (Maekawa et al., 2022). Moreover, it is worth noting that the MEN was suggested to function earlier in the cell cycle, such as in metaphase, in other processes unrelated to mitotic exit and cytokinesis. SPBs were notably reported to exploit the MEN as a way to drive age-dependent segregation. The spindle positioning protein Kar9 was shown to impact SPB segregation through preferential asymmetric enrichment to the older SPB in metaphase, a process requiring sustained Kar9 phosphorylation by the MEN kinases Dbf2 and Dbf20 (reviewed in Hotz and Barral, 2014). The SPB component Nud1 was also reported to further support the asymmetric enrichment of Kar9 on the old SPB and demonstrates that the MEN can impact cell cycle progression as early as in metaphase, through the establishment of asymmetric SPB inheritance (Hotz et al., 2012a; Hotz et al., 2012b). Importantly, the contribution of the MEN to early mitotic events was shown to be conserved across several eukaryotic species, including *S. pombe*, and suggests that this specific feature of the MEN is a commonly shared evolutive trait (Hachet and Simanis, 2008; Chiba et al., 2009; Chiyoda et

al., 2012; Grallert et al., 2012). Despite the fact that cell cycle progression is a collective function of Hippo-related kinases across many eukaryotic species, exceptions remain. The Hippo-related pathway in ciliates was notably reported to contribute to the regulation of cilia biology as well as to the establishment of cell polarity (Tavares et al., 2012; Soares et al., 2019). However, there is no clear evidence that Hippo-related kinases in ciliates regulate cell cycle progression the way it was reported in other species such as yeast and denotes a certain degree of functional variability in this otherwise conserved pathway.

In comparison to the vast body of knowledge collected on the MEN-SPBs relationship in budding yeast or the SIN-SPBs in fission yeast, the precise contribution of human centrosomes to mitotic exit remains relatively unexplored. The Hippo signaling pathway is an important regulator of cell proliferation and apoptosis in higher eukaryotes. Given its importance in chromosome segregation and cytokinesis, the Hippo pathway is thus considered to play a functionally analogous role to the MEN (Hergovich and Hemmings, 2012). Although no clear Tem1 homolog has been identified in humans so far, Ras has been proposed to play a Tem1-like role in mitotic exit. Other MEN components located at SPBs appear to be conserved in humans, for instance centriolin, a centriole-appendage protein that transiently locates at the centrosomes. Thus, centriolin may play a similar role to that of Nud1 in promoting mitotic exit through its protein-protein interactions involving human MEN components (Pereira and Schiebel, 2001; Gromley et al., 2003). The centrosomes appear to act as a scaffolding structure for a broader range of regulators in humans, thus involving them in a multitude of intertwined pathways and cellular processes (Mayor et al., 1999; Chavali et al., 2014).

1.7.3 Beyond MTOC and STOC roles of centrosomes/SPBs

The studies discussed above describe how SPBs/centrosomes act as essential signaling centers for many biological processes. However, multiple lines of evidence reveal the existence of additional non-canonical roles for centrosomes/SPBs. In this section, we describe how nature and evolution co-opt MTOCs into

fulfilling roles that go beyond their typical contribution to cell shape, intra-cellular transport and cell division. These roles require MTOC activity in some cases but utilize microtubules in ways that exceed and/or diverge from their primordial function in eukaryotic cells.

1.7.3.1 SPB-dependent membrane formation during sporulation

When facing environmental stresses or severe nutrient deprivation, organisms ranging from bacteria and protozoa to plants and fungi can undergo sporulation as a way to adapt to environmental changes and increase the likelihood of their survival. Certain eukaryotic species, such as budding yeast, have the capacity to initiate sporulation as a form of specialized meiosis. This meiotic process allows for cells to shuffle and partition their genomic contents into different combinations, thus increasing the likelihood of progeny survival. In yeast, vegetative cells enter into premeiotic S phase. After completion of S phase, replicated DNA is partitioned into four haploid nuclei, which constitute the backbones of the four daughter cells to be created (Figures 1.R6, steps A–C). Next, a membrane compartment, called the prospore membrane, matures and surrounds the four newly created daughter nuclei (Figure 1.R6D). This step is vital for spore maturation as it gives rise to thick spore walls required for chromatin compaction and protection of cells from harsh environmental conditions (Roeder and Shaw, 1996; Coluccio et al., 2004; Suda et al., 2007; Neiman, 2011). Finally, the remnants of the parental cell collapse around the dormant progeny (the asci) to give rise to four mature haploid cells (Figure 1.R6E) (Neiman, 2005).

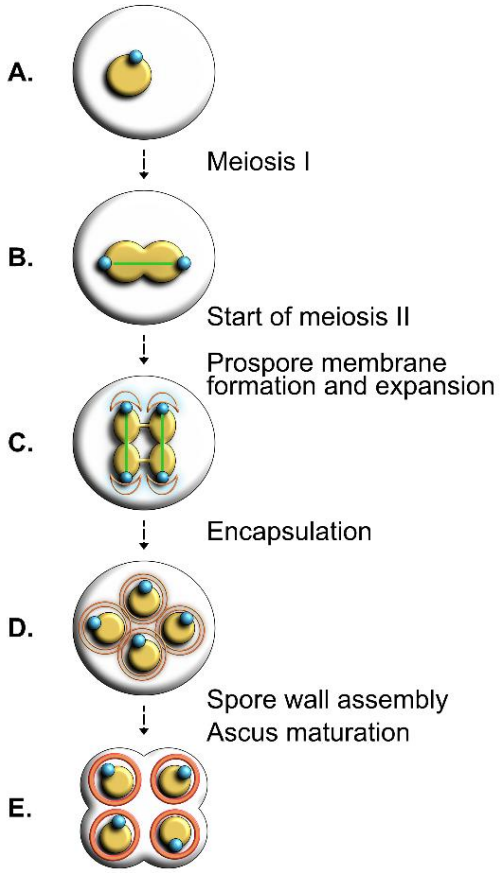

Figure 1.R6. Visual representation of sporulation and ascus formation in budding yeast. (A) In response to environmental stressors, diploid yeast cells initiate the sporulation program. **(B)** Completion of meiosis I nuclear division. **(C)** After the second round of chromosome segregation, the prospore membrane (shown in orange) forms and expands around each duplicated SPB (shown in blue). **(D)** The membrane grows and encapsulates each haploid nucleus in the tetrad. **(E)** Spore wall assembly begins and the remnants of the mother cell breaks down.

SPBs support the initial construction of the prospore membrane, but the developmental reprogramming of vegetative cells that leads to sporulation alters their composition and function. During meiosis I, SPB duplication is similar to the process observed during mitotic division, but meiosis II induces multiple changes in SPB constitution that turns this organelle into a focal point for membrane formation. Most of its outer plaque components are replaced with specific proteins required for sporulation. During meiosis II, Mpc54, Spo74 and Spo21/Mpc70, three meiotic plaque components, act as substitutes for Spc72 on the cytoplasmic face of the SPBs. Instead of interacting with microtubules, Mpc54 and Spo21/Mpc70 cooperate with Nud1, Cnm67 and Spc42. The mechanistic process underpinning prospore membrane

extension is not well understood, but we know that each prospore membrane surrounds its respective SPB in a semi-circular conformation prior to extension. Each membrane thus captures half of its corresponding nucleus, eventually forming walls englobing the entire nucleus (Neiman, 2011). In their research touching on prospore membrane formation, Knop and Strasser observed that levels of Mpc54 and Mpc70 peaked towards the end of meiosis II and plummeted shortly after, suggesting for a restricted role of these proteins exclusively in the formation of the meiotic plaque. Assembly of the prospore membrane was also shown to rely on Don1, a protein emerging towards the middle stages of meiosis I. Using immuno-electron microscopy, authors reported that Don1 localizes to the prospore wall during meiosis II and was proposed to be a marker for prospore membrane formation (Knop and Strasser, 2000). In a situation where meiotic SPB components are mutated or otherwise deficient, prospore membranes fail to engulf the four nuclei and the sporulation process collapses (Knop and Strasser, 2000), underscoring the essential nature of SPBs for this process.

1.7.3.2 MTOC as linchpins for cellular reprogramming of quiescent cells

Eukaryotic cells rely heavily on stimuli provided by their immediate surroundings to make cell proliferation decisions. In situations where nutrients become limiting and proliferation is impossible, cells have the ability to initiate stress survival programs that enable them to better face environmental hazards. A cellular state termed quiescence can also be induced when nutrient become scarce or in the presence of specific developmental cues.

Quiescence is a common dormant state in wildlife (Gray et al., 2004; Zhang et al., 2019). Upon entering quiescence, cells temporarily halt their division cycle, thus allowing time for the surrounding environment to replenish its resources (Sagot and Laporte, 2019). This process, routinely observed in unicellular eukaryotes, is also common in multi-cellular organisms including humans, where the quiescent state preserves and maintains embryonic stem cell pools in adult tissues until actively needed for

homeostasis or tissue repair (Cheung and Rando, 2013). In yeast, the decision to favor quiescence over other stress coping strategies can be determined by the availability and type of carbon source present in the environment. When ethanol is the predominant carbon source, sporulation and ascospores formation is the main stress coping strategy of budding yeast. Conversely, limited availability of a high-energy fermentable carbon source such as glucose makes quiescence the preferred route to maintain cellular homeostasis and redox equilibrium (Tomova et al., 2019).

Entry into quiescence induces major changes in cellular organization and physiology, including appearance of internal structures such as storage granules and actin bodies (Sagot et al., 2006; Narayanaswamy et al., 2009; Noree et al., 2010; Liu et al., 2012; Laporte et al., 2013; Shah et al., 2013; Sun and Gresham, 2020). The typical Rabl nuclear configuration, in which centromeres are clustered to one side of the nuclear envelope and concomitantly attached to the SPB, is replaced by a simplified nuclear arrangement in quiescent cells (Figure 1.R7) (Guacci et al., 1997; Jin et al., 1998; O'Toole et al., 1999; Jin et al., 2000; Bystricky et al., 2004; Laporte et al., 2013). This response is fully reversible because quiescent cells typically revert back to the standard Rabl configuration in less than an hour after nutrients are replenished in their immediate environment. This rapid response to environmental cues is highly beneficial for most unicellular organisms and is thought to provide cells with increased competing fitness and enhanced survival chances (Laporte and Sagot, 2014).

An important quiescence hallmark in yeast is the assembly of a long and highly stable array of nuclear microtubules (nMTs) which spans the entire length of the nucleus and consequently displaces the nucleolus (Laporte et al., 2013; Laporte and Sagot, 2014). Chromosomes, which remain tightly attached to the SPB-generated microtubules, become spread along the length of the newly formed nMTs array. Whilst the exact purpose of this nuclear rearrangement during quiescence remains unclear, this selective chromosomal relocation has been proposed to influence gene transcription and mRNA export efficiency (Taddei and Gasser, 2012; Laporte and Sagot, 2014).

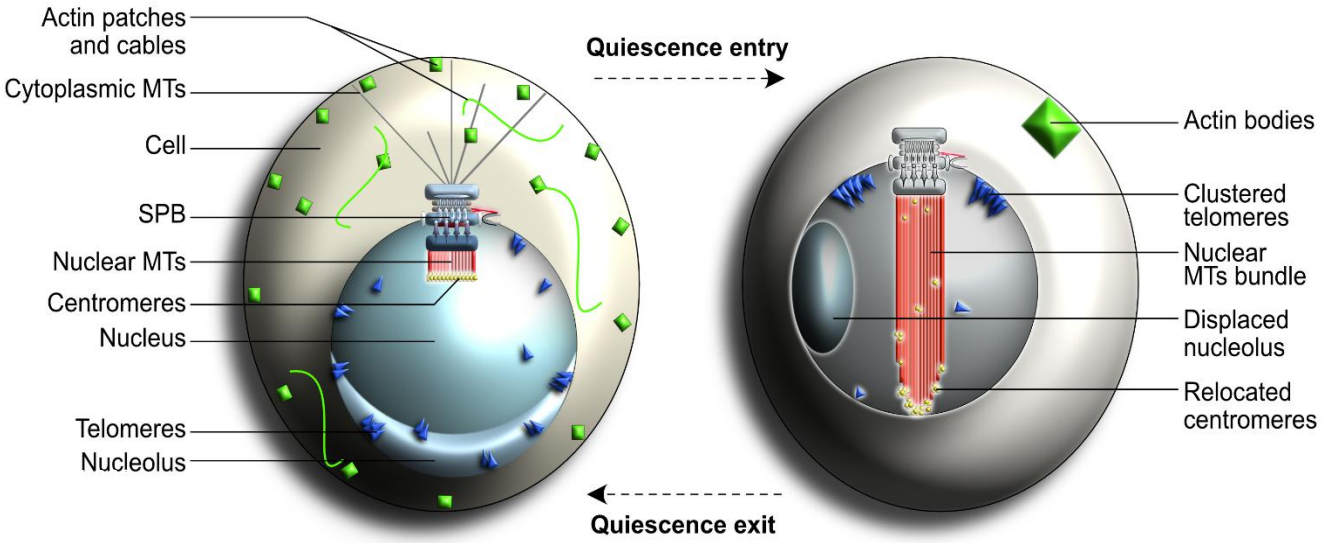

Figure 1.R7. Cellular changes associated with the quiescent state in yeast. These changes include the disappearance of cytoplasmic microtubules (MTs) and formation of a nuclear bundle of MTs (nMTs) that spans the entire nucleus. Centromeres (shown in yellow) normally cluster together at the end of nuclear MTs in interphase cells (left) but get redistributed along the length of the newly formed nMT bundle in quiescent cells (right). Chromosome arms are omitted from this figure to simplify the representation. See text for more details.

SPBs, that form the nMTs array in quiescent cells, are important executioners of the quiescence program. Accordingly, mutations that cause shifts in nMTs array length or stability impede quiescence-related nuclear reorganization and leads to quiescence defects, genomic instability and decreased likelihood of survival (Jin et al., 1998; Gray et al., 2004; Laporte and Sagot, 2014). Likewise, mutations affecting MT nucleation in SPB components, as well as in other organelles or transduction signal pathways involved in quiescence, may drastically reduce cell survival (Gray et al., 2004; Laporte and Sagot, 2014). *XRN1* (also known as *KEM1*) encodes an exonuclease involved in nutrient signaling. Mutated *xrn1* impaired relay of nutritional information to the SPBs, consistent with a possible role for the SPB as a signaling platform during quiescence (Werner-Washburne et al., 1993).

Although a few rare mutant cells survive and are capable of returning to a cycling state upon replenishment of environmental nutrients, the likelihood of survival of their offspring is greatly reduced; a

surviving quiescence mutant will confer genomic instability to its progeny, often resulting in cell death (Laporte and Sagot, 2014).

It is unclear if centrosomes play a similar role as SPBs in mammalian cell quiescence. The formation of a nMT array is unlikely to occur in mammalian cells because centrosomes are typically not embedded in the nuclear membrane in higher eukaryotes. However, centrosomes may act as a key docking platform to regulate protein kinase A (PKA) signaling in the early stages of quiescence, as suggested by Gray et al. (2004). Furthermore, the process of quiescence has often been correlated with the formation of a primary cilium in mammals (Tucker et al., 1979; Laporte and Sagot, 2014). Given the requirement for cilium resorption in differentiated cells prior to cell division, the presence of a primary cilium in quiescent cells has been proposed to act as an important biological checkpoint. This theory would satisfactorily correlate with a cell's need to assess the state of its external surroundings prior to reverting back to a cycling state (Kim and Tsiokas, 2011 as cited in; Laporte and Sagot, 2014). Further studies will be necessary to define more precisely the contribution of centrosomes to mammalian cell quiescence.

1.7.4 Closing remarks

Centrosomes and SPBs are cellular organelles mainly recognized for their role as microtubule nucleators (MTOCs) crucial for cell shape determination, intra-cellular transport and cell division. While there is little debate that this viewpoint is well justified, the importance of centrosomes/SPBs in other cellular processes must not be overlooked. Indeed, these organelles also act as key players in the transduction of several signalization events and in the implementation of important differentiation programs. Through their roles as intracellular docking platforms that enhance kinase-substrate interactions, centrosomes/SPBs effectively function as important STOCs. This role is achieved through the regulated formation of supramolecular protein assemblies on the surface of MTOCs. The scale and compositional complexities of these assemblies suggest that STOCs provide a unique regulatory environment for signaling events. Moreover, the dynamic

nature of their location/movements during the cell cycle suggest a capacity for decoding and translating spatio-temporal cues into transduction events. Overall, centrosomes/SPBs are indispensable to ensure cellular fitness and mutations in these organelles can lead to severe pathologies, ranging from microcephaly to cancer (Jaiswal and Singh, 2021). Given their versatile influence in cell proliferation and signaling events, future research efforts focused on the MTOC-independent roles of centrosomes could be a fruitful path for discovering therapeutic targets in the treatment of several diseases, including cancer.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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1.8 Yeast genetics: A powerful tool to study cell cycle regulation and checkpoint modulation

Separate from its well-known culinary applications in the production of fermentable goods such as bread and beer, the budding yeast *Saccharomyces cerevisiae* has, for decades, served in laboratory research as a pivotal tool to elucidate the eukaryotic cell cycle and its complexities (Mortimer 2000; Gaikani et al. 2024). *S. cerevisiae* has driven many of the most significant discoveries in cell cycle and checkpoint control, markedly contributing to the establishment of cyclin-CDKs as universal cell cycle engines through the seminal isolation of *CDC* genes (Hartwell 1967; Hartwell et al. 1970; 1973; 1974). The combination of its remarkably high doubling speed (i.e., 90 minutes) and inexpensive culture in simple media makes budding yeast one of the easiest model systems to maintain and manipulate in a laboratory setup (Salari and Salari 2017). Its well-documented genomic toolkit and high rates of successful HR-mediated DNA repair facilitates the process of designing, tagging, and accurately sequencing mutant strains (Gaikani et al. 2024). The exploitation of temperature-sensitive (ts) conditional mutants of essential genes is a powerful strategy to investigate gene function in budding yeast, as it enables transient functional inactivation at restrictive temperatures while circumventing lethality under permissive conditions (Z. Li et al. 2011). Similarly, a simple carbon source shift –i.e., substituting glucose with raffinose/galactose– can yield robust gene

overexpression via *GAL*-inducible promoters, making this technique a potent tool for conditional gene control (Weinhandl et al. 2014). Both in nature and under laboratory conditions, S.*cerevisiae* asexually propagates through an asymmetric division process termed budding (Chant and Herskowitz 1991). *MATα* and *MATα* haploids, as well as *MATα*/*MATα* diploids, can undergo this form of vegetative growth. Obtained through the conjugation of complementary haploid mating types, the diploid state offers a sizeable fitness advantage under limiting growth conditions by allowing for sporulation (Neiman 2011). Through this process, diploids that undergo meiosis give rise to four genetically diverse haploid spores (two *MATα*, two *MATα*), providing researchers with a great tool to assess conditional lethality. Highly tractable, kinetics of budding are also often used in yeast culture assessments as a morphological indicator of cell cycle progression in synchronized cultures or checkpoint-arrested cells. Budding yeast cells can be rapidly and reversibly synchronized using mating pheromones and analog-sensitive alleles of essential mitotic regulators. For example, α-factor induces a robust G1 arrest in *MATα* cells, while analog-sensitive or ts alleles –such as *cdc15-as1* and *cdc15-2*– enable telophase arrest, thereby allowing for the dissection of cell cycle processes with great precision (Breeden 1997; Zhou et al. 2021; Shou and Deshaies 2002).

The remarkable functional conservation of key pathways involved in cell cycle regulation and stress signaling positions budding yeast as an ideal model for elucidating complex biological mechanisms (Vanderwaeren et al. 2022; Archambault and Glover 2009; Cussiol et al. 2019). In the context of DNA damage research, the deployment of precise and quantifiable DNA lesion systems, such as inducible HO endonuclease-driven DSB formation or telomere uncapping in *cdc13-1* ts backgrounds, allows for the reliable assessment of checkpoint signals and adaptive responses (Toczyski 2006; Proctor et al. 2007; Garvik et al. 1995). In fact, these very tools were instrumental in the seminal discovery of DNA damage adaptation in yeast (Sandell and Zakian 1993; Toczyski et al. 1997; Lee et al. 1998). The genetic advantages associated with *S. cerevisiae* as a model organism, combined with the functional conservation of key proliferation and DNA damage signaling pathways across species, makes it an ideal system for dissecting the complexities of

DNA damage adaptation, genome maintenance and human disease mechanisms (Vanderwaeren et al. 2022; Pizzul et al. 2022).

1.9 Rationale and hypothesis

As master regulators of the cell cycle, Polo-like kinases (PLKs) function across a wide range of cycling conditions. Endowed with impressive functional flexibility, the budding yeast PLK Cdc5 carries out its diverse mitotic roles –including DNA damage checkpoint adaptation– via dynamic, PBD-regulated mechanisms. The core premise of this thesis is that Cdc5 expands its functional repertoire by leveraging organelle-specific localization and multi-surface substrate engagement to integrate diverse cellular inputs and coordinate cell cycle progression across both unperturbed and stress-induced conditions. While the functional relevance of PBD-mediated Cdc5 docking at SPBs during DNA damage adaptation is well established (Ratsima et al. 2016), the precise contribution of SPBs to checkpoint override remains obscure. We propose that yeast centrosomes function as active signalling hubs for Cdc5, integrating and relaying phosphorylation signals required for DNA damage adaptation. We further posit that PBD-driven combinatorial engagement of phospho-primed and unprimed targets in a concurrent manner broadens Cdc5 substrate spectrum, thereby shaping context-dependent cell cycle decisions.

1.10 Objectives

Objective I (Chapter II): Elucidate the structural mechanisms by which yeast centrosomes (SPBs) facilitate Cdc5-dependent DNA damage checkpoint adaptation;

Objective II (Chapter III): Determine how PBD-mediated combinatorial substrate engagement modulates Cdc5 activity in the essential coordination of mitotic events.

2 CHAPTER II: Manuscript #1

Yeast centrosomes act as organizing centers to promote Polo kinase-mediated adaptation to persistent DNA damage

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SIGNIFICANCE

During the adaptation response to persistent DNA damage, eukaryotic cells bearing moderate levels of unrepairable DNA lesions bypass a reversible checkpoint-mediated cell cycle arrest and resume cell division despite a compromised genome. While it is known that Polo-like kinases (PLKs) control this response by transiently localizing to microtubule-organizing centers (MTOCs) termed centrosomes, the specific contribution of centrosomes to PLK function during DNA damage adaptation remains unknown. Our work demonstrates that yeast centrosomes actively participate in cell fate decisions under genotoxic conditions by acting as supramolecular signaling platforms for the Cdc5 kinase. We show that upon Cdc5 PLK recruitment, structural components of the yeast centrosomes become dynamically modified to effectively transduce the Cdc5 phospho-signal and commit cells to the adaptation response.

2.1 ABSTRACT

The ability of cells to overcome cell cycle arrest and adapt to the presence of unrepairable DNA damage is under the control of Polo-like kinases (PLKs) in eukaryotes. How DNA damage checkpoints are silenced or bypassed during the adaptation response is unknown, but the process requires enrichment of the Cdc5 PLK to microtubule organizing centers (MTOCs), such as the yeast centrosomes or spindle pole bodies (SPBs). Here, we found that SPBs play an active role as supramolecular organizing centers that coordinate Cdc5 recruitment and signaling to downstream effectors during the adaptation response to DNA damage. We show that SPB components Nud1, Spc110, and Spc72 are key effectors of Cdc5 recruitment to SPBs in the presence of sustained DNA damage. Following recruitment, Cdc5 transduces a phospho-signal to key structural subunits of the SPB, including Cnm67 and Mps3. We demonstrate these phosphorylation events are required to bypass cell cycle checkpoint arrest and enable effective adaptation to DNA damage. This response is specific because it cannot be recapitulated by a generic inactivation of MTOC activity.

Collectively, our results indicate that centrosomes can act as supramolecular platforms to coordinate dynamic recruitment and substrate selection of PLKs during the DNA damage response (DDR).

2.2 INTRODUCTION

The maintenance of genomic integrity is an essential aspect of cell survival and homeostasis for all living species. In eukaryotic cells, the DNA damage response (DDR) integrates a highly conserved set of signaling pathways established to safeguard genomic integrity in the face of genotoxic stress (1, 2). Detection of DNA lesions in damaged cells induces a reversible checkpoint-mediated cell cycle arrest that allows time for repair of DNA lesions prior to DNA synthesis and/or mitotic reentry (3). Through a process termed recovery, cells that successfully complete DNA repair return to active proliferation by deactivation of checkpoint effectors (4). Cells incapable of repairing DNA lesions will engage in apoptosis when they carry high levels of persistent DNA damage, a behavior that protects organisms from genomic instability and oncogenesis. Interestingly, cells experiencing low or moderate levels of unrepairable DNA damage can bypass checkpoint-mediated cell cycle arrest and evade apoptosis through a specific process known as the adaptation response to persistent DNA damage. By turning off the G2/M checkpoint, cells can ignore persistent DNA damage and resume cycling despite a compromised genome (5–7). This evolutionarily conserved pathway can maximize cell survival (8) by making possible the repair of lingering DNA lesions in subsequent rounds of cell division (9), and/or by allowing asymmetric partition of damaged DNA to only one of the two daughter cells after mitosis (10, 11). To be successful, this adaptation response requires a careful coordination of checkpoint deactivation mechanisms with cell cycle reentry. This coordination is controlled by Polo-like kinases (PLKs) in all eukaryotes (11, 12).

PLKs are conserved serine/threonine protein kinases involved in the regulation of cell division and the maintenance of genomic integrity (13–15). Among the five PLKs found in humans (PLK1-PLK5), PLK1 is the most central in events related to cell cycle progression (16). The key structural features of PLKs include an

N-terminal kinase domain (KD) connected to a C-terminal phospho-serine/threonine-binding module known as the Polo-box domain (PBD). This family of kinases typically phosphorylates its targets via a two-stage process. First, the PBD of PLKs acts as a signal recognition module to identify and bind cellular targets that have been prephosphorylated by CDK1 in vivo (17–19). Second, the PBD-induced proximity of PLK targets to the KD of the enzyme leads to their hyperphosphorylation and amplification of the signal initiated by CDK1 phosphorylation. Ultimately, this cascade of events promotes phospho-signal transduction to stimulate downstream events critical for cell cycle progression (16).

Human PLK1 and its budding yeast homolog, Cdc5, are highly dynamic proteins that must enrich at various subcellular locations, such as the centrosome, to promote cell cycle progression (20, 21). The budding yeast spindle pole body (SPB) is analogous to the human centrosome in that it functions as the main microtubule-organizing center (MTOC) and represents an important scaffolding site for the enrichment of Cdc5 kinase. While best known for its MTOC function, the centrosome also plays an essential role as a supramolecular platform on which several kinases enrich to mediate key signaling events (12). Consistent with this notion, the PBD-mediated localization of Cdc5 at SPBs is crucial for mitotic exit, cytokinesis, maintenance of SPB integrity, and ploidy control (22). Moreover, recruitment of Cdc5 to SPBs is also essential for DNA damage adaptation and directly impacts cell survival (23, 24). A Cdc5 mutant defective for PBD-mediated substrate recognition does not localize to the SPB and fails to undergo DNA damage adaptation (23, 25). The PBD-mediated localization of Cdc5 to SPBs is therefore essential to modulate cell proliferation upon checkpoint activation and can directly impact cell homeostasis.

In this study, we explore the specific contribution of SPBs in Cdc5-dependent adaptation to DNA damage. We took advantage of the bipartite structure of PLKs to reveal the identity of two separate pools of effectors of Cdc5 at the SPB and found that several SPB constituents are direct targets for this kinase in damaged cells. Our work sheds light on the choreography of interactions connecting Cdc5 to diverse SPB

components during adaptation to DNA damage and reveals the specific sequence of events required to bypass checkpoint activation and return damaged cells to a cycling state.

2.3 RESULTS

Identification of Candidate Receptors for Cdc5 at the SPB.

We previously showed that PBD-mediated binding of Cdc5 to SPBs is essential to promote adaptation to DNA damage (23). To identify the putative SPB receptor protein(s) for Cdc5, we examined the sequence of SPB components for the presence of the conserved phospho-serine/threonine-binding motif recognized by Cdc5 PBD [i.e., S−(pS/pT)−(P/X)] (18, 19). *SI Appendix*, Fig. 2.S1 shows the number of PBD-binding motifs identified in each of the 17 components of the yeast SPB. This analysis revealed Nud1 as a top receptor candidate for Cdc5 because it contains the highest number of PBD-binding consensus sites (15) among all SPB components.

To assess the role of Nud1 as a SPB recruitment factor for Cdc5 during adaptation to DNA damage, we created three *nud1* temperature-sensitive (ts) alleles of this gene. The first allele, *nud1-28*, has lost the majority of putative Cdc5 PBD-binding sites in *NUD1* (Fig. 2.1 *A*, *Top*). The second allele, *nud1-92*, is also a PBD site mutant of *NUD1*, but a milder version than the *nud1-28* allele that contains only six PBD-binding mutations along with a G585E substitution (26) (Fig. 2.1 *A*, *Middle* schematic). Finally, the third *nud1* allele corresponds to the *cdc18-1* mutation originally described by Hartwell and colleagues (27, 28) and carries a G706D point substitution (Fig. 2.1 *A*, *Bottom* schematic). For clarity, the *cdc18-1* allele will be referred to as *nud1-18*. Consistent with the essential nature of *NUD1*, all three alleles we tested conferred varying degrees of temperature sensitivity to yeast in serial dilution growth assays both at high (Fig. 2.1*A*) and low (*SI Appendix*, Fig. 2.S2*A*) temperatures.

Aside from Nud1, two core SPB components—namely Spc110 and Spc72—have been reported to show affinity for Cdc5 (29, 30), making them plausible candidate receptors for Cdc5 during adaptation to

DNA damage. Spc110 and Spc72 contain seven and eight PBD-binding motifs (*SI Appendix*, Fig. 2.S1), respectively, and can bind to Cdc5 PBD in vitro (30). To evaluate their contribution as SPB recruitment factors for Cdc5, we constructed the *spc110-7* and *spc72-8* alleles in which all the consensus PBD-binding motifs were rendered inactive by mutation of the putative phospho-serine/threonine residues to alanines (Fig. 2.1 *B*, *Top*). Neither *spc110-7* nor *spc72-8* mutants showed proliferation defects at 37 °C (Fig. 2.1 *B*, *Bottom*), indicating that removal of PBD-specific binding sites in these structural components of the SPB does not interfere with the essential/MTOC function of the organelle.

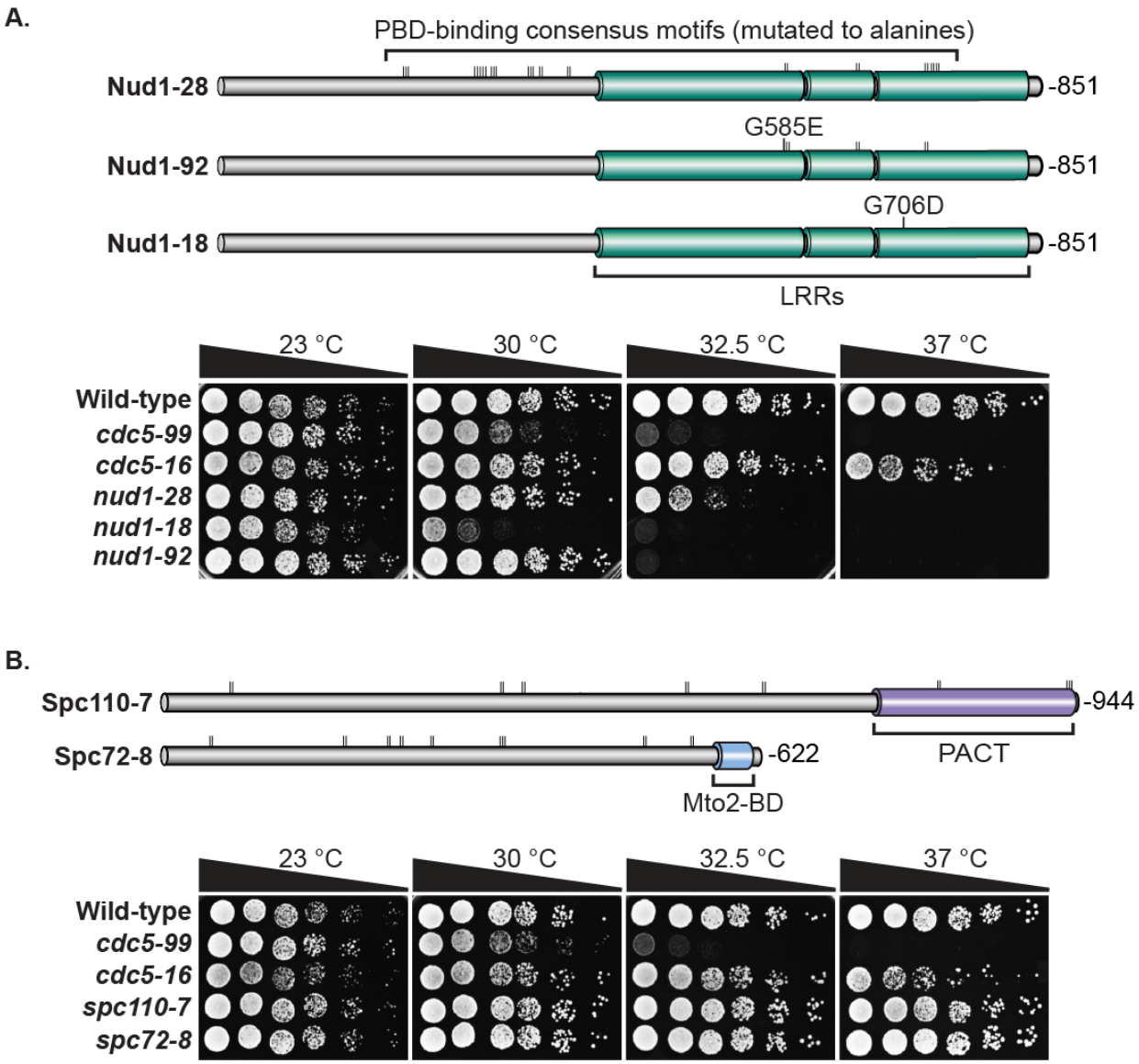

Figure 2.1. Growth phenotype of novel SPB mutants. (A) Schematic representation of Nud1 protein domains and mutations (Top). The linear domain architecture and location of mutations (vertical lines) are shown to scale. Protein length is shown on the Right side of each structure. LRRs: Leucine-rich repeats. All mutations are serine/threonine to alanine unless stated otherwise. The proliferation phenotype of *nud1* mutants at various temperatures is shown under the protein schematics. Cells were spotted on YPD media and grown at the indicated temperatures. (n = 3). **(B)** Domain architecture and position of mutations inserted in Spc110-7 and Spc72-8 proteins (Top). Representation of domains and mutations are as described in panel A. PACT: Pericentrin-AKAP450 centrosomal targeting domain. Mto2-BD: Mto2-binding domain. Growth properties of *spc110-7* and *spc72-8* mutants (Bottom) were assessed on rich YPD media at 23, 30, 32.5, and 37 °C. (n = 3)

Nud1 Contributes to the Adaptation Response to Persistent DNA Damage.

Using the mutants described above, we next investigated the contribution of Nud1 to the adaptation response of yeast experiencing unrepairable DNA damage. Persistent DNA damage was generated by temperature-induced (31 to 33 °C) inactivation of the *cdc13-1* allele, resulting in a sustained DDR and G2/M checkpoint-mediated cell cycle arrest (31). The duration of this DNA damage-induced cell cycle arrest normally ranges from 8 to 10 h, after which cells adapt to the presence of DNA damage and reenter a proliferative state (6). As previously observed, *cdc13-1* mutant cells showed a typical large-budded morphology 2.5 h after exposure to 32.5 °C, reflecting a robust G2/M checkpoint arrest (Fig. 2.2*A*). These cells eventually form microcolonies comprising at least 5 cell bodies of normal size 24 h after formation of DNA lesions, consistent with an effective adaptation response to DNA damage. In contrast, *nud1* mutants showed a strongly reduced microcolony formation capacity and a steady increase in cell size over the course of the experiment. This morphological feature, also reported in the adaptation mutant *cdc5-16* (23), represents a consequence of an active metabolism in nondividing cells (32). Adaptation rates in *nud1* mutants only reached 30 to 50% of the rate observed in a *NUD1 cdc13-1* control strain (Fig. 2.2*B*). Similar results were obtained when *nud1* mutants were tested for adaptation in response to a sustained HO endonuclease-induced double-strand break (DSB) (*SI Appendix*, Fig. 2.S3). Importantly, the fact that *nud1* mutants remain significantly defective for microcolony formation when DNA damage/HO induction occurs at permissive temperature (i.e., 30 °C; a permissive condition for the growth of *nud1*-

28 and *nud1-92*) further highlights the dependency of the adaptation process on Nud1 activity. Collectively, these results indicate that Nud1 is a key contributor to the adaptation response to genotoxic stress and its effects are observed with different alleles and forms of DNA damage.

To verify that the adaptation phenotype detected in *nud1* mutants was specific to the adaptation pathway and did not stem from other proliferation defects, we evaluated the microcolony formation properties of *nud1* strains growing at 32.5 °C in the absence of DNA damage (i.e., in a *CDC13* background). While appearance of microcolonies was slightly delayed in *nud1* mutants compared to wild-type cells, we observed that *nud1* mutants could form microcolonies at rates ranging from 70 to 90% of wild-type rates under nondamaging conditions (Fig. 2.2*D*). Consistent with this, assessing cell cycle kinetics and microtubule morphology in the slowest-proliferating *nud1-92* mutant confirmed that these cells are capable of assembling a functional bipolar spindle, completing nuclear separation (*SI Appendix*, Fig. 2.S4), and successfully undergoing cell separation (*SI Appendix*, Fig. 2.S5 *B* and *C*) at 32.5 °C in the absence of DNA damage. Importantly, strains carrying the *nud1-28* allele showed only partial temperature sensitivity for growth at 32.5 °C (Fig. 2.1*A*) and could form microcolonies at nearly wild-type frequency in the absence of DNA damage (Fig. 2.2*D*), despite showing a strong adaptation defect in the presence of DNA damage (i.e., with *cdc13-1* and HO; Fig. 2*B* and *SI Appendix*, Fig. 2.S3*C*). Therefore, the strong microcolony formation defect of PBD-binding site mutants of *nud1* reveals itself specifically during the adaptation response to DNA damage and not under normal growth conditions.

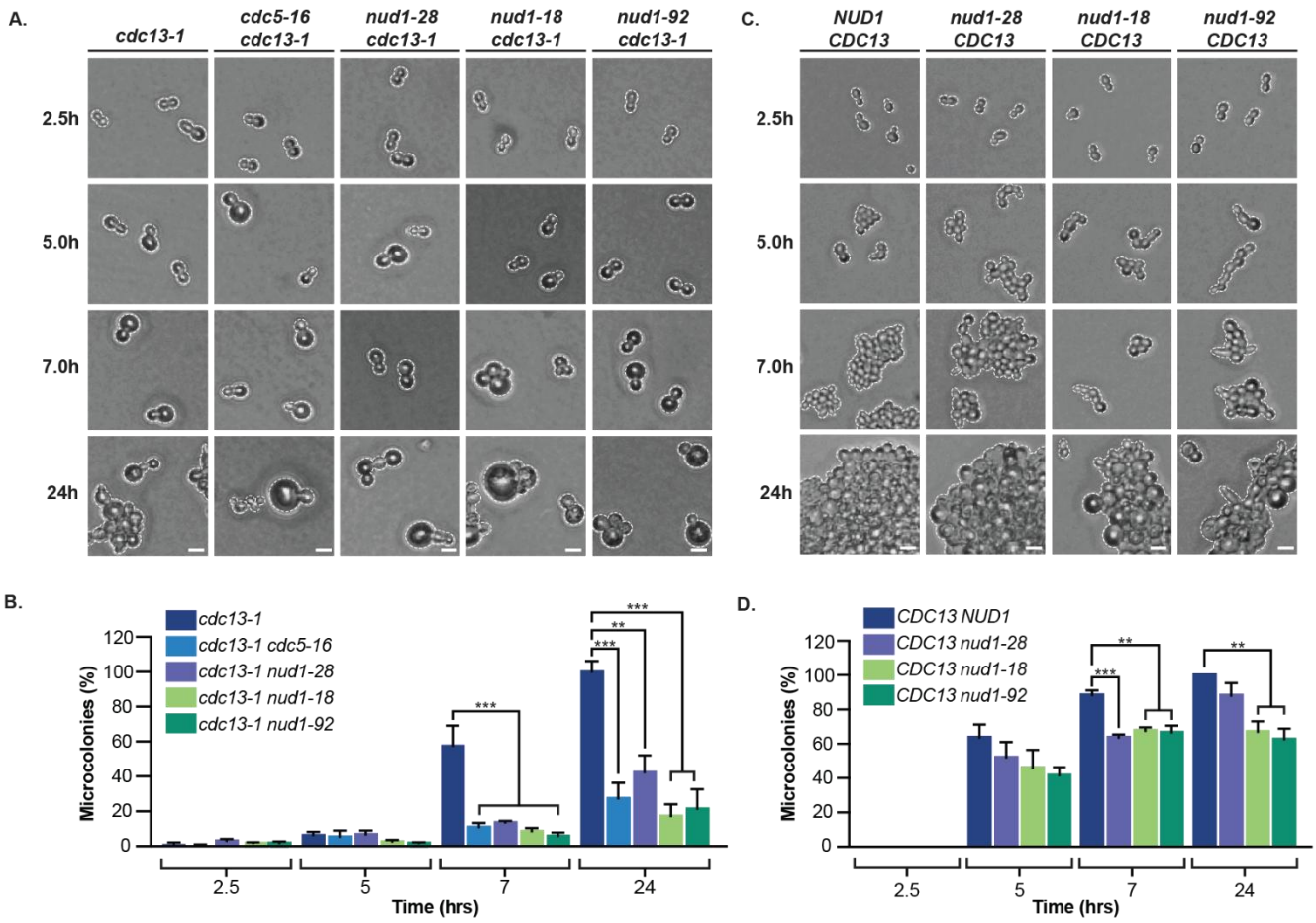

Figure 2.2. Nud1 is required for the adaptation response to persistent DNA damage. (A) *nud1* mutants were assayed for microcolony formation after inactivation of *cdc13-1* at restrictive temperature (32.5 °C) and formation of unrepairable DNA damage at telomeres. All *nud1* mutants showed reduced rates of adaptation to DNA damage relative to the control *NUD1* strain. Scale bars correspond to 10 µm. Outlines of cells and microcolonies are marked with dashed lines to facilitate visualization. Strain genotypes are described in the panel headings. **(B)** Quantification of the fraction of individual cells growing into microcolonies for each time point tested in panel A. A minimum of 100 cells per strain was counted for each time point. All values were normalized to 100% based on the T24 h mean value of the *cdc13-1* control strain. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (**P ≤ 0.01 and ***P ≤ 0.001, respectively). **(C)** Microcolony formation assays performed with *nud1* mutants in the absence of DNA damage (i.e., wild-type *CDC13* background at 32.5 °C). Scale bars correspond to 10 µm. **(D)** Quantification of *CDC13* cells forming microcolonies at each time point shown in panel C. A minimum of 100 cells per strain was counted for each time point. Data represented as mean values and SEM of at least 3 independent experiments. Analysis of statistical significance is as described in panel B.

Nud1 Operates Upstream of Cdc5 During the DDR.

To understand how Nud1 contributes to Cdc5-mediated adaptation to DNA damage, we next aimed to determine the relative position of Nud1 in this pathway. To achieve this, we used *CDC5* overexpression as a tool to place Nud1 upstream or downstream of Cdc5 point-of-action, as previously performed by us and others (23, 33). Wild-type *CDC5* was overexpressed under the *GAL1* promoter in *cdc13-1* cells defective for *nud1* and the kinetics of adaptation were monitored in the presence of DNA damage. As seen in *SI Appendix*, Fig. 2.S6*A*, Cdc5 overexpression allowed for markedly increased adaptation kinetics in *nud1* mutants relative to nonoverexpressing cells (c.f., Fig. 2.2*B*). *nud1* mutants overexpressing *CDC5* showed an abridged G2/M cell cycle arrest and adaptation rates mirroring or exceeding those observed in *NUD1* control cells 24 h after induction of DNA damage (*SI Appendix*, Fig. 2.S6*B*). The suppression of the *nud1* adaptation defect by *CDC5* overexpression indicates that this protein operates upstream of (or in parallel to) Cdc5 during the cellular response to DNA damage.

Spc110 and Spc72 Assist Nud1 in Cdc5-Dependent Adaptation to DNA Damage.

Previous reports have shown that Cdc5 PBD can bind in vitro to other SPB components aside from Nud1 (29, 30). In particular, SPB inner and outer plaque components Spc110 and Spc72 are promising candidates as SPB receptors for Cdc5 because they contain multiple consensus sites for PBD binding (*SI Appendix*, Fig. 2.S1). To test this hypothesis, we designed *spc110-7* and *spc72-8* mutants in which all putative Cdc5 phospho-binding sites were mutated from serines/threonines to alanines (Fig. 2.1*B*). Testing these mutants for adaptation to DNA damage showed that neither *spc110-7* nor *spc72-8* are defective in microcolony formation following a G2/M checkpoint arrest (*SI Appendix*, Fig. 2.S7). As previous literature highlighted the preferential binding of Cdc5 PBD to Nud1 at the SPB in vitro (29), we projected that the contribution of other SPB components as Cdc5 recruitment factors in adaptation might be better assessed in a Nud1-defective background. Interestingly, double mutants carrying *nud1-28* and *spc72-8* alleles could not be obtained due

to systematic reversion of *spc72-8* to wild-type following sporulation of heterozygous diploids. Instead, we used a milder PBD-defective allele, *nud1-92*, to create a triple mutant with *spc110-7* and *spc72-8*. Growth assays on solid medium indicated that *spc110-7* and *spc72-8* single mutants proliferate akin to wild-type control at all temperatures tested (cf., Fig. 2.1*B*). In contrast, combining these alleles with *nud1-92* reduced cell proliferation rates at room temperature (23 °C) (Fig. 2.3*A*) and led to considerable cold sensitivity at 18 °C (*SI Appendix*, Fig. 2.S2*B*), reflecting a clear synthetic effect associated with the combination of PBD mutations in several SPB components.

Next, we assessed the adaptation behavior of the *nud1-92 spc110-7 spc72-8* triple mutant strain. This experiment was carried out at a lower restrictive temperature of 31 °C to account for the enhanced temperature sensitivity of the triple mutant and to widen the scope of detectable phenotypic variations between *nud1-92* and the triple mutant. The microcolony assay depicted in Fig. 2.3 shows that a combination of PBD-binding mutations (i.e., *nud1-92 spc110-7 spc72-8)* led to adaptation defects twice as severe as those observed in the *nud1-92* single mutant and recapitulated the adaptation phenotype observed in *cdc5-16* cells with a 70% decrease in adaptation rates relative to *cdc13-1* control (Fig. 2.3*C*). To validate that this adaptation phenotype was unrelated to impaired cell cycle progression, we measured dynamics of microcolony formation at 31 °C in absence of DNA damage *(CDC13)* in wild-type control cells, *nud1-92* mutant, and triple SPB mutant. Fig. 2.3 *D* and *E* shows that while the triple mutant proliferated with slightly slower kinetics relative to wild-type control, the ability of this strain to undergo several cell divisions was maintained in the absence of DNA damage and resulted in effective formation of microcolonies containing at least 5 cell bodies. Measuring budding kinetics and dynamics of nucleus separation in synchronous liquid cultures at 31 °C without DNA damage induction confirmed that the triple mutant is proficient for anaphase progression, albeit with a slight delay (i.e., 15 min) relative to control (*SI Appendix*, Fig. 2.S8*A*). While minor cytokinetic defects were detectable in the triple mutant, this trait did not hinder cell proliferation and physically fused cells successfully rebudded to undergo consecutive cell

cycles (*SI Appendix*, Fig. 2.S8 *B*, *Bottom Right* panel). As the combination of PBD-binding mutations into the triple mutant exacerbated *nud1-92* adaptation phenotype without obstructing interphase or mitosis, we conclude that Nud1, Spc110, and Spc72 are required to produce a full adaptation response to DNA damage.

To gain additional insight into the DDR occurring in the *nud1-92 spc110-7 spc72-8* triple mutant, we monitored Mec1/Tel1-dependent DNA damage checkpoint activation through Rad53 phosphorylation dynamics. To achieve this, we followed the phosphorylation state of HA-tagged Rad53 upon DNA damage induction in *cdc13-1* control and triple mutant cells over the course of 40 h. As seen in Fig. 2.3*F*, both control and SPB triple mutant strains underwent full Rad53 hyperphosphorylation 2 h post DNA damage induction. The *cdc13-1* control maintained Rad53 in a hyperphosphorylated state until dephosphorylation gradually occurred at the end of the timecourse (Fig. 2.3 *F*, *Left* panel). In striking contrast, the *nud1-92 spc110-7 spc72-8* mutant begun rapid Rad53 dephosphorylation less than 16 h after exposure to DNA damage (Fig. 2.3 *F*, *Right* panel). The SPB mutant phenotype is remarkable because it recapitulates the behavior of the *cdc5-16* adaptation-defective PBD mutant, in which Rad53 undergoes an abrupt and premature dephosphorylation within 12 h of DNA damage exposure (23). Taken together, these results suggest that proper enrichment of Cdc5 at SPBs is necessary to sustain Rad53 hyperphosphorylated state after exposure to DNA damage. Moreover, the observation that Rad53 can rapidly lose its hyperphosphorylated state in cells experiencing adaptation defects implies that Rad53 dephosphorylation does not always correlate with cell cycle reentry after DNA damage.

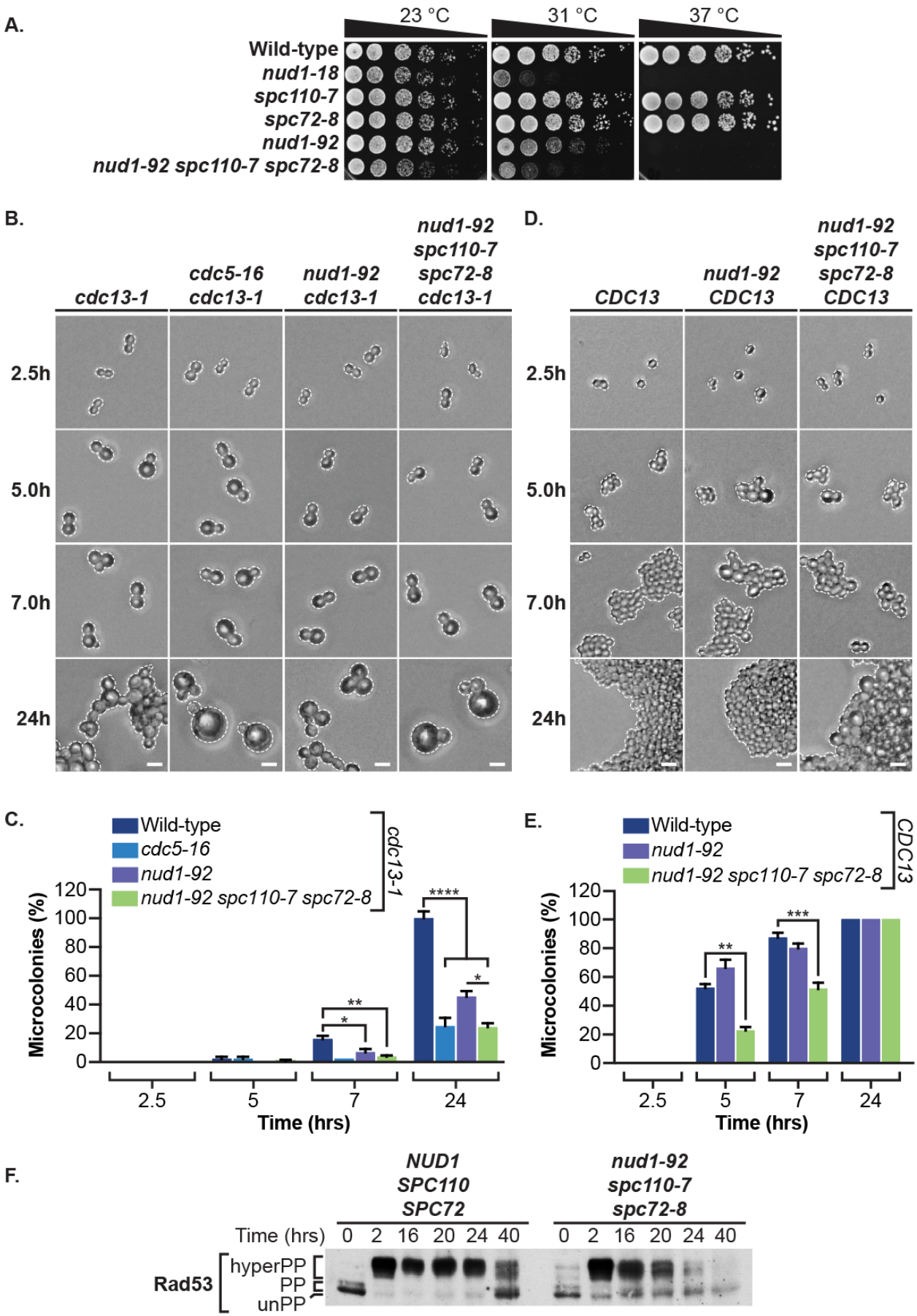
A.
23 °C
31 °C
37 °C
Wild-type
nud1-18
spc110-7
spc72-8
nud1-92
nud1-92 spc110-7 spc72-8
B.
cdc13-1
cdc5-16 cdc13-1
nud1-92 cdc13-1
nud1-92 spc110-7 spc72-8 cdc13-1
2.5h
5.0h
7.0h
24h
D.
CDC13
nud1-92 CDC13
nud1-92 spc110-7 spc72-8 CDC13
C.
Wild-type
cdc5-16
nud1-92
nud1-92 spc110-7 spc72-8
cdc13-1
Microcolonies (%)
Time (hrs)
E.
CDC13
F.
NUD1 SPC110 SPC72
nud1-92 spc110-7 spc72-8
Time (hrs) 0 2 16 20 24 40
Rad53
hyperPP
PP
unPP

Figure 2.3. Spc110 and Spc72 contribute to Cdc5-dependent adaptation to DNA damage. (A) Proliferation properties of *spc110-7, spc72- 8,* and *nud1- 92* mutants at various temperatures. Cells were spotted on the surface of solid YPD medium and grown at 23, 31, and 37 °C. The combination of the three SPB alleles into a triple mutant exacerbated the growth phenotype of *nud1-92* (n = 3). **(B)** Impact of *spc110-7* and *spc72-8* on the adaptation defect of *nud1-92*. Microcolony formation following DNA damage induction was assessed at 31 °C, a restrictive temperature for *cdc13-1* (34). The adaptation defect of the triple mutant was twice as severe relative to *nud1-92* and recapitulated the adaptation phenotype of *cdc5-16* PBD-binding mutant. Scale bars, genotype representation, and cell outlines are as described in Fig. 2. **(C)** Quantification of individual cells growing into microcolonies for each time point in controls, *nud1-92*, and triple mutant strains in the adaptation test shown in panel B. A minimum of 100 cells per strain was counted for each time point. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. An unpaired *t* test on normally distributed values was used to assess the statistical significance of differences observed between *nud1-92* and *nud1-92 spc110-7 spc72-8* strains at T24 h. Statistical significance is depicted on the graph with asterisks (*P ≤ 0.05, **P ≤ 0.01, and ****P≤ 0.0001, respectively). **(D)** Microcolony formation assay performed at 31 °C with control cells, *nud1-92* single mutant, and SPB triple mutant in the absence of DNA damage (*CDC13*). **(E)** Quantification of microcolony formation phenotypes shown in panel D. A minimum of 100 cells per strain was counted for each time point. Data represented as mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (**P ≤ 0.01 and ***P ≤ 0.001, respectively). **(F)** Phosphorylation status of Rad53 monitored at 31 °C in a *cdc13-1* control and a *cdc13-1 nud1-92 spc110-7 spc72-8* SPB triple mutant strain experiencing persistent DNA damage. Aliquots were collected and processed for immunoblot analysis at the specified time points for a total duration of 40 h. While inactivation of *cdc13-1* resulted in effective Rad53 hyperphosphorylation in both control and triple mutant strains (i.e., hyperPP), Rad53 begun rapid dephosphorylation less than 16 h after initial exposure to DNA damage in the *nud1-92 spc110-7 spc72-8* triple mutant (i.e., PP and unPP). A minimum of 3 independent experiments were performed and a representative immunoblot is shown.

Generic Inactivation of SPBs Does Not Cause a Specific Defect in the Adaptation Response to DNA Damage.

Given the role played by Nud1, Spc110, and Spc72 in the DDR, we next asked whether generic inactivation of any SPB component would produce equivalent defects in the microcolony formation assay. We introduced the previously described Q110P substitution into Spc42 (35), a core SPB component, to create the *spc42-10* ts mutant (*SI Appendix*, Fig. 2.S9*A*). This mutant is fully defective in the essential MTOC activity of the SPBs (35). To test whether a generic/unspecific loss of SPB activity in a *spc42-10* strain could induce a specific defect in adaptation, we monitored the dynamics of microcolony formation in a *cdc13-*

1 background at restrictive temperature (32.5 °C). Under these conditions, the *spc42-10* mutant showed a uniform population of large-budded cells arrested in G2/M 2.5 h after inactivation of *cdc13-1*, consistent with a robust checkpoint-induced arrest, and these cells ultimately formed microcolonies at 40% of the rates observed in a control *SPC42* strain exposed to DNA damage for 24 h (*SI Appendix*, Fig. 2.S9 *B* and *C*). Interestingly, very similar results were observed when the *spc42-10* mutant was tested in the absence of DNA damage (*CDC13*; *SI Appendix*, Fig. 2.S9 *D* and *E*). Since the cell cycle arrest and microcolony formation defect occurred regardless of DNA damage in *spc42-10* cells, we conclude that generic inactivation of a SPB core component leads to a loss of MTOC activity that interferes with further cell division. We corroborated this by evaluating cell cycle progression and spindle formation in synchronous liquid cultures at 32.5 °C without DNA damage, as more than 70% of *spc42-10* cells failed to progress through anaphase and experienced a uniform cell cycle arrest in metaphase with undivided nuclei and monopolar spindles (*SI Appendix*, Fig. 2.S10). The terminal phenotype of *spc42-10* SPB mutant is clearly distinct from that of *nud1* mutants, which can undergo consecutive cell cycles and form quantifiable microcolonies in the absence of DNA damage. Taken together, these results indicate that the DNA damage-induced microcolony phenotype observed with PBD-binding mutants of Nud1 (alone or in combination with *spc110-7* and *spc72-8*) reflects a specific role for this protein in adaptation to DNA damage.

Cdc5 Association With SPBs Is Impaired in Adaptation-Defective SPB Mutants.

Since PBD-mediated enrichment of Cdc5 at the SPB is essential to promote checkpoint adaptation (23) and the SPB triple mutant described above is defective for this pathway, we next asked whether the reduced number of available PBD-binding sites in this strain impairs Cdc5 recruitment at SPBs. Typically, Cdc5 enrichment at SPBs begins in S phase to support SPB duplication and peaks in mid-mitosis, where Cdc5 colocalizes with 1 or both SPBs to promote mitotic events and cell division (22). To test whether the SPB triple mutant maintains a typical mitotic enrichment of Cdc5 at SPBs, we monitored GFP-Cdc5 localization

at the SPB in wild-type, *nud1-92*, and *nud1-92 spc110-7 spc72-8* triple mutant strains expressing the SPB marker Spc29-RFP. We assessed Cdc5-SPB colocalization before DNA damage, in presence of DNA damage, and after recovery from DNA damage by transiently inactivating *cdc13-1* at 31 °C. Whereas control and *nud1-92* strains showed typical SPB behavior and Cdc5 colocalization, the triple mutant exhibited severe Cdc5 delocalization and supernumerary Spc29-RFP foci, possibly indicating SPB fragmentation (Fig. 2.4 and *SI Appendix*, Fig. 2.S15*C*). These defects, extending to at least 50% of triple mutant cells, encompassed Cdc5 delocalization in the form of diffuse Cdc5 cellular signal (with or without SPB fragmentation), mislocalization of Cdc5 in puncta independent from SPBs (Fig. 4*B*), or supernumerary Spc29-RFP foci with Cdc5 colocalization (*SI Appendix*, Fig. 2.S15*C*) (see *SI Appendix*, Fig. 2.S11 for classification). Impaired Cdc5 colocalization did not correlate temporally with the DDR, as the atypical phenotypes were observed both before and after DNA damage induction. Importantly, the adaptation and SPB colocalization defects of the *nud1-92 spc110-7 spc72-8* mutant recapitulated the behavior of the *cdc5-16* PBD binding mutant (23) (*SI Appendix*, Fig. 2.S12 *A-B* and *SI Appendix*, Fig. 2.S15*A*). Consistent with the functional overlap of multiple MTOC factors in facilitating Cdc5 enrichment at SPBs (29, 30), a *nud1-28* mutant alone was unable to phenocopy the Cdc5 delocalization phenotype of the *nud1-92 spc110-7 spc72-8* triple mutant and showed a colocalization behavior mirroring that of the *cdc13-1* control (*SI Appendix*, Figs. 2.S12 *C* and *D* and 2.S15*B*). The fact that Cdc5-SPB mislocalization depends on the loss of multiple SPB components dovetails nicely with the observation that Cdc5 enriches at two spatially distinct sites on SPBs; namely, the inner (Spc110) and outer (Nud1/Spc72) plaques of the organelle (36). Together, these results indicate that PBD-mediated enrichment of Cdc5 at the SPB is a precondition to maintain SPB function during adaptation to DNA damage.

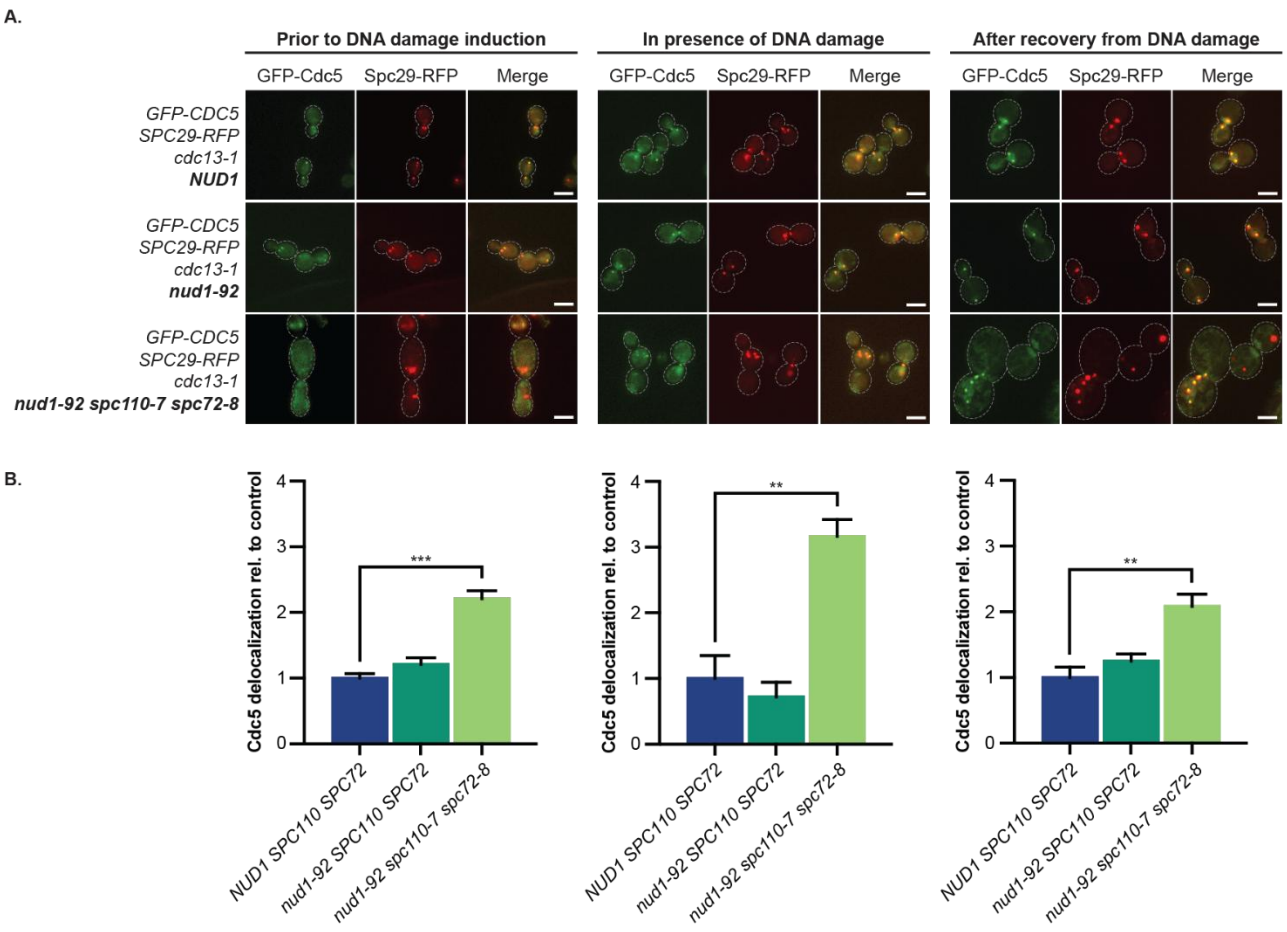

Figure 2.4. Cdc5 enrichment at SPBs depends on the PBD-binding sites of Nud1, Spc72, and Spc110. (A) A timecourse experiment was performed with yeast cells carrying *cdc13-1, GFP-CDC5*, and *SPC29-RFP* to assess Cdc5 localization at SPBs. Control cells as well as a *nud1-92* single mutant and a *nud1-92 spc110-7 spc72-8* triple mutant were analyzed prior to DNA damage induction (Left panel, room temperature), during (Middle panel), and after recovery from DNA damage (Right panel, room temperature). Mitotic cells were monitored for SPB morphology and Cdc5 colocalization with Spc29-RFP. Significant delocalization of Cdc5 away from SPBs was observed in the triple mutant. Genotypes corresponding to each strain are shown on the Left side. Scale bars correspond to 5 µm. Outlines of single cells marked with a dashed line. **(B)** Quantification of the fraction of mitotic cells showing Cdc5 delocalization for the experiment shown in panel A. Phenotypes categorized as Cdc5 delocalization included: diffuse Cdc5 cellular signal (with or without supernumerary Spc29/Spc72 foci), and mislocalization of Cdc5 in GFP puncta unrelated to SPBs. See SI Appendix, Fig. S15C for a quantification of cells displaying supernumerary Spc29 foci with Cdc5 colocalization. A minimum of 100 cells per strain was counted for each time point. Data expressed as fold change in Cdc5 delocalization relative to the *cdc13-1* control group and represented as mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (**P ≤ 0.01 and ***P ≤ 0.001, respectively).

SPB Components Cnm67 and Mps3 Are Substrates of Cdc5 In Vivo.

We next asked whether SPB proteins are substrates of Cdc5 kinase during the adaptation response to DNA damage. To achieve this goal, we mined phosphorylation datasets (37) and analyzed SPB proteins for the presence of consensus motifs for Cdc5 kinase activity (38). Results from this analysis revealed the outer plaque component Cnm67 and the nuclear envelope (NE) protein Mps3 as putative substrates for Cdc5. Both Cnm67 and Mps3 share a key function in SPB dynamics by regulating SPB assembly (39) and SPB duplication events (40), respectively. To assess whether Cdc5 phosphorylates Cnm67 in vivo, we monitored the electrophoretic behavior of HA-tagged Cnm67 (Cnm67-3xHA) during a synchronous round of cell division in the presence and absence of functional Cdc5 kinase activity. We used a ts allele of Cdc5 defective for substrate phosphorylation (i.e., *cdc5-99*) (41) as well as a *cdc15-2* control (42) to allow effective comparison of the phosphorylation levels observed in each strain (i.e., both mutants have a terminal arrest in telophase). As seen in Fig. 2.5*A*, the hyperphosphorylation shift normally detected on Cnm67-3xHA in late mitosis (120 min; *Left* panel) was absent in the kinase-defective *cdc5-99* strain (*Right* panel), leaving only Cdc28-mediated priming phosphorylation events on Cnm67-3xHA (43). Next, we created the *cnm67-16A* mutant, a phospho-defective allele that has lost all Cdc5 consensus sites for phosphorylation (38) (Fig. 2.5*B*). Timecourse analyses of Cnm67-3xHA and Cnm67-16A-3xHA electrophoretic behavior revealed that the Cnm67-16A mutant is completely defective for phosphorylation-induced gel retardation relative to the wild-type protein (Fig. 2.5*C*).

As we showed that Nud1, Spc110, and Spc72 behave as Cdc5 recruitment factors at SPBs and that Cnm67 is dynamically modified in situ upon Cdc5 enrichment, we asked whether a *nud1-92 spc110-7 spc72-8* SPB triple mutant would display reduced Cnm67 phosphorylation as a result of lower Cdc5 docking at SPBs. To test this, Cnm67-3xHA phosphorylation kinetics were monitored in control and SPB triple mutant strains at 31 °C. Remarkably, Cnm67 remained largely unphosphorylated throughout the entire duration of the timecourse in the *nud1-92 spc110-7 spc72-8* SPB triple mutant (i.e., unPP), contrasting with the rapid

accumulation of Cnm67 hyperphosphorylated species (i.e., hyperPP) visible in a control strain bearing functional PBD-binding sites on SPBs (Fig. 2.5*D*). This result further substantiates our hypothesis and suggests that Cnm67 is a target of Cdc5 that becomes dynamically modified following Nud1-, Spc110-, and Spc72-mediated recruitment of Cdc5 at SPBs.

Similar results were obtained when performing an analysis of phosphorylation kinetics with HA-tagged Mps3 (Mps3-3xHA). Specifically, a loss of hyperphosphorylation was detected on Mps3-3xHA in late anaphase when Cdc5's KD activity was impaired (*cdc5-99)* (120 min; Fig. 2.5 *E*, *Right* panel). Only basal/Cdc28-mediated phosphorylation remained on Mps3 after inactivation of Cdc5 (see "PP'' isoform in Fig. 2.5). Assessing the electrophoretic behavior of the Mps3-3A phospho-mutant (Fig. 2.5*F*) after SDS-PAGE revealed its inability to undergo hyperphosphorylation, as all detectable Mps3-3A-3xHA species were evenly distributed between an unphosphorylated state (i.e., unPP) and a basal/Cdc28-mediated phosphorylated state (i.e., PP; Fig. 2.5*G*) (44, 45). Taken together, these results show that effective phosphorylation of Cnm67 and Mps3 requires full Cdc5 activity.

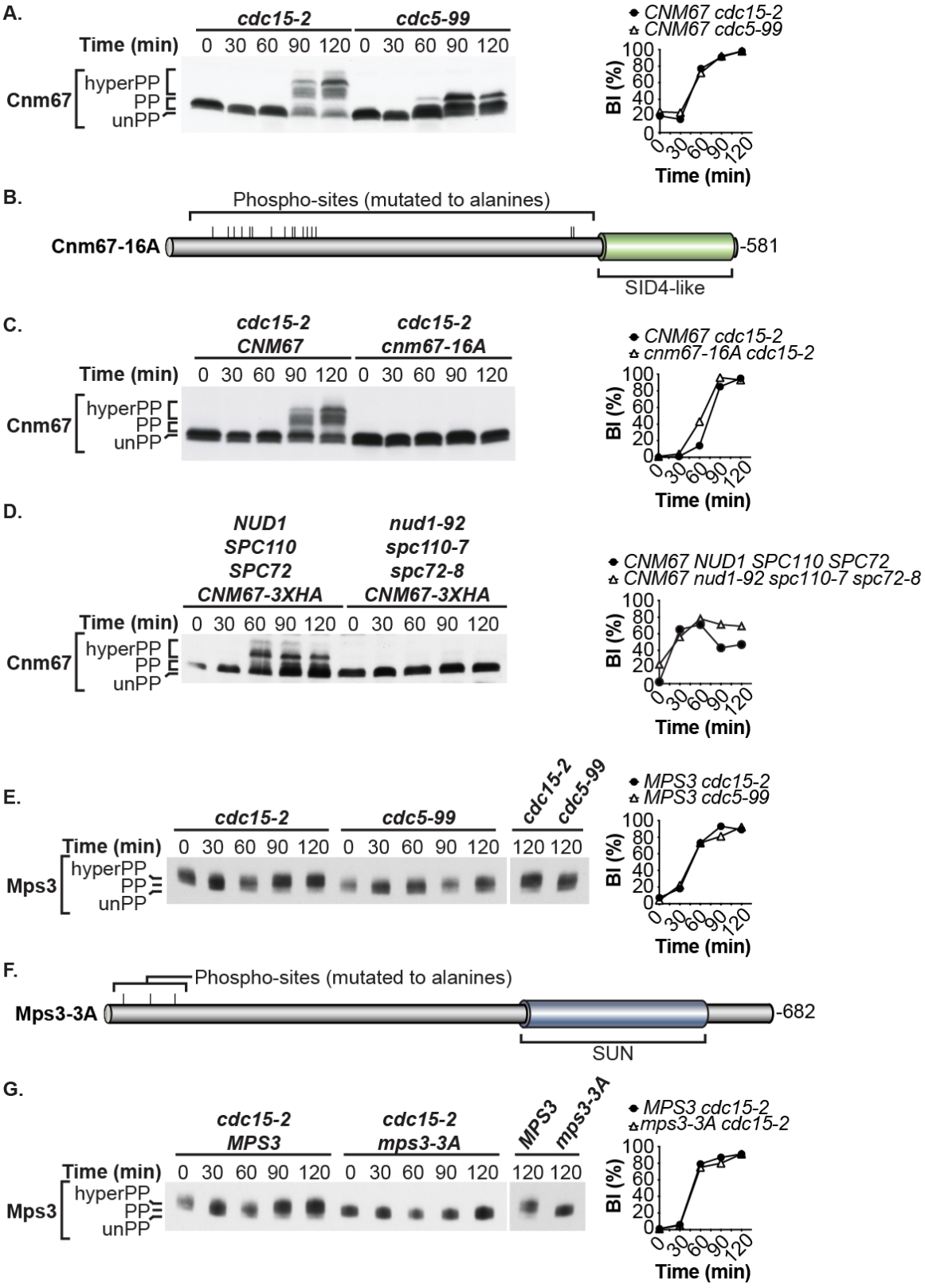
A.
cdc15-2
cdc5-99
Time (min)
0 30 60 90 120 0 30 60 90 120
Cnm67
hyperPP
PP
unPP
CNM67 cdc15-2
CNM67 cdc5-99
BI (%)
Time (min)
B.
Phospho-sites (mutated to alanines)
Cnm67-16A
-581
SID4-like
C.
cdc15-2
CNM67
cdc15-2
cnm67-16A
CNM67 cdc15-2
cnm67-16A cdc15-2
D.
NUD1
SPC110
SPC72
CNM67-3XHA
nud1-92
spc110-7
spc72-8
CNM67-3XHA
CNM67 NUD1 SPC110 SPC72
CNM67 nud1-92 spc110-7 spc72-8
E.
cdc15-2
cdc5-99
120 120
Mps3
MPS3 cdc15-2
MPS3 cdc5-99
F.
Phospho-sites (mutated to alanines)
Mps3-3A
-682
SUN
G.
cdc15-2
MPS3
cdc15-2
mps3-3A
MPS3
mps3-3A
MPS3 cdc15-2
mps3-3A cdc15-2

Figure 2.5. SPB components Cnm67 and Mps3 are substrates for Cdc5 in vivo. (A) Impact of Cdc5 kinase on Cnm67 phosphorylation. Cells synchronized in G1 with α-factor were released into a synchronous cell cycle at restrictive temperature for *cdc15-2* and *cdc5-99* (37 °C) and samples were collected at regular intervals to monitor the electrophoretic behavior of HA-tagged Cnm67. The control yeast strain carries the *cdc15-2* mutation to elicit a telophase arrest similar to that of *cdc5-99* mutants (41). Loss of Cnm67 hyperphosphorylation, as evidenced by the faster migration of the protein after SDS-PAGE, was observed in absence of functional Cdc5 kinase activity. Budding index was monitored to assess the synchrony of cell cycle progression in cultures of *cdc15-2* and *cdc5-99* mutants (i.e., graph on the right of the immunoblots). A total of 100 cells per strain was counted for each time point, and a minimum of 3 independent experiments were performed. A representative immunoblot with corresponding budding index analysis is presented. **(B)** Schematic representation of phosphorylation site mutations inserted in Cnm67-16A protein. Key structural domains and position of mutations (vertical lines) are represented to scale. **(C)** Cnm67 and Cnm67-16A phosphorylation dynamics were compared in *cdc15-2* strains progressing synchronously in the cell cycle. Assessment of phosphorylation and budding index were carried out as described in panel A. All detectable forms of phosphorylation were lost on Cnm67-16A-3xHA. **(D)** Comparison of Cnm67 phosphorylation dynamics in control and *nud1-92 spc110-7- spc72-8* mutant cell populations growing synchronously at 31 °C. Cnm67 phosphorylation-induced gel shift was strongly reduced in the SPB mutant, as evidenced by the presence of largely unphosphorylated/fast-migrating Cnm67 species throughout the time course (i.e., unPP—Right panel). Note that both cell populations in this experiment carried wild-type *CDC15* and were grown at 31 °C, thus explaining the different levels of Cnm67 phosphorylation in the control strain compared to those shown in panels A and C. A minimum of 3 independent experiments were performed. Other details are as described in panel A. **(E)** Mps3 phosphorylation status in the presence and absence of functional Cdc5 kinase. The experiment was performed as in panel A. **(F)** Schematic representation of phosphorylation site mutations inserted in Mps3-3A protein. Other details are as described in panel B. **(G)** Phosphorylation state of Mps3-3A during a synchronous cell cycle. Analysis of Mps3 phosphorylation and cell culture synchrony are essentially identical to the experiment described in panel A. Hyperphosphorylation was lost on Mps3-3A-3xHA, resulting in the protein being evenly distributed between an unphosphorylated state (i.e., unPP), and a basal/Cdc28-mediated phosphorylated state (i.e., PP).

Phosphorylation of Cnm67 and Mps3 Is Required to Induce an Adaptation Response to DNA Damage.

The observation that Cdc5 modulates Cnm67 and Mps3 phosphorylation levels at SPBs suggests that these modifications might be physiologically relevant for the adaptation response to DNA damage. To test this notion, we performed a microcolony formation assay with *cnm67-16A* and *mps3-3A* mutants carrying *cdc13-1* (Fig. 2.6*B*). Both *cnm67-16A* and *mps3-3A* were impaired for microcolony formation in response to DNA damage induction, with a 30 to 40% reduction relative to control after 24 h of growth (Fig. 2.6*C*). The adaptation response to persistent DNA damage is therefore compromised in *cnm67-16A* and *mps3-3A* phospho-mutants. Similar results were obtained when testing microcolony formation in

response to a sustained HO-induced DSB (*SI Appendix*, Fig. 2.S13). Importantly, the *cnm67-16A* and *mps3-3A* phospho-defective alleles do not show detectable proliferation defects under a wide range of temperatures (Fig. 2.6*A*) and remain functional for Cdc5 localization at SPBs (*SI Appendix*, Figs. 2.S14 and 2.S15 *D* and *E*), indicating that they are proficient in the essential MTOC function of SPBs. Therefore, their adaptation defect is mechanistically distinct from the core function of SPBs in microtubule nucleation.

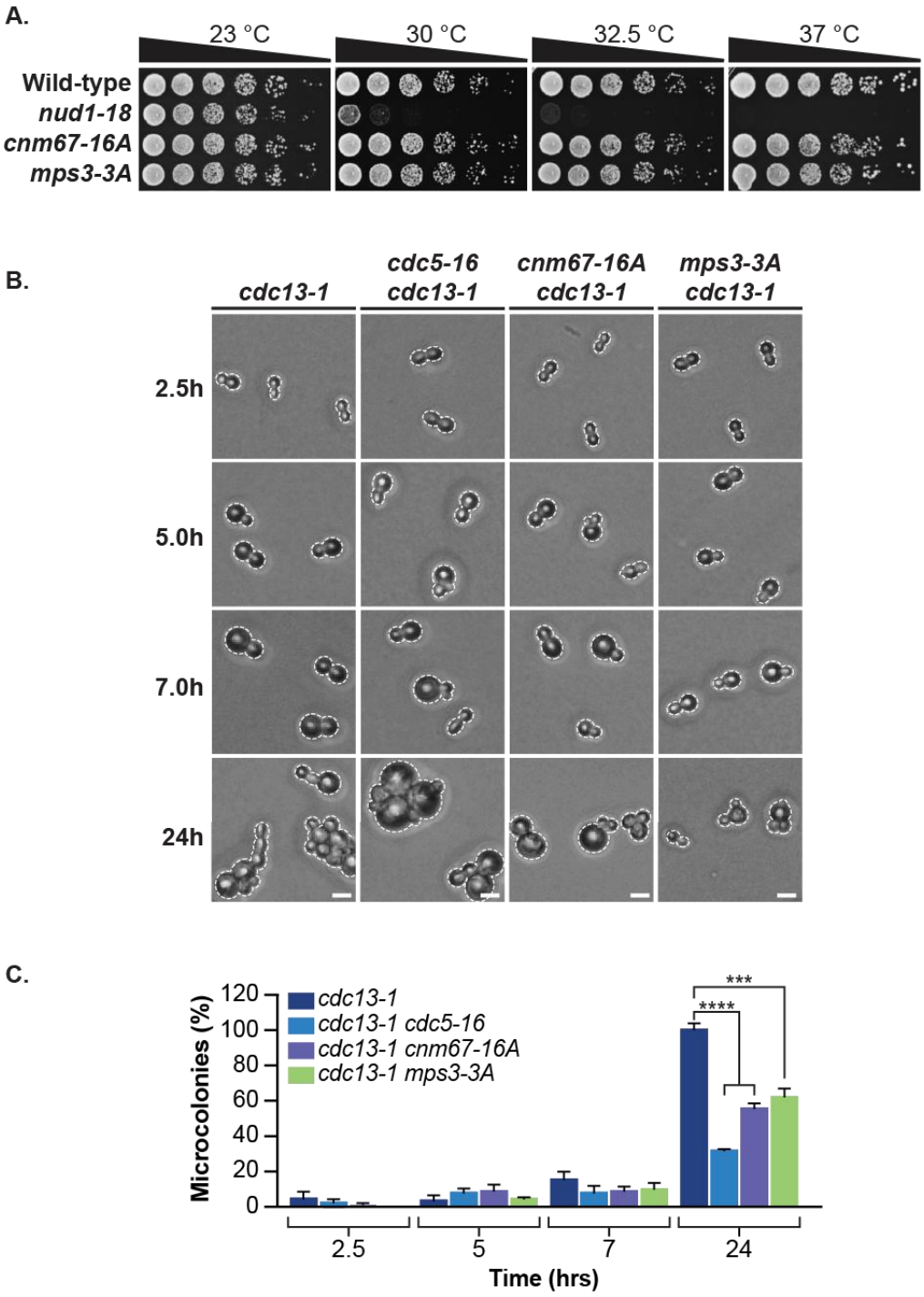

Figure 2.6. Cdc5-dependent phosphorylation of Cnm67 and Mps3 is required to signal adaptation to DNA damage. (A) Proliferation of phospho-mutants at various temperatures was assessed by serial dilution assay on solid media and growth at 23, 30, 32.5, and 37 °C. *cnm67-16A* and *mps3-3A* phospho-mutants proliferated normally at all temperatures tested (n = 3). **(B)** The ability of *cnm67-16A* and *mps3-3A* phospho-mutants to elicit an adaptation response after inducing *cdc13*-dependent/unrepairable DNA damage was assessed at 32.5 °C using a standard microcolony formation assay. *cnm67-16A* and *mps3-3A* both showed defects in adaptation to DNA damage relative to control strain. Scale bars correspond to 10 µm. Outlines of cells and microcolonies marked with a dashed line. **(C)** Quantification of the fraction of individual cells growing into microcolonies for the strains tested in panel B. A minimum of 100 cells per strain was counted for each time point. All values were normalized to 100% based on the T24 h mean value of the *cdc13-1* control strain. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (***P ≤ 0.001 and ****P ≤ 0.0001, respectively).

2.4 DISCUSSION

The capacity of cells to proliferate in the presence of unrepairable DNA damage is an intriguing biological property from the perspective of genome stability and organism homeostasis. While the adaptation pathway promotes cell survival in the short term, it also increases the risk of genome instability, which is an underlying cause of cellular dysfunction and cancer development. In this context, it appears likely that the adaptation response to DNA damage has been finely honed by evolution to optimize cell survival without dramatically increasing cancer occurrence in multicellular organisms. Polo kinases are the most important regulators of this adaptation response in eukaryotes, and their activity is tightly regulated to ensure the careful balance between cell survival and organism homeostasis. We have recently shown that centrosomes are key components of yeast PLK/Cdc5 kinase regulation during the cellular response to DNA damage (23). Until now, the issue of how SPBs contribute to cellular adaptation to DNA damage has remained a key unanswered question. We found in this study that Cdc5 takes advantage of essential SPB components to promote DNA damage adaptation, adding unexpected players and mechanistic insights into our understanding of Cdc5's role in this pathway. In particular, our findings shed light on the nature of Cdc5 interactome at SPBs and highlight how essential processes involved in cell cycle progression are modulated to promote cell survival under challenging conditions for genome stability.

Our study revealed a central role for Nud1 as a platform that coordinates the action of Cdc5 at SPBs during the adaptation response to DNA damage. What might be the extent of Nud1 input in this process? Nud1 may contribute to this pathway by 1) recruiting Cdc5 at SPBs to facilitate transduction of a crucial signal that overrides DNA damage checkpoint activation, and 2) acting as a MEN scaffold that induces mitotic exit when the G2/M checkpoint is silenced and cells become receptive to cell cycle reentry with lingering DNA damage (46, 47). The observation that a hyperactive Nud1 allele (i.e., *nud1-A308T)* can trigger partial spindle assembly checkpoint (SAC) bypass and robust spindle position checkpoint (SPoC) bypass through Cdc5-independent constitutive recruitment of the Hippo-like Cdc15 kinase is consistent with this hypothesis (48). In addition, it is possible that other SPB receptors for Cdc5—like Spc72—would contribute to the adaptation response via their roles in the sequential and asymmetric recruitment of SPB components to the organelle (49).

Results obtained from our genetic interaction analyses with the SPB triple mutant *nud1-92 spc110-7 spc72-8* confirmed that Cdc5 uses more than one recruitment factor to mediate its enrichment at the SPB during DNA damage adaptation. Mutating PBD-binding sites in the triple mutant significantly exacerbated *nud1-92* adaptation phenotype and led to microcolony formation defects equivalent in severity to the ones of the PBD-defective allele *cdc5-16*, without impairing essential mitotic processes. It is noteworthy that the essential function of SPBs in microtubule organization was maintained in *nud1, spc110, and spc72* PBD mutants, likely reflecting a lower concentration/activity threshold required for these proteins to sustain spindle formation relative to the levels necessary to promote DNA damage adaptation. Assessing Cdc5-SPB colocalization in the PBD triple mutant demonstrated that Cdc5 requires a significant number of independent PBD-binding motifs to mediate its effective recruitment to SPBs, implying a multivalent mode of interaction based on weak and/or transient associations with several PBD-binding sites at the organelle. Consistent with this view, Cdc5 localization at SPBs was only impaired when a substantial number of PBD-binding sites were removed from Nud1, Spc110, and Spc72. Importantly, this colocalization

defect was associated with aberrant SPB foci formation (with or without Cdc5 colocalization), diffuse Cdc5 cellular signal, and mislocalization of Cdc5 in aggregates distinct from SPBs. Together, these observations suggest that multisite recruitment of Cdc5 at SPBs is important to mediate and/or modulate the function of PLKs at centrosomes.

As Nud1 behaves as a sensor for cytoplasmic microtubule organization throughout the cell cycle and actively contributes to mitotic exit via the MEN (50), it is tempting to speculate that Cdc5 might fine-tune cell cycle progression by interacting with a fixed group of SPB components to coordinate spatial cues with the timing of mitotic events both under normal conditions and upon checkpoint activation. The identification of Spc110 and Spc72 as additional Cdc5 SPB scaffolds/interactors during DNA damage adaptation supports this notion. While Spc110 regulates mitotic spindle formation (51) and promotes timely mitotic exit (52), Spc72 is a key SPB component required for spindle function (53) and SPoC regulation (54). Cdc5 also phosphorylates the SAC factor Mad3 at kinetochores under normal conditions and especially upon SAC activation (55), suggesting that the pattern of Cdc5 spatiotemporal enrichment across the cell might remain the same regardless of checkpoint stimulus. Such a biological system would facilitate Cdc5 dynamic activity in promoting mitotic progression regardless of cycling conditions, or the nature of the checkpoint involved.

The finding that Cdc5-mediated phosphorylation of the conserved Sad1-UNC-84 (SUN) nuclear envelope protein Mps3 is important for the adaptation response points to a potential role for Mps3 in facilitating DNA damage tolerance in cells exposed to persistent DNA damage. Aside from its essential contribution to SPB duplication and organization (40, 56), Mps3 tethers both telomeres and DSBs to the nuclear envelope in a way that supports chromosome movements and genome stability (57–59). As Mps3-mediated tethering of damaged DNA to the nuclear envelope occurs in S and G2 phases of the cell cycle in response to DDR-driven remodeling of chromatin at sites of damage (60, 61), these DSB relocation events are likely to be pivotal in the success of DNA damage adaptation. By spatially sequestering DSBs to the

nuclear periphery, Mps3 inherently reduces chances of aberrant recombination and contributes to the formation of an optimal environment enriched in DNA repair factors (62). This physical compartmentalization event may support checkpoint modulation by dampening the intensity of DNA damage signals, consequently promoting cell cycle reentry despite substantial levels of unresolved DNA breaks. In alignment with this, removing the Mps3 N-terminal region in a *mps3Δ75-150* deletion mutant generates increased sensitivity to DNA damaging agents (58). Remarkably, this Mps3-dependent relocation of damaged DNA at the nuclear periphery is contingent on the dynamic Cdc14-mediated regulation of Spc110 phosphorylation at SPBs (61), potentially linking DNA damage processing and SPB function to mitotic exit and checkpoint bypass in DNA damage adaptation. The observation that a regulatory event exerted on Spc110 at SPBs is necessary to ensure DSB relocation at perinuclear Mps3 anchorage sites (61) suggests that other factors involved in the maintenance of SPB function may have similar roles during DNA damage adaptation. Consistent with this notion, our work has shown that Mps3 and Cnm67 are substrates of Cdc5 during DNA damage adaptation. Interestingly, our results do not preclude the possibility of a dual role for these SPB components as upstream SPB recruitment factors and downstream targets of Cdc5 in checkpoint bypass. In fact, a *cnm67Δ* deletion mutant was shown to significantly impede rates of Cdc5 enrichment on the organelle (29). Our work with Cdc5 phospho-site mutants has, however, only probed the role of Cnm67 and Mps3 as substrates of Cdc5, not putative receptors. In terms of cellular fitness, Cdc5 multifaceted exploitation of SPB factors as docking structures, structural sensors, and phospho-targets during DNA damage adaptation is expected to enhance the robustness of the adaptation pathway while enabling the integration of elaborate signaling events required to withstand challenging proliferation conditions.

Our work demonstrates that SPBs actively partake in the regulation of cell division under strenuous DNA damage conditions, which falls directly in line with the essential role of these organelles as signal transduction organizing centers (STOCs) (63). Previous work has established centrosomes/SPBs as central hubs capable of coordinating complex signaling events via transient recruitment of kinases, cell cycle

regulators, and cell fate effectors relevant to diverse biological functions (12). In the context of our work, it is remarkable that eukaryotic cells have leveraged SPBs/centrosomes as STOCs to coordinate the complex sequence of events necessary to bypass checkpoint arrest. Cdc5-mediated phosphorylation of the canonical MEN substrate Bfa1 represents another good example of the STOC role of SPBs relevant to DNA damage conditions (46, 47). Taken together with previous work (23), our study establishes centrosomes/SPBs as active participants in cell fate decisions leading to cell cycle reentry in the presence of persistent DNA damage. Our data support a model whereby SPBs act as structural platforms on which spatial and temporal cues relevant to DNA damage are integrated and signaled to downstream cell cycle effectors.

Overall, our findings shed light on the nature of Cdc5 interactome at SPBs and highlight how essential processes involved in cell cycle progression are modulated to promote cell survival under DNA damage conditions. Gaining a better understanding of how PLK function is regulated at centrosomes and its impact on the proliferation of cells carrying unrepairable DNA damage has important ramifications for human health. For instance, the poor prognosis associated with PLK1-overexpressing cancers (64–66) and their acute resistance to radiotherapy (67–69) or drug treatments (70) may be directly linked to the enhanced recruitment of PLK1 observed at centrosomes in cancer cells and tumor tissues (71). It is tempting to speculate that some of the tumorigenic effects associated with *PLK1* overexpression are the consequence of an enhanced adaptation response to DNA damage, similar to the phenotype observed with *CDC5* overexpression in yeast cells. In this context, the development of inhibitors specifically designed to impede PLK1 action at centrosomes might be effective in preventing tumor cell proliferation in the presence of radiotherapy/drug-induced DNA damage. Future work in mammalian cell models will determine whether this therapeutic strategy holds promise for the treatment of human cancers.

Materials and Methods

All yeast strains used in this study are derived from a W303 background (see *SI Appendix*, Table 2.S1 for a detailed list of strain genotypes). For yeast culture and genetic manipulations (including mating, sporulation, and dissection), standard procedures were used (72). For the generation of mutant alleles as well as epitope tagging, PCR amplification and yeast transformation were used as detailed in previous literature (73). Standard conditions were used to conduct microcolony formation assays (6, 7, 33), GFP/RFP/CFP/tubulin-specific microscopy (25), as well as electrophoresis and immuno-blotting procedures (41, 74, 75). All quantifications in this study represent the means ± SEM of at least three independent replicates. GraphPad Prism 9 for MacOS (version 9.5.1) was used to process data and run all statistical tests. An extensive list of all materials and methods can be found in *SI Appendix*, *Supplemental Experimental Procedures*.

Data, Materials, and Software Availability

There are no data underlying this work.

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Author contributions

L.L.-L. and D.D. designed research; L.L.-L. performed research; L.L.-L. analyzed data; and L.L.-L. and D.D. wrote the paper.

Competing interests

The authors declare no competing interest.

2.5 MATERIALS AND METHODS

Supplemental Experimental Procedures

Yeast proliferation assays. Strains were incubated overnight at room temperature (23 °C), diluted to 0.25 OD_{600nm} the next morning and grown until they reached exponential phase at permissive temperature (23 °C). Five-fold dilution series with the first dilution corresponding to an OD_{600nm} of 0.2 were spotted on solid YEPD medium (yeast extract, peptone, D-glucose) and grown in temperature-controlled incubators (Binder) for 30-168 hours prior to scanning and processing.

Microcolony formation assays. To monitor yeast cell adaptation to unrepairable DNA damage, we performed microcolony assays under DNA damage conditions induced by inactivation of the telomere-capping protein Cdc13 (i.e., *cdc13-1*) at restrictive temperature (between 31 °C and 33 °C) (1). Cells were inoculated in liquid YEPD medium, grown at room temperature overnight and diluted to 0.25 OD_{600nm} the next morning. After reaching mid-log phase (0.3 OD_{600nm}), cultures were transferred into a shaking water bath at restrictive temperature (T=0 h) for a duration of 2 hours to activate the DNA damage checkpoint in response to *cdc13-1* inactivation. Then, each culture was plated on the surface of 4 YEPD plates (one for each time point analyzed) in a temperature-controlled room to maintain DNA damage-inducing conditions. Stacks for each tested strain comprised 4 plates (plates: T=2.5 h, T=5 h, T=7 h, T=24 h) and were incubated at restrictive temperature for the indicated time frame. Cell populations in each plate were assessed for DNA damage adaptation at its corresponding time point. Cell morphology was documented using digital microscopic photography on a tetrad dissection microscope (Nikon Eclipse 50i) equipped with a DS-Fi1 camera (Nikon). Raw images were adjusted using the Fiji/ImageJ software (National Institutes of Health) to

optimize brightness and contrast. Microcolonies were defined as an interconnected group of at least 5 cell bodies with less than half of the cells displaying a diameter above 10 µm. A minimum of 100 cells per strain was counted in each replicate. In all cases, a minimum of three independent experiments were performed. In microcolony assays in which CDC5 was overexpressed, the same experimental procedure was used with a minor modification. Cells were incubated overnight in raffinose-containing medium (YEPR – 2%) and diluted in YEPR the next morning. Cells were plated on solid YEP medium containing 2% galactose (YEPG).

In microcolony assays using the HO endonuclease, cells were genetically modified to generate a sustained double-strand break using an inducible *GAL1* promoter to modulate HO expression and activity (*PGAL1-HO*) (2). In these assays, cultures of cells were grown overnight at room temperature and diluted the next morning at 0.25 OD_{600nm} in fresh YEP-2% raffinose medium. For *nud1* mutants, cells were grown until they reached mid-log phase, after which each culture was directly plated on the surface of solid YEP-2% galactose medium (T=0 h). Plates were incubated at 30 °C for the entire duration of the assay and monitored for microcolony formation at various time points (T=2 h, T=8 h, T=24 h, T=30 h). The different duration and sample collection time frames of this adaptation experiment compared to the *cdc13-1* protocol reflect the differences in proliferation kinetics of yeast cells in the presence of galactose and HO-induced DNA damage. For *cnm67-16A* and *mps3-3A* mutants, cells were maintained at a temperature of 23 °C throughout the HO adaptation experiments. For *cnm67-16A*, cells were placed with a dissection needle in a grid-like arrangement on the surface of solid medium and monitored for adaptation rates (2). The same plate was monitored at each time point to assess microcolony formation. Prior to adaptation tests, "micro-manipulation" survival ratios were established for the control strain and *cnm67-16A* mutant by plating 100 cells of each strain with a dissection needle on solid YEPD media (i.e., without DNA damage) and assessing microcolony formation after 6 hours of growth. This control was necessary to determine if yeast strains were sensitive to needle manipulation. Survival to needle manipulation was defined as the mean value of the percentage of cells surviving needle plating from three independent experiments. Values obtained in

the adaptation tests were normalized to the micro-manipulation survival ratio to accurately reflect rates of adaptation. Survival rates in response to the physical stress induced by the dissection needle in *nud1* and *mps3-3A* mutants were too low to yield quantifiable survival values. To circumvent this limitation, *nud1* and *mps3-3A* mutants were plated onto solid medium using a traditional carpet-plating technique and analyzed for adaptation to persistent DNA damage as previously described.

When applicable, values were normalized to 100% based on the T24 h or T48 h mean value of the control strain/strain showing the highest mean value to maintain comparable results across experiments and facilitate visual comparisons between figures. Figure legends are reflective of data normalization when applied.

Protein phosphorylation analysis. The phosphorylation levels of Cnm67-3xHA, Cnm67-16A-3xHA, Mps3 3xHA, Mps3-3A-3xHA and Rad53-3xHA were assessed after electrophoresis and immuno-blotting (3). Total cell extracts for each time point were processed using the TCA glass beads method (4) and loaded on a 10% homemade low-bis 150:1 ratio gel (5), or a 8% SDS-page for Rad53-3xHA (6). Proteins were transferred onto a PVDF membrane using a wet transfer procedure and visualized by immuno-blotting using the mouse monoclonal antibody 12CA5 (Cnm67-3xHA, Cnm67-16A-3xHA and Rad53-3xHA) (Roche, #11583816001) at a dilution of 1:5000 in PBS-T supplemented with 3% milk, or the 16B12 epitope (Mps3-3xHA and Mps3-3A 3xHA) at a dilution of 1:2500 in PBS-T supplemented with 3% milk. When applicable, small samples of each cell culture were collected (in parallel to samples dedicated to protein analysis) to determine the budding index (BI) as cells progressed in the time course experiment. Specifically, fractions of the cell cultures (10 ml per time point) were pelleted and resuspended in 250 µl 20% TCA. A volume of 50 µl was aliquoted into a separate tube, pelleted and resuspended in 1 ml 70% ethanol (dedicated to BI counting) while the remaining 200 µl was processed for electrophoresis and immuno-blotting using the TCA glass beads method. The

fraction of budded cells at each time point was counted on a light microscope (Nikon *Eclipse 50i*), as previously described (3). In all cases, a minimum of three independent experiments were performed.

Cell separation assay. To assess cell separation (i.e., mitotic exit and cytokinesis), control and mutant yeast cells carrying the *cdc15-as1* (L99G) allele were inoculated in liquid YEPD medium at room temperature and diluted to 0.25 OD_{600nm} the following morning. To synchronize cells in telophase, 1NM-PP1 (Sigma-Aldrich, #529606-1MG) was added to yeast cultures grown to mid-log phase (0.4-0.5 OD_{600nm}) at a final concentration of 10 µM. Cell cultures were incubated to reach synchrony at room temperature for a total duration of 3 hours, washed 3 times with pre-warmed YEP medium (32.5 °C), and released at 32.5 °C in fresh pre-warmed YEPD medium containing 5 µm α-factor. To prevent yeast escape from the G1 arrest, α-factor was re-added to liquid cultures 60 minutes after telophase release at a concentration of 2 µm. T0 and T90 minutes aliquots (1 ml) were centrifuged and fixed overnight at 4 °C with 1 ml 70% ethanol. The following day, aliquots were resuspended in 200 µl of a 0.1 M potassium phosphate buffer solution (pH 7) and 5 µl were spotted on a microscope slide for observation. The fraction of budded cells was assessed using a light microscope (Nikon Eclipse 50i) as previously stated. Images were processed and scale bars were added using the Fiji/ImageJ software. A minimum of 100 cells was scored for each time point. The experiment was repeated 3 times.

Fluorescence imaging. To monitor SPB dynamics and Cdc5 colocalization in vivo, Cdc5-GFP and a SPB marker (Spc29-RFP or Spc72-yCFP, as specified) were introduced in mutant strains carrying *cdc13-1*. The colocalization of Cdc5-GFP with an SPB marker was assessed in a time course experiment prior to, during, and after recovery from DNA damage induction. Briefly, cells were grown overnight and diluted at 0.25 OD_{600nm} the next morning at room temperature (23 °C). After two hours at 23 °C, the first sample (T=0 h – before DNA damage induction; 1 ml) was aliquoted and cultures were transferred into a shaking water bath

for 2 hours at restrictive temperature to inactivate *cdc13-1* and generate DNA damage. After 2 hours in the water bath, a second sample (T=2 h – during DNA damage; 1 ml) was collected. Then, cultures were transferred at room temperature and grown for another 2 hours before collecting the final sample (T=4 h – after recovery from DNA damage; 1 ml). Cell culture samples were processed for fluorescence microscopy imaging using a standard procedure. Briefly, cells were pelleted and resuspended in a 0.1 M potassium phosphate solution (pH 6.4) containing 3.7% of formaldehyde. Cells were fixed in this solution for 20 min. Samples were then washed twice with a 0.1 M potassium phosphate solution (pH 7). Final samples were resuspended in 100 µl potassium phosphate buffer (pH 7) and 5 µl was spotted on a microscope slide for observation. Samples were analyzed using a Nikon Eclipse T*i*2 inverted microscope equipped with a 100x/NA 1.45 objective. A minimum of 100 cells was scored for each time point and SPB behavior/Cdc5-SPB colocalization was monitored using *NIS elements* software (Nikon Instruments Inc., NY, USA). Phenotypes categorized as Cdc5 delocalization include: 1) diffuse Cdc5 cellular signal (with or without SPB fragmentation), and 2) mislocalization of Cdc5 in GFP puncta unrelated to SPBs. Phenotypes classified as SPB fragmentation (Figure S15) were defined by the presence of supernumerary (>2) Spc29-RFP or Spc72-yCFP foci that colocalize with cellular Cdc5 signal. Raw images were processed, and scale bars were added using Fiji/ImageJ. In all figures showing colocalization microscopy assays, values for all tested groups were expressed as fold change in atypical localization relative to control group *cdc13-1*. The experiment was repeated 3 times.

For microtubule staining, control and mutant strains carrying the *cdc15-as1* (L99G) allele were grown overnight at 23 °C in liquid YEPD medium and diluted to 0.25 OD_{600nm} the next day. Mid-log cells were synchronized in G1 using 5 µM α-factor, washed 3 times with pre-warmed YEP medium (32.5 °C), and released at 32.5 °C in fresh YEPD medium supplemented with 10 µM 1NM-PP1. Yeast culture aliquots (1.5 ml) were taken at T0 min (G1), T52 min (metaphase) and T120 min (telophase), pelleted, and resuspended in a 0.1 M potassium phosphate solution (pH 7) supplemented with 3.7% formaldehyde. Cells were fixed

overnight at 4 °C, washed 3 times with a 0.1 M potassium phosphate solution (pH 7) and once with an IF sorbitol solution. To digest cell walls, samples were spheroplasted for a duration of 40 minutes at 30 °C using a glusulase- and zymolase-containing IF sorbitol solution. Cells were washed once with an IF sorbitol solution, resuspended in that same solution, and attached onto the polylysine-coated wells of microscopy slides. Slides were prepared for anti–α-tubulin labeling as previously described (3, 7), and 10 µl of a 4',6-diamidino-2-phenylindole (DAPI) Vectashield antifade mounting media solution (Vector laboratories, #H-1200-10) was added to each well prior to sealing. Indirect immunofluorescence imaging of yeast mitotic spindles was performed with an anti α-tubulin antibody [YOL1/34] (Abcam, #ab6161) at a dilution of 1:200 in PBS-BSA. Samples were examined using a Nikon Eclipse T*i*2 inverted microscope. A minimum of 100 cells was scored for each time point. Cells were monitored using *NIS elements* software and processed using the Fiji/ImageJ software, as previously stated. Cells were assessed for budding index, nuclear mass separation and microtubule morphology. Yeast nucleus morphology was classified as mononucleated or binucleated, and microtubule morphology was classified as 1) monopolar, 2) bipolar short, or 3) bipolar long. The experiment was repeated 3 times.

To evaluate the cell cycle behavior of the *nud1-92 spc110-7 spc72-8* triple mutant (Figure S8), SPB tagged strains (*SPC29-RFP*) were monitored for budding index, anaphase progression and nuclear mass division. Growth conditions and cell synchronization/release are as previously described (8). Yeast culture aliquots (1 ml) were taken every 15 minutes after an initial α-factor release for a total of 135 minutes. To prevent cells from entering a new cell division cycle after completion of the first cycle, α-factor was re-added to liquid cultures 60 minutes post-G1 release. Cells were harvested by centrifugation, fixed with 1 ml 70% ethanol for 24 hours, and resuspended in 200 µL of a 0.1 M potassium phosphate buffer solution (pH 7) supplemented with 10 µl of a 4',6-diamidino-2-phenylindole (DAPI) Vectashield antifade mounting media solution (Vector laboratories, #H-1200-10). Samples were left to incubate at 4 °C for at least 20 minutes and 5 µl was spotted on a microscope slide for observation. Assessment of bud morphology and nuclear mass

division was performed on a Nikon Eclipse Ti2 inverted microscope and raw images were processed using the Fiji/ImageJ software as previously stated. The following criteria were used to classify yeast nucleus morphology: 1) mononucleated, 2) binucleated with or without minor anaphase bridges, and 3) abnormal/missegregated nuclei within the same parental bud. 100 cells were scored at each time point. The experiment was repeated 3 times.

Statistical analyses. All statistical analyses were performed using GraphPad Prism 9 for macOS (version 9.5.1). Normal data distribution was assessed using the Shapiro-Wilk test in which a $W \geq 0.7500$ represented data fitting standard normal quantiles. Q-Q plots were analyzed and used as an auxiliary supporting method to evaluate data distribution. To test for statistical significance, an ordinary one-way ANOVA was performed on normally distributed values followed by a Dunnett's multiple comparisons post hoc test to report statistically significant differences between control group and mutants. When only two groups were compared, a *t* test (paired or unpaired, as specified in figure legends) on normally distributed values was performed. Statistical significance was represented as asterisks on graphs (* for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$; **** for $p \leq 0.0001$).

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2.7 SUPPLEMENTARY FIGURES

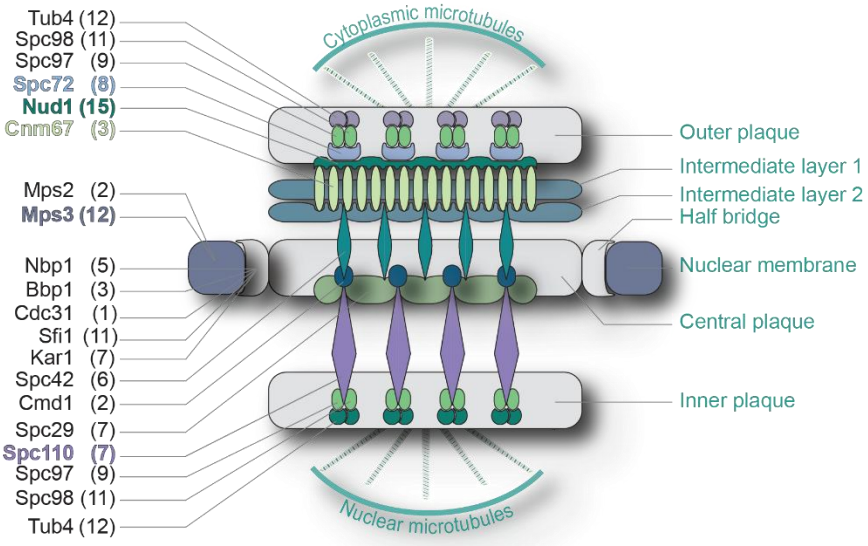

Figure 2.S1. Schematic representation of the yeast SPB and its physical components. Number of consensus binding sites for Cdc5 PBD are reported for each SPB protein (see number in brackets). Subunit arrangement within the SPB is derived from Klenchin (9), Jaspersen and Winey (10), and Kilmartin (11).

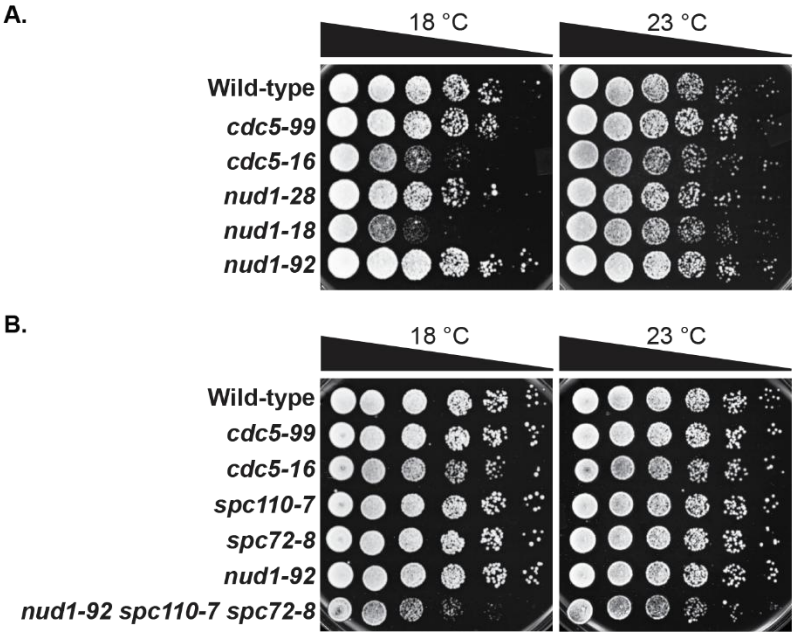

Figure 2.S2. Cell proliferation phenotype of SPB mutants at low temperatures. (A) Cold sensitivity was assessed in *nud1* mutants growing on rich YPD media at 18 °C and 23 °C (n=3). Both *nud1-28* and *nud1-18* showed reduced growth rates at 18 °C relative to control. **(B)** Cold sensitivity of the *nud1-92 spc110-7 spc72-8* SPB triple mutant was assessed at 18 °C and 23 °C (n=3). The SPB triple mutant showed severe cold sensitivity at 18 °C relative to wild-type cells.

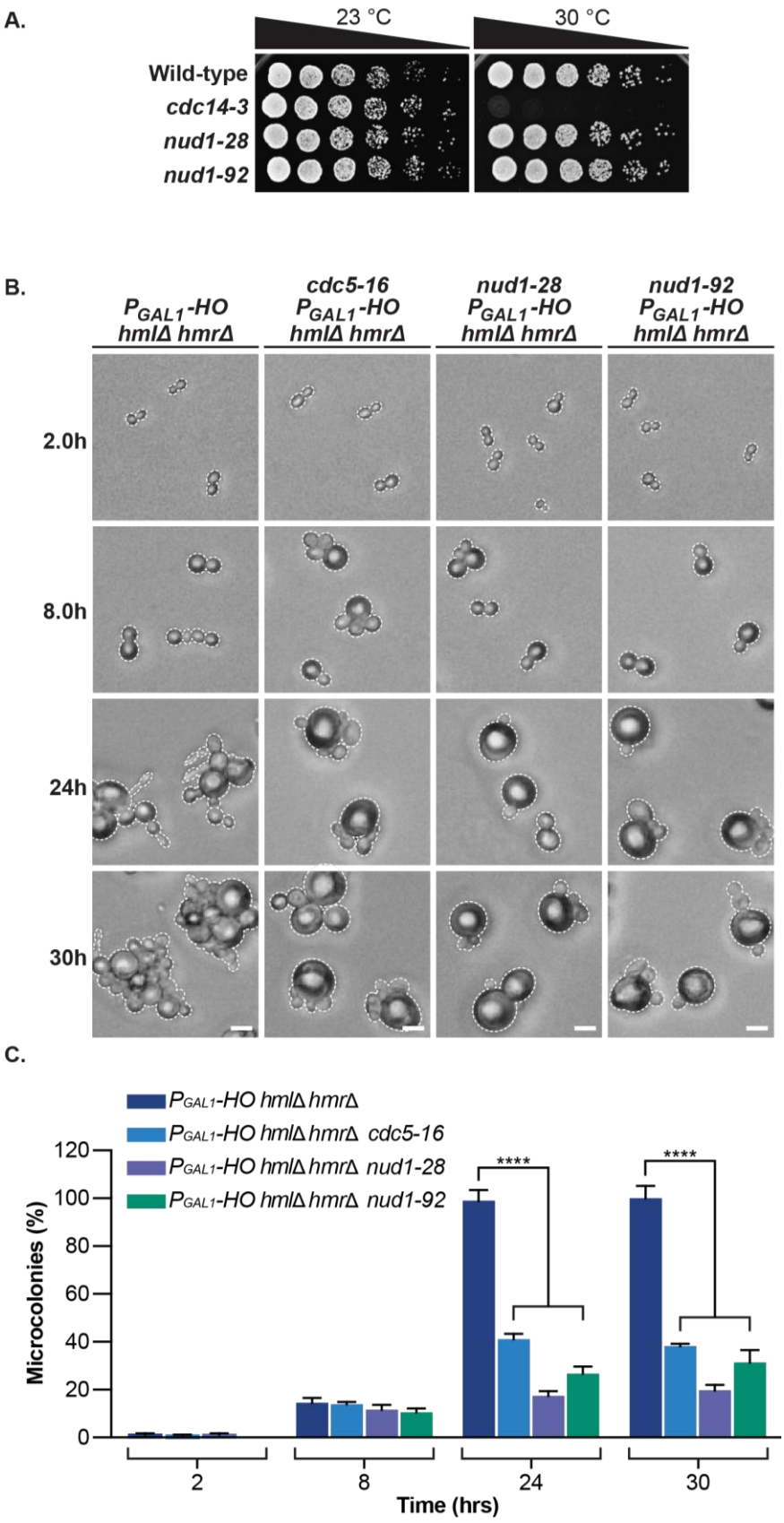
A.
23 °C
30 °C
Wild-type
cdc14-3
nud1-28
nud1-92
B.
PGAL1-HO hmlΔ hmrΔ
cdc5-16 PGAL1-HO hmlΔ hmrΔ
nud1-28 PGAL1-HO hmlΔ hmrΔ
nud1-92 PGAL1-HO hmlΔ hmrΔ
2.0h
8.0h
24h
30h
C.
PGAL1-HO hmlΔ hmrΔ
PGAL1-HO hmlΔ hmrΔ cdc5-16
PGAL1-HO hmlΔ hmrΔ nud1-28
PGAL1-HO hmlΔ hmrΔ nud1-92
Microcolonies (%)
120
100
80
60
40
20
0
****
****
2
8
24
30
Time (hrs)

Figure 2.S3. *nud1* mutants are defective for adaptation to a sustained HO-induced double-strand break (DSB). (A) Proliferation phenotype of *nud1-28* and *nud1-92* mutants spotted on YPD media and incubated at 23 °C and 30 °C. Both mutants proliferated with kinetics mirroring the wild-type strain at those temperatures. The *cdc14-3* mutant was included as a control that displays temperature sensitivity at 30 °C. (n=3) **(B)** Microcolony formation assay performed at 30 °C using overexpression of HO endonuclease (*PGAL1-HO*) as a source of persistent DNA damage. Strains were grown in YEPR (2% raffinose), plated on solid YEPG (2% galactose) to induce DNA damage (see methods for a detailed description of the assay), and incubated at 30 °C for the entire duration of the assay. Rates of microcolony formation following DNA damage induction were significantly lower in nud1 mutants relative to the control strain and mirrored the adaptation rates of the *cdc5-16* PBD mutant. Scale bars correspond to 10 μm. Outlines of cells and microcolonies marked with a dashed line. Differences in genotypes for each strain described in panel headings. **(C)** Quantification of the fraction of cells growing into microcolonies for each strain and time point shown in panel B. A minimum of 100 cells per strain was counted for each time point. All values were normalized to 100% based on the T30 h mean value of the control strain. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (**** indicates $p \leq 0.0001$).

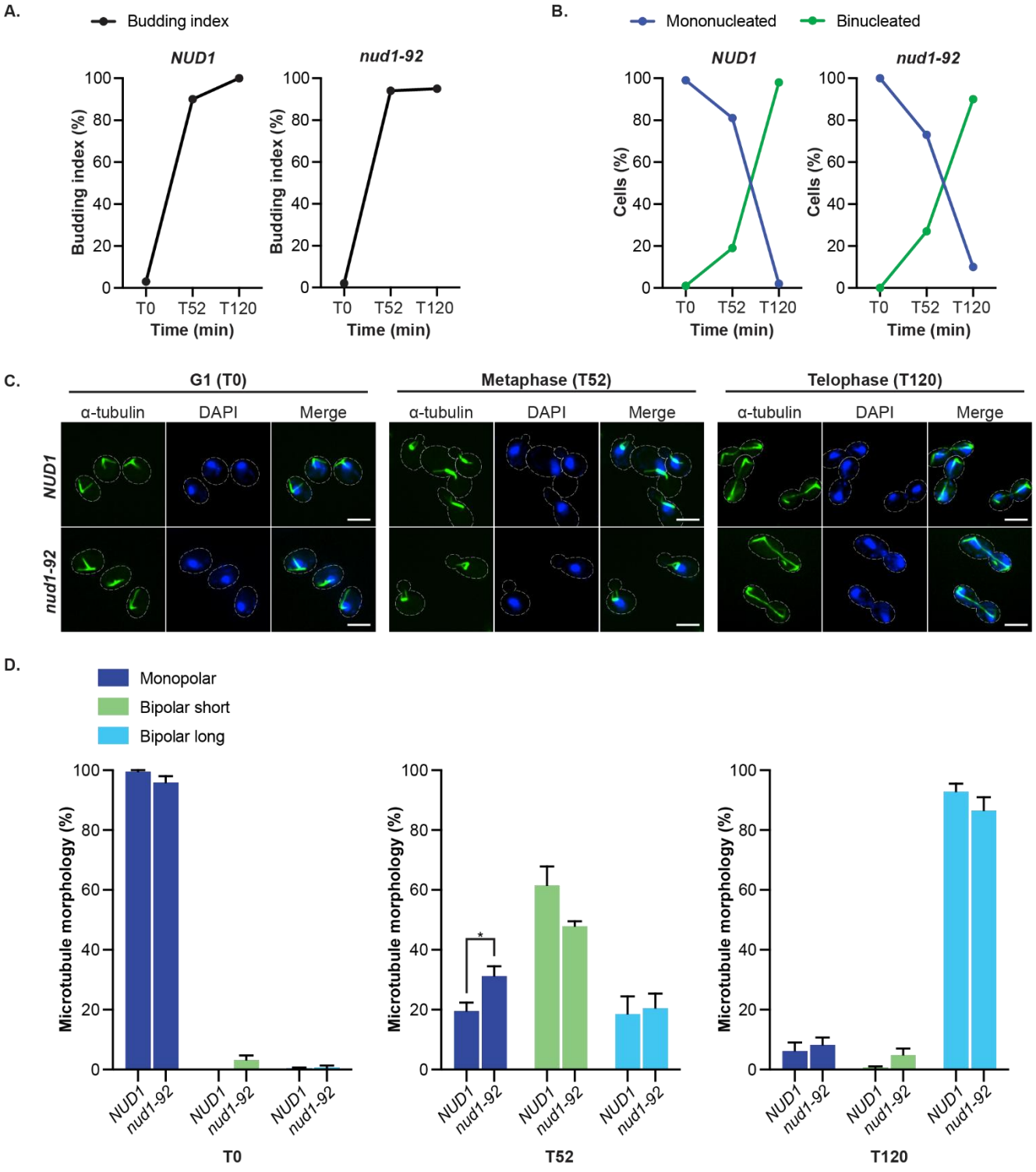
A.
Budding index
NUD1
nud1-92
Budding index (%)
Time (min)
T0
T52
T120
B.
Mononucleated
Binucleated
Cells (%)
C.
G1 (T0)
Metaphase (T52)
Telophase (T120)
α-tubulin
DAPI
Merge
NUD1
nud1-92
D.
Monopolar
Bipolar short
Bipolar long
Microtubule morphology (%)
T0
T52
T120

Figure 2.S4. The *nud1-92* mutant displays normal spindle formation and anaphase progression. Exponential cultures of *nud1-92* mutant and control cells carrying *cdc15-as1* (L99G) were synchronized in G1 at 23 °C using α-factor and released in fresh YPAD medium at 32.5 °C. 1NM-PP1 was added to the release medium at a concentration of 10 μm to impede Cdc15 activity and restrict cells to a single cycle from G1 to telophase. Aliquots were collected at T0 min (G1), T52 min (metaphase), and T120 min (telophase) and analyzed for cell cycle landmarks. **(A)** Analysis of bud formation in control (left panel) and *nud1-92* (right panel) strains across a single cell cycle. Budding kinetics in *nud1-92* mirrored those of the control strain. **(B)** Analysis of nucleus separation in control (left panel) and *nud1-92* (right panel) cells in a single round of cell division. Yeast nuclear morphologies were classified as mononucleated or binucleated. Wild-type cells and *nud1-92* mutants successfully progressed through anaphase. A minimum of 100 cells per strain was counted for each time point. **(C)** Micrographs of representative microtubule and nuclear morphologies for control and *nud1-92* strains tested at the indicated times (T0 min, T52 min, T120 min) during the time course experiment. Nuclei are shown in blue (DAPI) whereas microtubule morphology (α-tubulin) is shown in green (GFP). Outlines of cells were marked with a dashed line. Scale bars correspond to 5 μm. A minimum of 3 independent experiments were performed. **(D)** Quantification of microtubule morphology in control and *nud1-92* strains for the experiment shown in panel C. Microtubule staining was classified in three distinct categories reflecting normal kinetics of cell cycle progression from G1 to metaphase, followed by anaphase/telophase: monopolar organization, short bipolar spindle array, and long bipolar spindle. Spindle formation in *nud1-92* was comparable to control. A minimum of 100 cells per strain was counted for each time point. Data represented as mean values and SEM of at least 3 independent experiments. Statistical analysis performed using an unpaired *t* test on normally distributed values. Statistical significance is shown on graph with asterisks (* indicates $p \leq 0.05$).

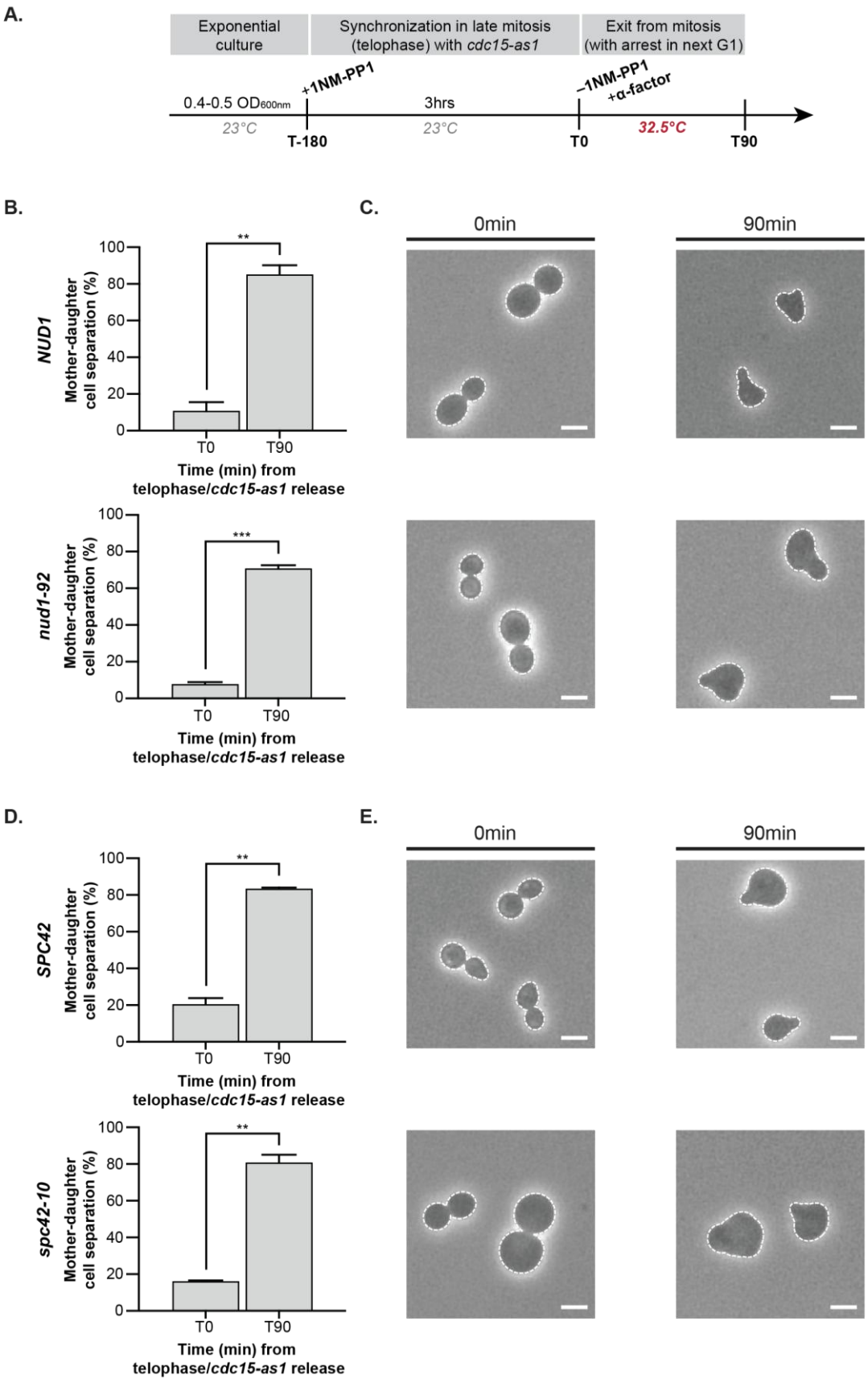
A.
Exponential culture
Synchronization in late mitosis (telophase) with cdc15-as1
Exit from mitosis (with arrest in next G1)
0.4-0.5 OD600nm
23°C
T-180
+1NM-PP1
3hrs
23°C
T0
–1NM-PP1
+α-factor
32.5°C
T90
B.
NUD1
Mother-daughter cell separation (%)
**
T0
T90
Time (min) from telophase/cdc15-as1 release
nud1-92
***
C.
0min
90min
D.
SPC42
spc42-10
E.

Figure 2.S5. Telophase-synchronized/binucleated *nud1-92* and *spc42-10* mutants can complete cell division at 32.5 °C. (A) Schematic representation of experimental details including synchronization, release and sampling points. Exponential cultures of SPB mutants and control cells carrying *cdc15-as1* (L99G) were synchronized in telophase at 23 °C using 10 µm of 1NM-PP1 and released in fresh YPAD medium at 32.5 °C. The release medium was supplemented with α-factor to prevent cell cycle re-entry following cell separation and progression into G1. Aliquots of cells were collected at the indicated times (T0 min and T90 min) and analyzed for cell separation, a phenotype that depends on effective execution of mitotic exit and cytokinesis. **(B)** Analysis of mother/daughter cell separation in control (top panel) and *nud1-92* (bottom panel) cells following release from a telophase/*cdc15-as1* arrest. Cell separation kinetics in *nud1-92* mirrored those of the control strain. Genotypes are indicated to the left of each panel. A minimum of 100 cells per strain was counted for each time point. Data represented as mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a paired *t* test on normally distributed values. Statistical significance is shown on graph with asterisks (** and *** indicates $p \leq 0.01$ and $p \leq 0.001$, respectively). **(C)** Micrographs of representative cell morphology observed in each strain tested at the indicated times (T0 min, T90 min) during the time course experiment. Outlines of cells were marked with a dashed line. Scale bars correspond to 5 µm. A minimum of 3 independent experiments were performed. **(D)** Analysis of cell separation in control (top panel) and *spc42-10* (bottom panel) strains following mitotic exit. *spc42-10* exhibited kinetics of cell separation similar to the control strain. Other details are as described in (B). **(E)** Micrographs of representative cell morphology observed in each strain tested at the indicated times (T0 min, T90 min) during the time course experiment. Other experimental details are as described in the legend to panel (C).

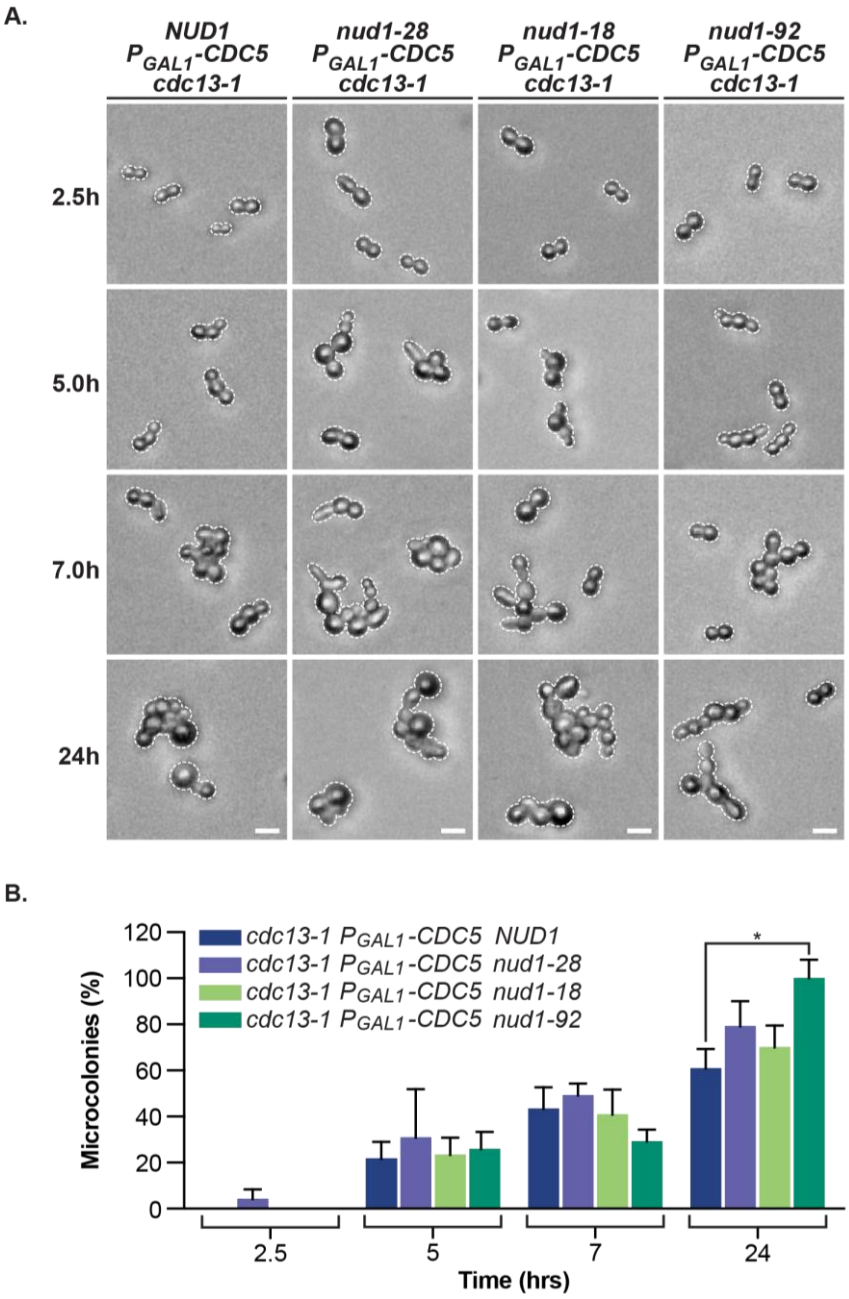

Figure 2.S6. Nud1 operates upstream of Cdc5 in the adaptation response to DNA damage. (A) *nud1* strains overexpressing *CDC5* (*PGAL1-CDC5*) were assayed for microcolony formation in a DNA damage adaptation test using *cdc13-1* inactivation at 32.5 °C. Cells were plated on solid YEP medium containing 2% galactose (YEPG). Overexpression of *CDC5* in *nud1* mutants rescued the adaptation phenotype and led to an abridged G2/M cell cycle arrest. Scale bars, genotype representation and cell outlines are as described in Figure 2. **(B)** Quantification of the fraction of cells growing into microcolonies for each strain tested in panel A. A minimum of 100 cells per strain was counted for each time point. All values were normalized to 100% based on the T24 h mean value of the *PGAL1-CDC5 cdc13-1 nud1-92* mutant strain, as it presented the highest adaptation rates observed in this experimental dataset. Data represented as mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (* indicates $p \leq 0.05$).

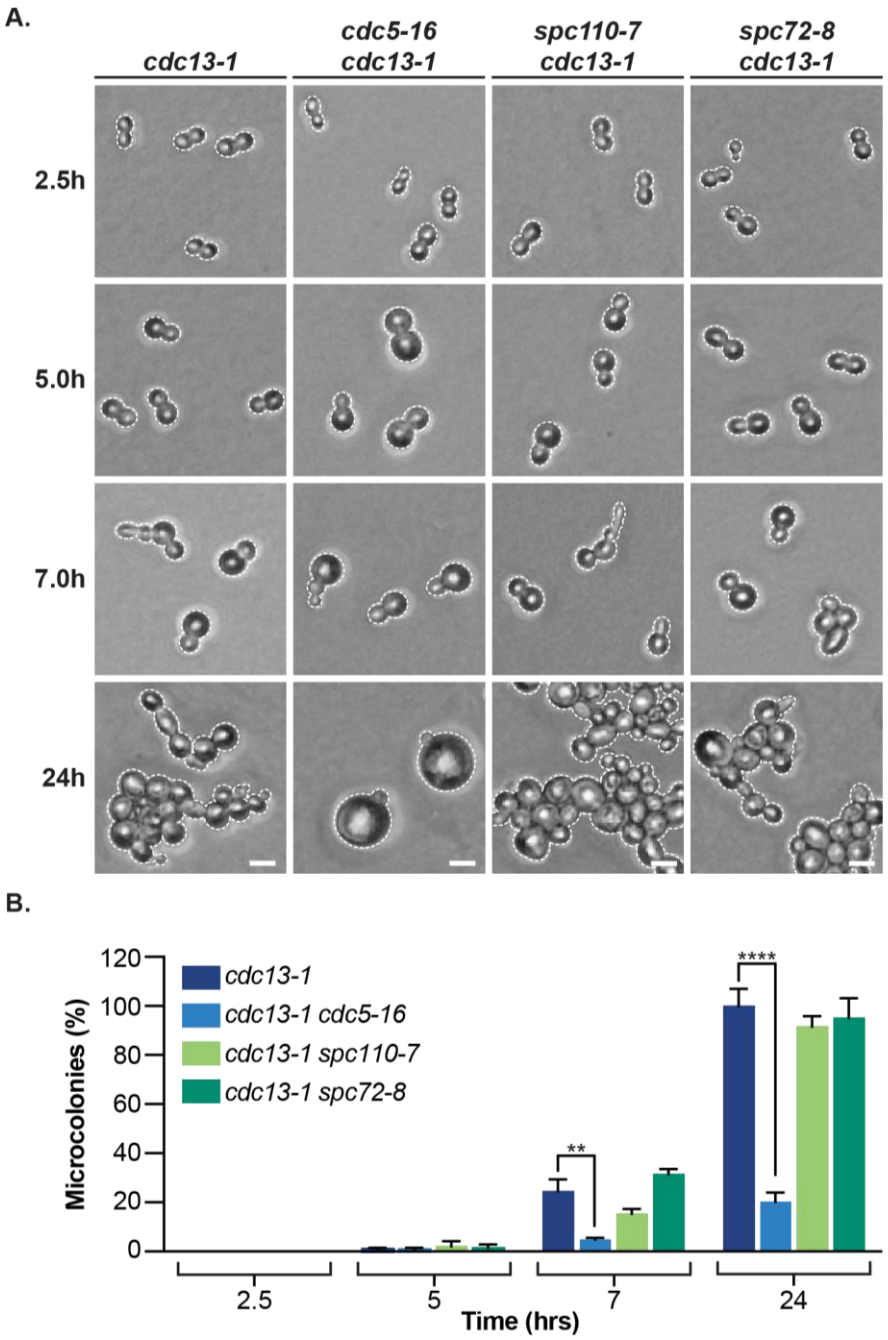

Figure 2.S7. Strains carrying *spc110-7* or *spc72-8* alleles are functional for adaptation to DNA damage. **(A)** Mutant strains carrying *spc110-7* or *spc72-8* were assayed for microcolony formation following DNA damage induction at 31 °C. Both mutants formed microcolonies with kinetics mirroring a *cdc13-1* control. Scale bars, genotype notations and cell outlines are as described in Figure 2. **(B)** Quantification of the fraction of cells growing into microcolonies for each time point in strains tested in panel A. A minimum of 100 cells per strain was counted for each time point. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (** and **** indicate $p \leq 0.01$ and $p \leq 0.0001$, respectively).

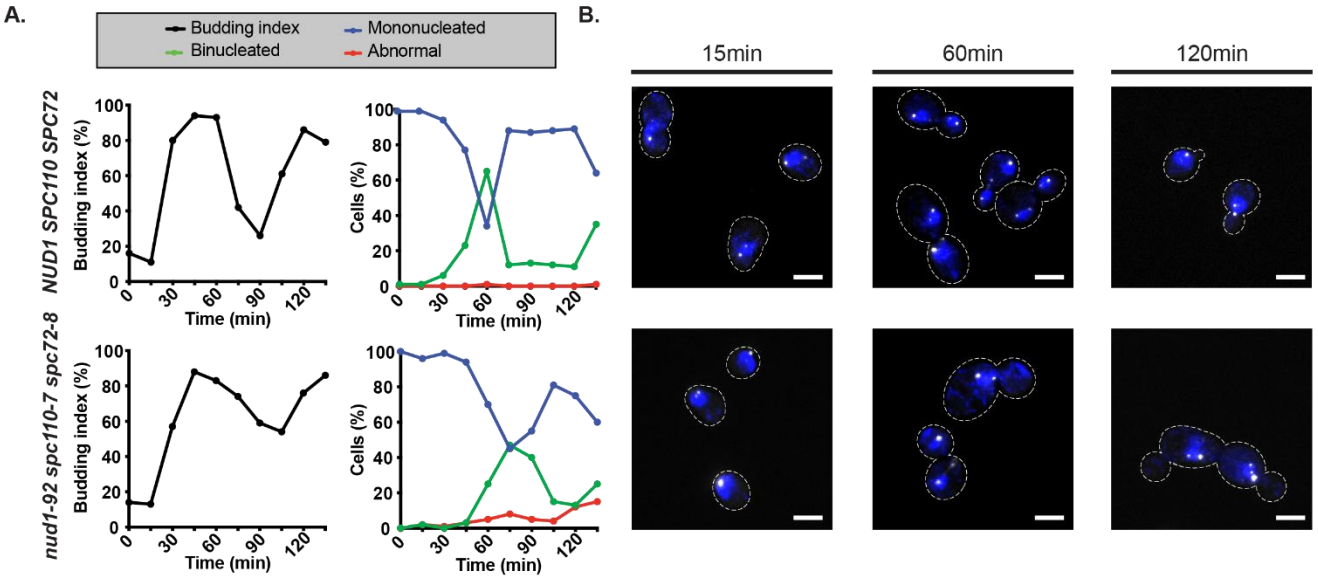

Figure 2.S8. The *nud1-92 spc110-7 spc72-8* triple mutant is functional for anaphase progression. Exponential cultures of *nud1-92 spc110-7 spc72-8* triple mutant and control cells carrying *SPC29-RFP* were synchronized in G1 at 23 °C using α-factor and released into fresh YPAD medium at 31 °C. Aliquots were collected at regular intervals (15 min) and analyzed for cell cycle landmarks. **(A)** Analysis of bud formation (left panel) and nucleus separation (right panel) across a single cell cycle. The *nud1-92 spc110-7 spc72-8* SPB triple mutant progresses through anaphase with a small subset of its population showing a weak cytokinesis delay. **(B)** Micrographs of representative nuclear morphologies for each strain tested at the indicated times (T15 min, T60 min, T120 min) during the time course experiment. Strain genotype labeling and other experimental details are as described in the legend to Figure S4.

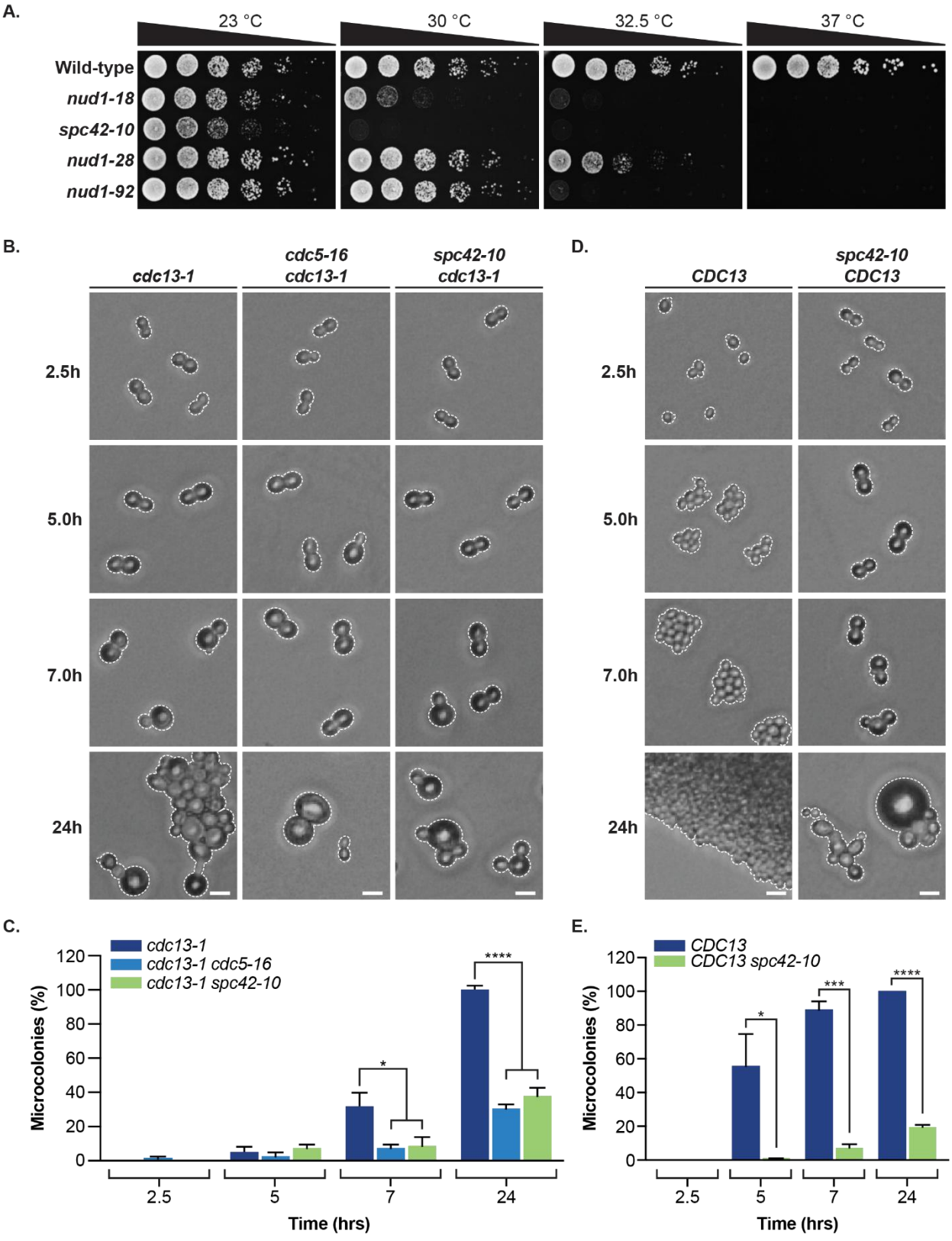
A.
23 °C
30 °C
32.5 °C
37 °C
Wild-type
nud1-18
spc42-10
nud1-28
nud1-92
B.
cdc13-1
cdc5-16 cdc13-1
spc42-10 cdc13-1
2.5h
5.0h
7.0h
24h
D.
CDC13
spc42-10 CDC13
2.5h
5.0h
7.0h
24h
C.
cdc13-1
cdc13-1 cdc5-16
cdc13-1 spc42-10
Microcolonies (%)
120
100
80
60
40
20
0
*
****
2.5
5
7
24
Time (hrs)
E.
CDC13
CDC13 spc42-10
Microcolonies (%)
120
100
80
60
40
20
0
*
***
****
2.5
5
7
24
Time (hrs)

Figure 2.S9. Generic inactivation of MTOC activity is not associated with a specific defect in adaptation to DNA damage. (A) Growth properties of a yeast mutant carrying *spc42-10*. Cell cultures were serially diluted, spotted on solid medium and grown at 23 °C, 30 °C, 32.5 °C and 37 °C. The *spc42-10* mutant demonstrated a severe temperature sensitive growth phenotype. (n=3) **(B)** A yeast strain expressing *spc42-10* was assayed for microcolony formation after inactivation of *cdc13-1* at restrictive temperature (32.5 °C). Scale bars, genotype notations and cell outlines are as described in Figure 2. **(C)** Quantification of the fraction of cells growing into microcolonies for each time point in strains tested in panel B. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using a one-way ANOVA test on normally distributed values with Dunnett's multiple comparisons post hoc test. Statistical significance is shown on graph with asterisks (* and **** indicate $p \leq 0.05$ and $p \leq 0.0001$, respectively). **(D)** Microcolony formation ability of the *spc42-10* mutant growing at 32.5 °C in the absence of DNA damage (*CDC13*). Notice how a global inactivation of SPB/MTOC activity causes a permanent mitotic arrest independently of DNA damage in the *spc42-10* mutant. A similar defect was not observed in *nud1* mutants. **(E)** Quantification of the fraction of *CDC13* cells forming microcolonies at each time point of the experiment shown in panel D. A minimum of 100 cells per strain was counted for each time point. Data represented as mean values and SEM of at least 3 independent experiments. Statistical analysis performed using an unpaired *t* test on normally distributed values. Statistical significance is shown on graph with asterisks (*, *** and **** indicate $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.0001$, respectively).

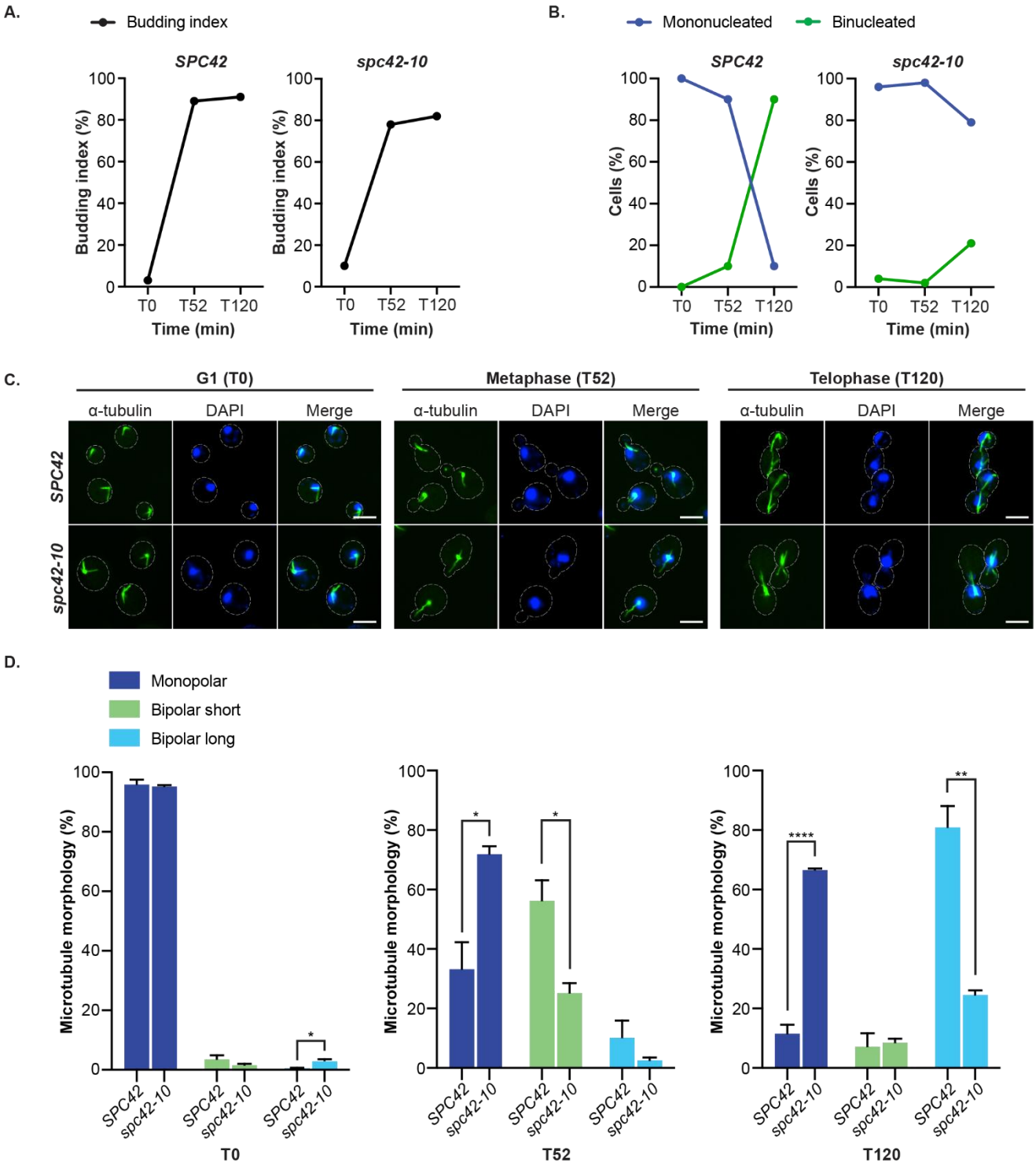
A.
Budding index
SPC42
spc42-10
Budding index (%)
Time (min)
T0
T52
T120
B.
Mononucleated
Binucleated
Cells (%)
C.
G1 (T0)
Metaphase (T52)
Telophase (T120)
α-tubulin
DAPI
Merge
SPC42
spc42-10
D.
Monopolar
Bipolar short
Bipolar long
Microtubule morphology (%)
T0
T52
T120

Figure 2.S10. Defective MTOC function in *spc42-10* mutants induces a terminal cell cycle arrest in metaphase. Exponential cultures of *spc42-10* mutant and control cells carrying *cdc15-as1* (L99G) were synchronized in G1 at 23 °C using α-factor and released in fresh YPAD medium supplemented with 10 µm 1NM-PP1 at 32.5 °C. Other experimental details are as described in the legend to Figure S4. **(A)** Budding index was analyzed over a single cell cycle in strains carrying *SPC42* (left panel) and *spc42-10* (right panel) alleles. The budding kinetics of the *spc42-10* mutant was comparable to that of the control strain. **(B)** Analysis of nucleus separation across a synchronous cell cycle in cultures of cells carrying *SPC42* (left panel) and *spc42-10* (right panel) alleles. At least 70% of cells in the *spc42-10* population experienced a terminal arrest in metaphase with undivided nuclei. **(C)** Micrographs of representative microtubule and nuclear morphologies for control and *spc42-10* strains tested at the indicated times in a time course experiment. Over 70% of cells in the *spc42-10* population failed to form a bipolar spindle. Other details are as described in Figure S4C. **(D)** Quantification of microtubule morphology in control and *spc42-10* strains for the experiment shown in panel C. Statistical significance is shown on graph with asterisks (*, ** and **** indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.0001$, respectively).

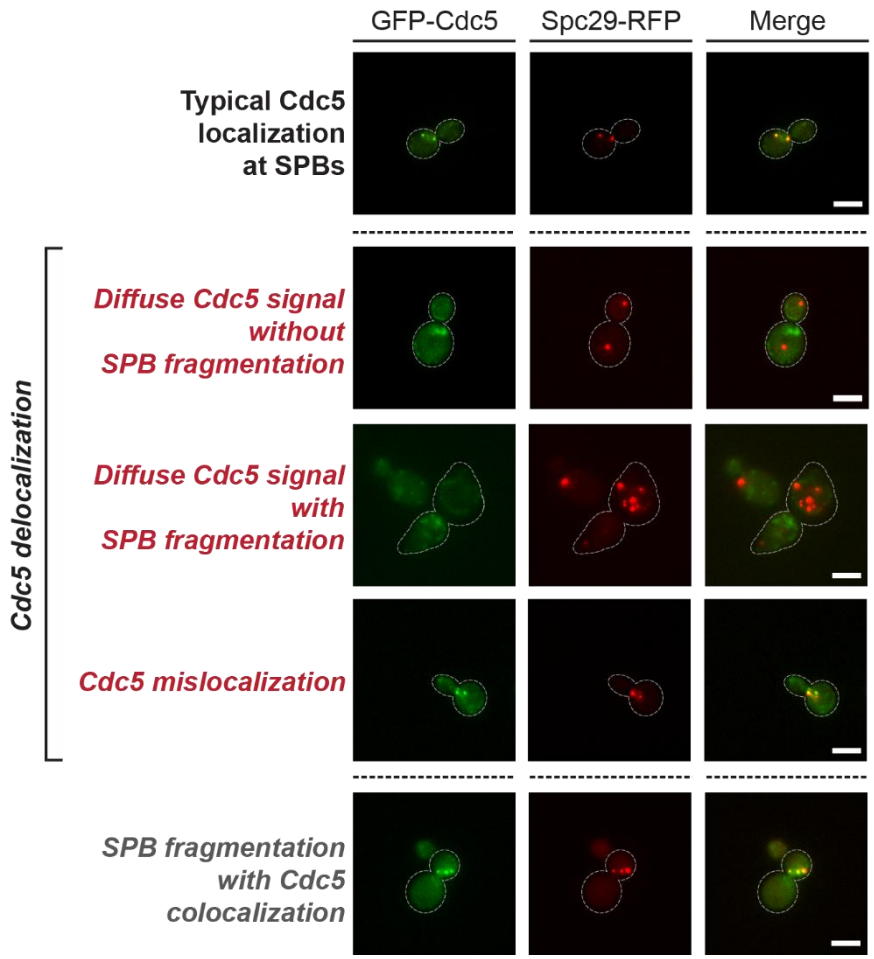

Figure 2.S11. Representative examples of atypical Cdc5 localization phenotypes observed in fluorescence imaging assays. Micrographs show Cdc5 normal localization at SPBs, as well as three distinct Cdc5 delocalization phenotypes, and a final atypical phenotype associated with supernumerary Spc29/Spc72 foci colocalizing with Cdc5, likely reflecting SPB fragmentation. Imaging was performed on cells carrying *cdc13-1*, *GFP-CDC5* and a SPB marker (*SPC29-RFP* or *SPC72-yCFP*, as specified). Top panels: Typical Cdc5 localization at SPBs, defined by 1 or 2 Cdc5 puncta colocalizing with SPBs. Second row of panels: Diffuse Cdc5 signal with otherwise normal SPB appearance, defined by an absence of Cdc5 puncta and 1-2 SPB foci/cell. Third row of panels: Diffuse Cdc5 signal in cells carrying supernumerary SPB signal, defined by an absence of Cdc5 puncta in cells containing >2 Spc29/Spc72 foci. Fourth row of panels: Cdc5 mislocalization, defined by the presence of at least one GFP-Cdc5 punctum formed independently of Spc29/Spc72 foci. Bottom panels: SPB fragmentation with Cdc5 colocalization, defined by the presence of at least three SPB foci (where the two smallest foci are of similar size). In this category, Cdc5 colocalization to supernumerary Spc29/Spc72 foci is observed. For clarity, this last category was quantified separately and is available in Figure S15 for all strains tested. Scale bars correspond to 5 µm. Outlines of individual cells are marked with a dashed line.

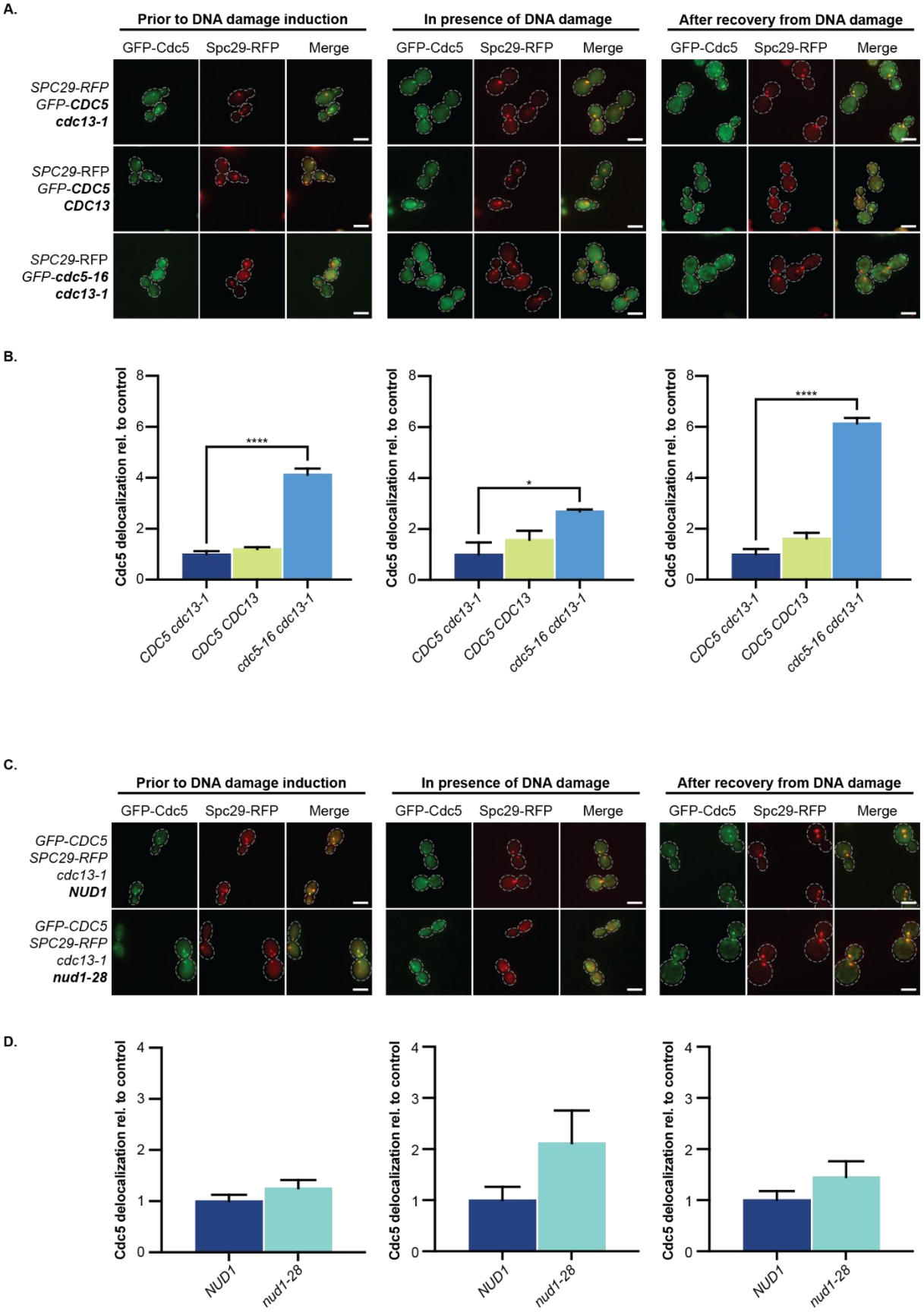
A.
Prior to DNA damage induction
In presence of DNA damage
After recovery from DNA damage
GFP-Cdc5
Spc29-RFP
Merge
SPC29-RFP
GFP-CDC5
cdc13-1
SPC29-RFP
GFP-CDC5
CDC13
SPC29-RFP
GFP-cdc5-16
cdc13-1
B.
Cdc5 delocalization rel. to control
****
*
CDC5 cdc13-1
CDC5 CDC13
cdc5-16 cdc13-1
C.
Prior to DNA damage induction
In presence of DNA damage
After recovery from DNA damage
GFP-Cdc5
Spc29-RFP
Merge
GFP-CDC5
SPC29-RFP
cdc13-1
NUD1
GFP-CDC5
SPC29-RFP
cdc13-1
nud1-28
D.
Cdc5 delocalization rel. to control
NUD1
nud1-28

Figure 2.S12. (A) The *cdc5-16* adaptation-defective PBD mutant is unable to colocalize at SPBs. A control time course experiment was performed to assess the impact of the *cdc13-1* mutation and Cdc5 PBD function on Cdc5 localization at SPBs. Yeast cells carrying *SPC29-RFP* and a combination of either wild-type *CDC13* or *cdc13-1*, and *GFP-CDC5* or *GFP-cdc5-16* were analyzed prior to DNA damage induction (left panel, room temperature), during (middle panel), and after recovery from DNA damage (right panel, room temperature). Mitotic cells were monitored for SPB morphology and Cdc5 colocalization with Spc29-RFP. The presence or absence of the *cdc13-1* mutation (i.e., *cdc13-1* vs *CDC13*) used for DNA damage induction had no impact on the rate of atypical localization. Defects in Cdc5 PBD function led to abnormal localization in 80-100% of *cdc13-1 cdc5-16* PBD mutant cells across the time course. As previously reported, a large fraction of the *cdc5-16* PBD mutant population experienced fragmented SPBs in cells lacking Cdc5 signal (12). Other details are as described in Figure 4A. **(B)** Quantification of the fraction of mitotic cells showing Cdc5 delocalization for the experiment shown in panel A. Phenotypes categorized as Cdc5 delocalization included: mislocalization of Cdc5 in GFP puncta unrelated to SPBs, and diffuse Cdc5 cellular signal (with or without SPB fragmentation). See Figure S15A for the fraction of cells displaying supernumerary Spc29 foci with Cdc5 colocalization. Other details are as described in Figure 4B (* and **** indicate $p \leq 0.05$ and $p \leq 0.0001$, respectively). **(C)** Nud1 PBD-binding sites redundantly support Cdc5 localization at SPBs. A time course experiment was performed with yeast cells expressing *cdc13-1, GFP-CDC5* and *SPC29-RFP* to assess Cdc5 localization at SPBs. Control cells and a *nud1-28* single mutant were analyzed prior to DNA damage induction (left panel, room temperature), during (middle panel), and after recovery from DNA damage (right panel, room temperature). Other details are as described in (A). **(D)** Quantification of the fraction of mitotic cells showing Cdc5 delocalization for the experiment shown in panel C. Nud1 PBD-binding sites contribute redundantly to Cdc5 localization at SPBs alongside other receptors, as mutation of Nud1 PBD sites alone was insufficient to phenocopy the Cdc5 delocalization phenotype observed in the *nud1-92 spc110-7 spc72-8* SPB triple mutant (see Figure 4). Data representation is as shown in panel (B). See Figure S15B for the fraction of cells displaying supernumerary Spc29 foci with Cdc5 colocalization. Statistical analysis performed using an unpaired *t* test on normally distributed values.

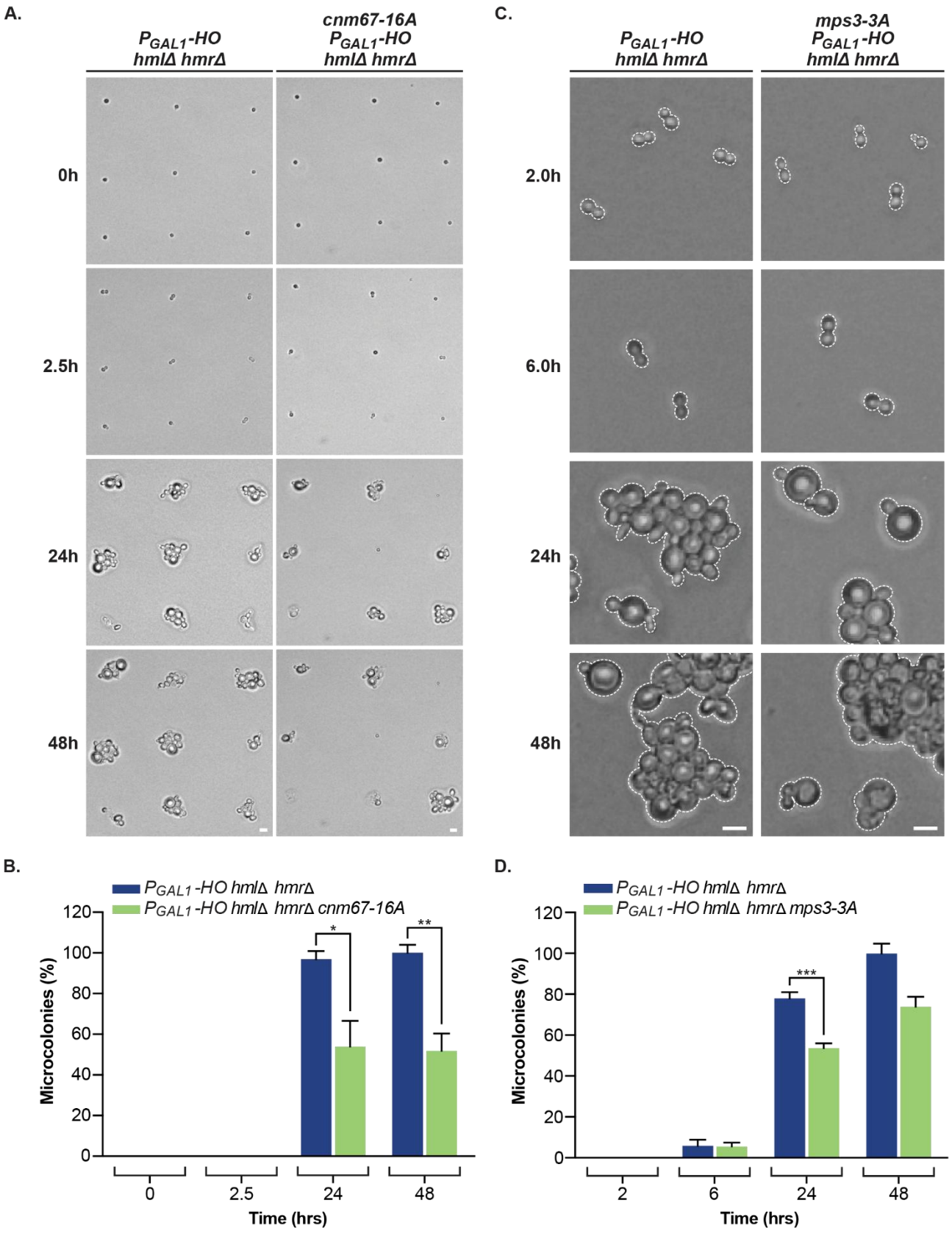
A.
PGAL1-HO hmlΔ hmrΔ
cnm67-16A PGAL1-HO hmlΔ hmrΔ
0h
2.5h
24h
48h
C.
PGAL1-HO hmlΔ hmrΔ
mps3-3A PGAL1-HO hmlΔ hmrΔ
2.0h
6.0h
24h
48h
B.
PGAL1-HO hmlΔ hmrΔ
PGAL1-HO hmlΔ hmrΔ cnm67-16A
Microcolonies (%)
120
100
80
60
40
20
0
*
**
0
2.5
24
48
Time (hrs)
D.
PGAL1-HO hmlΔ hmrΔ
PGAL1-HO hmlΔ hmrΔ mps3-3A
Microcolonies (%)
120
100
80
60
40
20
0
***
2
6
24
48
Time (hrs)

Figure 2.S13. *cnm67-16A* and *mps3-3A* SPB phospho-mutants are defective for the adaptation response to a sustained double-strand break (DSB). (A) Microcolony formation in the presence of persistent DNA damage induced by HO endonuclease (*PGAL1-HO*). Strains were grown in YEPR (2% raffinose) and plated on solid YEPG (2% galactose) to induce DNA damage. 100 cells were plated in a grid-like pattern on the surface of solid medium using a dissection needle. The ability of *cnm67-16A* to adapt to a persistent DSB was reduced relative to control strain. Scale bars, genotype notations and cell outlines are as described in Figure 2. **(B)** Quantification of the fraction of individual cells growing into microcolonies for each time point in the strains shown in panel A. A minimum of 100 cells per strain was counted for each time point. All values are normalized to a relevant micro-manipulation survival ratio (see methods for details) and expressed on a percent basis relative to the value obtained at the T48 h time point for the control strain. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using an unpaired *t* test on normally distributed values. Statistical significance is shown on graph with asterisks (* and ** indicate $p \leq 0.05$ and $p \leq 0.01$, respectively). **(C)** Microcolony formation assay in the presence of HO endonuclease (*PGAL1-HO*) overexpression at 23° C. Strains were grown in YEPR (2% raffinose) and plated on solid YEPG (2% galactose) to induce DNA damage. Rates of microcolony formation following DNA damage induction were lower in *mps3-3A* relative to the control strain. **(D)** Quantification of microcolony formation for the strains tested in panel C. A minimum of 100 cells per strain was counted for each time point. All values were normalized to 100% based on the T48 h mean value of the control strain. Data represented as normalized mean values and SEM of at least 3 independent experiments. Statistical analysis performed using an unpaired *t* test on normally distributed values. Statistical significance is shown on graph with asterisks (*** indicates $p \leq 0.001$).

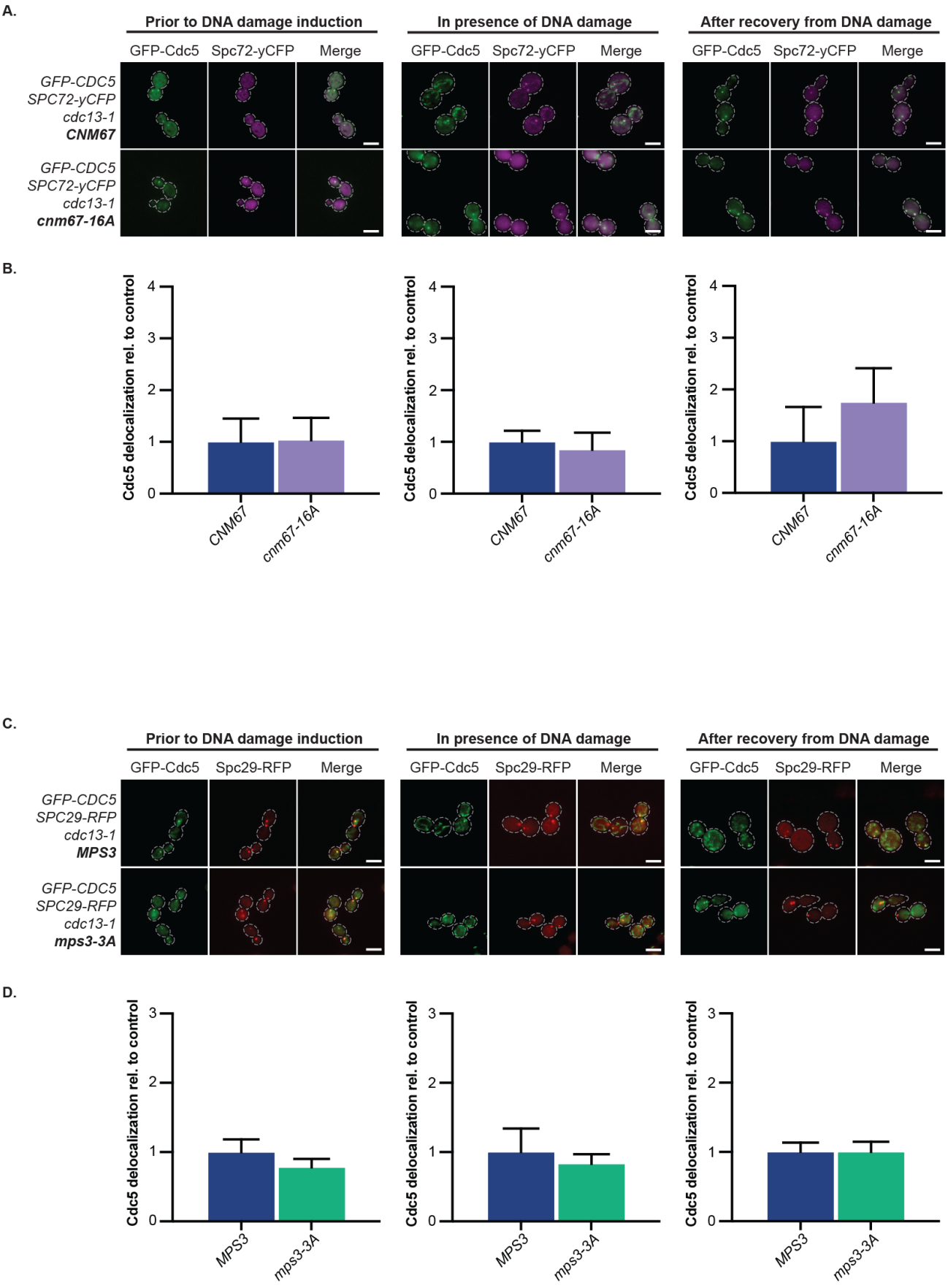
A.
Prior to DNA damage induction
In presence of DNA damage
After recovery from DNA damage
GFP-Cdc5
Spc72-yCFP
Merge
GFP-CDC5
SPC72-yCFP
cdc13-1
CNM67
cnm67-16A
B.
Cdc5 delocalization rel. to control
CNM67
cnm67-16A
C.
Spc29-RFP
MPS3
mps3-3A
D.

Figure 2.S14. (A) Cdc5 enrichment at SPBs does not require Cnm67 phosphorylation. A time course experiment was performed with yeast cells carrying *cdc13-1, GFP-CDC5* and *SPC72-yCFP* to assess Cdc5 localization at SPBs. Control cells and a *cnm67-16A* phospho-mutant were analyzed prior to (left panel, room temperature), during (middle panel), and after exposure to DNA damage (right panel, room temperature), as detailed in the legend to Figure 4A. Mitotic cells were monitored for SPB morphology and Cdc5 colocalization with Spc72-yCFP. The localization behavior of Cdc5 at SPBs in *cnm67-16A* was typical and mirrored that of the *cdc13-1* control strain. **(B)** Quantification of the fraction of mitotic cells showing Cdc5 delocalization for the experiment shown in panel A. See Figure S15D for the fraction of cells displaying supernumerary Spc72 foci with Cdc5 colocalization. Data representation and statistical analysis shown in this Figure are as described in the legend to Figure S12D. **(C)** Cdc5 colocalization with SPBs does not depend on Mps3 phosphorylation. A time course experiment was conducted with yeast cells expressing *cdc13-1, GFP-CDC5* and *SPC29-RFP* to monitor Cdc5 enrichment at SPBs. Control *cdc13-1 MPS3* cells and a *cdc13-1 mps3-3A* phospho-mutant were analyzed prior to (left panel, room temperature), during (middle panel), and after exposure to DNA damage (right panel, room temperature), as detailed in the legend to Figure 4A. Mitotic cells were assessed for SPB morphology and Cdc5 colocalization with Spc29-RFP. The localization pattern of Cdc5 at SPBs in *cdc13-1 mps3-3A* cells was typical and mirrored that of the *cdc13-1 MPS3* control strain. **(D)** Quantification of the fraction of mitotic cells showing Cdc5 delocalization for the experiment in panel C. See Figure S15E for the fraction of cells displaying supernumerary Spc29 foci with Cdc5 colocalization. Data representation and statistical analysis shown in this figure are as described in the legend to Figure S12D.

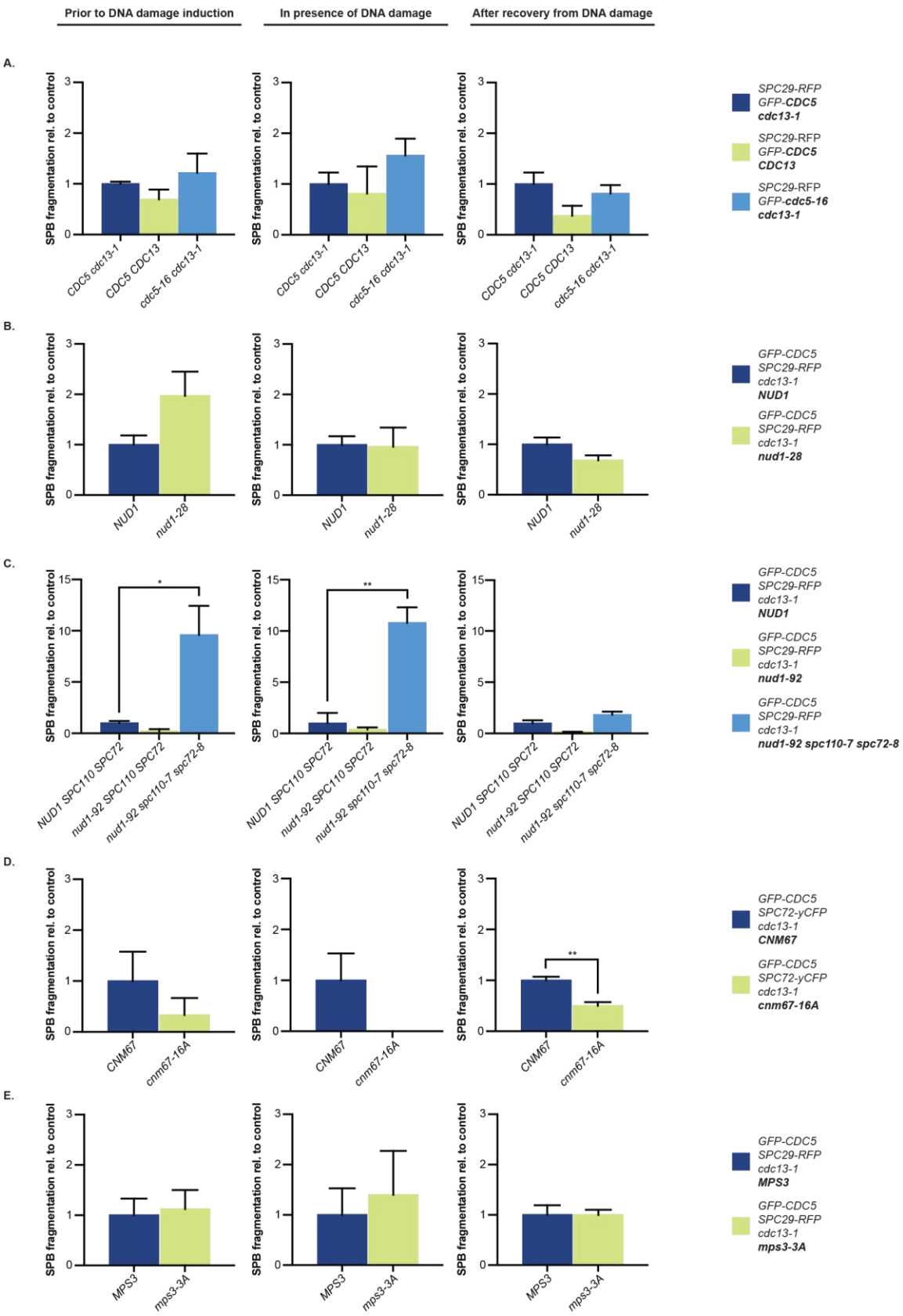
Prior to DNA damage induction
In presence of DNA damage
After recovery from DNA damage
A.
SPB fragmentation rel. to control
CDC5 cdc13-1
CDC5 CDC13
cdc5-16 cdc13-1
SPC29-RFP GFP-CDC5 cdc13-1
SPC29-RFP GFP-CDC5 CDC13
SPC29-RFP GFP-cdc5-16 cdc13-1
B.
NUD1
nud1-28
GFP-CDC5 SPC29-RFP cdc13-1 NUD1
GFP-CDC5 SPC29-RFP cdc13-1 nud1-28
C.
NUD1 SPC110 SPC72
nud1-92 SPC110 SPC72
nud1-92 spc110-7 spc72-8
GFP-CDC5 SPC29-RFP cdc13-1 NUD1
GFP-CDC5 SPC29-RFP cdc13-1 nud1-92
GFP-CDC5 SPC29-RFP cdc13-1 nud1-92 spc110-7 spc72-8
D.
CNM67
cnm67-16A
GFP-CDC5 SPC72-yCFP cdc13-1 CNM67
GFP-CDC5 SPC72-yCFP cdc13-1 cnm67-16A
E.
MPS3
mps3-3A
GFP-CDC5 SPC29-RFP cdc13-1 MPS3
GFP-CDC5 SPC29-RFP cdc13-1 mps3-3A

Figure 2.S15. Fraction of SPB mutants displaying supernumerary Spc29/Spc72 foci positive for Cdc5 signal. Quantification of the fraction of mitotic cells displaying supernumerary (>2) Spc29/Spc72 foci with overlapping Cdc5 signal in colocalization experiments. Other details are as described in Figure S12B-D. The following genotypes were used: **(A)** *CDC5 cdc13-1; CDC5 CDC13; cdc5-16 cdc13-1* (related to Figure S12A-B). **(B)** *NUD1; nud1-28* (related to Figure S12C-D). **(C)** *NUD1 SPC110 SPC72; nud1-92 SPC110 SPC72; nud1-92 spc110-7 spc72-8* (related to Figure 4) (* and ** indicate $p \leq 0.05$ and $p \leq 0.01$, respectively). **(D)** *CNM67; cnm67-16A* (related to Figure S14A-B) (** indicates $p \leq 0.01$). (E) *MPS3; mps3-3A* (related to Figure S14C-D).

2.8 SUPPLEMENTARY TABLES

Table 2.S1. Yeast strains used in this study.

Figure 1A		
	D4107	M*AT**a***
	D777	M*AT**a** cdc5-99::HIS3MX6*
	D1465	M*AT**a** cdc5-16::HIS3MX6*
	D6800	M*AT**a** nud1-28A::URA3MX6*
	D67	M*AT**a** nud1-18 (cdc18-1)*
	D6762	M*AT**a** nud1-92::URA3MX6*
Figure 1B		
	D4107	M*AT**a***
	D777	M*AT**a** cdc5-99::HIS3MX6*
	D1465	M*AT**a** cdc5-16::HIS3MX6*
	D8798	M*AT**a** spc110-7::kanMX6*
	D6797	M*AT**a** spc72-8::kanMX6*
Figure 2A		
	D2884	M*AT**a** cdc13-1 RAD53-3xHA::TRP1*
	D3378	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 cdc5-16::HIS3MX6*
	D6412	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 nud1-28A::URA3MX6*
	D6810	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 nud1-18 (cdc18-1)*
	D6788	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 nud1-92::URA3MX6*
Figure 2C		
	D6850	M*AT**a** RAD53-3xHA::TRP1*
	D6892	M*AT**a** RAD53-3xHA::TRP1 nud1-28A::URA3MX6*
	D6852	M*AT**a** RAD53-3xHA::TRP1 nud1-18 (cdc18-1)*
	D6853	M*AT**a** RAD53-3xHA::TRP1 nud1-92::URA3MX6*
Figure 3A		
	D4107	M*AT**a***
	D67	M*AT**a** nud1-18 (cdc18-1)*
	D8798	M*AT**a** spc110-7::kanMX6*
	D6797	M*AT**a** spc72-8::kanMX6*
	D6762	M*AT**a** nud1-92::URA3MX6*
	D8936	M*AT**a** nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure 3B		
	D2884	M*AT**a** cdc13-1 RAD53-3xHA::TRP1*
	D3378	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 cdc5-16::HIS3MX6*
	D6788	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 nud1-92::URA3MX6*
	D9000	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure 3D		
	D6850	M*AT**a** RAD53-3xHA::TRP1*
	D6853	M*AT**a** RAD53-3xHA::TRP1 nud1-92::URA3MX6*

	D9086	M*AT**a** RAD53-3xHA::TRP1 nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure 3F		
	D2884	M*AT**a** cdc13-1 RAD53-3xHA::TRP1*
	D9000	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure 4A		
	D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
	D6808	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR nud1-92::URA3MX6*
	D8940	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure 5A		
	D8648	M*AT**a** CNM67-3xHA::URA3MX6 cdc15-2*
	D8757	M*AT**a** CNM67-3xHA::URA3MX6 cdc5-99::HIS3MX6*
Figure 5C		
	D8648	M*AT**a** CNM67-3xHA::URA3MX6 cdc15-2*
	D8650	M*AT**a** cnm67-16A-3xHA::TRP1 cdc15-2*
Figure 5D		
	D8479	M*AT**a** CNM67-3xHA::URA3MX6*
	D9926	M*AT**a** CNM67-3xHA::HIS3MX6 nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure 5E		
	D9440	M*AT**a** MPS3-3xHA::kanMX6 cdc15-2*
	D9443	M*AT**a** MPS3-3xHA::kanMX6 cdc5-99::HIS3MX6*
Figure 5G		
	D9440	M*AT**a** MPS3-3xHA::kanMX6 cdc15-2*
	D9446	M*AT**a** mps3-3A-3xHA::URA3MX6 cdc15-2*
Figure 6A		
	D4107	M*AT**a***
	D67	M*AT**a** nud1-18 (cdc18-1)*
	D7034	M*AT**a** cnm67-16A::kanMX6*
	D8065	M*AT**a** mps3-3A::kanMX6*
Figure 6B		
	D2884	M*AT**a** cdc13-1 RAD53-3xHA::TRP1*
	D3378	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 cdc5-16::HIS3MX6*
	D7971	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 cnm67-16A::kanMX6*
	D8088	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 mps3-3A::kanMX6*
Figure S2A		
	D4107	M*AT**a***
	D777	M*AT**a** cdc5-99::HIS3MX6*
	D1465	M*AT**a** cdc5-16::HIS3MX6*
	D6800	M*AT**a** nud1-28A::URA3MX6*
	D67	M*AT**a** nud1-18 (cdc18-1)*
	D6762	M*AT**a** nud1-92::URA3MX6*
Figure S2B		
	D4107	M*AT**a***
	D777	M*AT**a** cdc5-99::HIS3MX6*
	D1465	M*AT**a** cdc5-16::HIS3MX6*
	D8798	M*AT**a** spc110-7::kanMX6*
	D6797	M*AT**a** spc72-8::kanMX6*
	D6762	M*AT**a** nud1-92::URA3MX6*
	D8936	M*AT**a** nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure S3A		
	D4107	M*AT**a***
	D46	M*AT**a** cdc14-3*
	D6800	M*AT**a** nud1-28A::URA3MX6*
	D6762	M*AT**a** nud1-92::URA3MX6*
Figure S3B		
	D6871	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6*
	D9907	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6 cdc5-16::HIS3MX6*
	D7205	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6 nud1-28A::kanMX6*
	D7122	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6 nud1-92::kanMX6*
Figure S4C		

	D10102	M*AT**a** RAD53-3xHA::TRP1 SPC29-RFP::HYGR cdc15-as1::URA3*
	D10105	M*AT**a** RAD53-3xHA::TRP1 SPC29-RFP::HYGR cdc15-as1::URA3 nud1-92::CaURA3MX6*
Figure S5C		
	D10102	M*AT**a** RAD53-3xHA::TRP1 SPC29-RFP::HYGR cdc15-as1::URA3*
	D10105	M*AT**a** RAD53-3xHA::TRP1 SPC29-RFP::HYGR cdc15-as1::URA3 nud1-92::CaURA3MX6*
Figure S5E		
	D10126	M*AT**a** RAD53-3xHA::TRP1 SPC72-yEmCFP::URA3MX6 cdc15-as1::URA3*
	D10128	M*AT**a** RAD53-3xHA::TRP1 SPC72-yEmCFP::URA3MX6 cdc15-as1::URA3 spc42-10::URA3MX6*
Figure S6A		
	D9665	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::Pgal1-CDC5::URA3*
	D7088	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::Pgal1-CDC5::URA3 nud1-28A::URA3MX6*
	D6811	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::Pgal1-CDC5::URA3 nud1-18 (cdc18-1)*
	D9666	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::Pgal1-CDC5::URA3 nud1-92::URA3MX6*
Figure S7A		
	D2884	M*AT**a** cdc13-1 RAD53-3xHA::TRP1*
	D3378	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 cdc5-16::HIS3MX6*
	D9139	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 spc110-7::kanMX6*
	D9141	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 spc72-8::kanMX6*
Figure S8B		
	D9840	M*AT**a** RAD53-3xHA::TRP1 SPC29-RFP::HYGR*
	D9915	M*AT**a** RAD53-3xHA::TRP1 SPC29-RFP::HYGR nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure S9A		
	D4107	M*AT**a***
	D67	M*AT**a** nud1-18 (cdc18-1)*
	D8340	M*AT**a** spc42-10::URA3MX6*
	D6800	M*AT**a** nud1-28A::URA3MX6*
	D6762	M*AT**a** nud1-92::URA3MX6*
Figure S9B		
	D2884	M*AT**a** cdc13-1 RAD53-3xHA::TRP1*
	D3378	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 cdc5-16::HIS3MX6*
	D8834	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 spc42-10::URA3MX6*
Figure S9D		
	D6850	M*AT**a** RAD53-3xHA::TRP1*
	D8800	M*AT**a** RAD53-3xHA::TRP1 spc42-10::URA3MX6*
Figure S10C		
	D10126	M*AT**a** RAD53-3xHA::TRP1 SPC72-yEmCFP::URA3MX6 cdc15-as1::URA3*
	D10128	M*AT**a** RAD53-3xHA::TRP1 SPC72-yEmCFP::URA3MX6 cdc15-as1::URA3 spc42-10::URA3MX6*
Figure S12A		
	D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
	D9794	M*AT**a** CDC13 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
	D9802	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-cdc5-16::HIS3MX6::URA3 SPC29-RFP::HYGR*
Figure S12C		
	D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
	D6543	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR nud1-28A::CaURA3MX6*
Figure S13A		
	D6871	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6*
	D7276	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6 cnm67-16A::kanMX6*
Figure S13C		
	D6871	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6*
	D8097	M*AT**a** hml::LEU2 hmr::URA3MX6 Pgal1-HO::URA3MX6 mps3-3A::kanMX6*
Figure S14A		
	D7988	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC72-yEmCFP::URA3MX6*
	D8014	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC72-yEmCFP::URA3MX6 cnm67-16A::kanMX6*
Figure S14C		
	D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
	D9810	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR mps3-3A::kanMX6*
Figure S15A		
	D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*

D9794	M*AT**a** CDC13 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
D9802	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-cdc5-16::HIS3MX6::URA3 SPC29-RFP::HYGR*
Figure S15B	
D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
D6543	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR nud1-28A::CaURA3MX6*
Figure S15C	
D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
D6808	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR nud1-92::URA3MX6*
D8940	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR nud1-92::URA3MX6 spc110-7::kanMX6 spc72-8::kanMX6*
Figure S15D	
D7988	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC72-yEmCFP::URA3MX6*
D8014	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC72-yEmCFP::URA3MX6 cnm67-16A::kanMX6*
Figure S15E	
D6542	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR*
D9810	M*AT**a** cdc13-1 RAD53-3xHA::TRP1 ura3-1::3xGFP-CDC5::HIS3MX6::URA3 SPC29-RFP::HYGR mps3-3A::kanMX6*

3 CHAPTER III: Manuscript #2

Distinct surfaces on Cdc5/PLK Polo-box domain orchestrate combinatorial substrate recognition during cell division

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3.1 ABSTRACT

Polo-like kinases (Plks) are key cell cycle regulators. They contain a kinase domain followed by a polo-box domain that recognizes phosphorylated substrates and enhances their phosphorylation. The regulatory subunit of the Dbf4-dependent kinase complex interacts with the polo-box domain of Cdc5 (the sole Plk in *Saccharomyces cerevisiae*) in a phosphorylation-independent manner. We have solved the crystal structures of the polo-box domain of Cdc5 on its own and in the presence of peptides derived from Dbf4 and a canonical phosphorylated substrate. The structure bound to the Dbf4-peptide reveals an additional density on the surface opposite to the phospho-peptide binding site that allowed us to propose a model for the interaction. We found that the two peptides can bind simultaneously and non-competitively to the polo-box domain in solution. Furthermore, point mutations on the surface opposite to the phosphopeptide binding site of the polo-box domain disrupt the interaction with the Dbf4 peptide in solution and cause an early anaphase arrest phenotype distinct from the mitotic exit defect typically observed in *cdc5* mutants. Collectively, our data illustrates the importance of non-canonical interactions mediated by the polo-box domain and provide key mechanistic insights into the combinatorial recognition of substrates by Polo-like kinases.

3.2 INTRODUCTION

Processes that drive mitotic progression are under strict cellular regulation to ensure the faithful propagation of newly replicated genetic material. Cellular defects that arise during mitosis – such as sister chromatid mis-alignment or spindle pole mis-positioning – can lead to chromosome segregation defects and give rise to polyploid and aneuploid daughter cells[1–3]. It is during these events that the cell activates signaling cascades known as checkpoints to inhibit mitotic processes[4]. Misregulation of mitotic checkpoints gives cells a proliferative advantage – one of the hallmarks of carcinogenesis[3,5].

A conserved family of kinases known as Polo-like kinases (Plks) control mitotic events[6]. Plks function in mitotic entry, spindle pole dynamics, chromosome condensation[7], sister-chromatid segregation[8], and cytokinesis[2,6,9]. Plk1 is upregulated in multiple human tumors and, therefore, has become an attractive anti-cancer target[10,11]. The structural similarity with other cellular kinases, however, has limited the potential of inhibiting Plk1 due to off-target effects. Some of the most selective drugs against Plk1 also inhibit Plk2 and Plk3 with similar potency[12,13]. Therefore, targeting interactions that regulate kinase activity provide a promising approach to specifically target Plk1.

Much of our understanding of Plk1 comes from Cdc5, the sole Plk in budding yeast[4,14,15]. Cdc5 promotes the release of Cdc14 from the nucleolus and regulates the mitotic exit network (MEN)[9,16–18] as well as cytokinesis[19–21]. Full release of Cdc14 leads to the downstream activation of the anaphase promoting complex, cyclin destruction, mitotic spindle disassembly, and displacement of the septin ring during cytokinesis[16,18,22]. Cdc5 is inhibited during the spindle position checkpoint (SPOC), which is activated in response to mis-aligned and/or damaged spindle poles[23]. One of the components of SPOC is the Dbf4-dependent kinase (DDK) complex[23]. DDK is a heterodimer formed by the association of the Cdc7 kinase and its regulatory subunit, Dbf4, necessary at multiple steps of the cell cycle[23–26]. During SPOC, Dbf4 binds to Cdc5 and promotes Cdc7-mediated phosphorylation of Cdc5, which then presumably prevents Cdc5 from recognizing its substrates in the MEN pathway[23,27]. However, DDK can also activate Cdc5[28,29]. Cdc5, along with DDK and CDK1, hyper-phosphorylate the structure-specific nuclease Mus81-Mms4 and this, in turn, activates Mus81-Mms4 to resolve joint DNA molecules at the onset of mitosis[29]. Full activation of Mus81-Mms4 depends on the Cdc5-Dbf4 interaction, indicating that DDK and Cdc5 work together during this process[29].

Like Plks, Cdc5 contains a Ser/Thr kinase domain followed by a polo-box domain that mediates substrate recognition and sub-cellular localization[10,30]. The polo-box domain is made up of two polo boxes that together define the p(S/T)-binding pocket. Polo-box domains recognize substrates harboring a X-S-p(S/T)-

(P/X) consensus phosphorylated site[30,31]. However, polo-box domains can also mediate phosphorylation-independent interactions[27,32,33]. For instance, the drosophila microtubule-associated protein Map205 interacts with the polo-box domain of Polo (the drosophila homolog of Plk1) at the pS/T-binding pocket[33], despite lacking the consensus phosphorylated motif. Dbf4 also lacks the phosphorylated consensus sequence. Instead, it uses a unique polo-interacting motif (^{83}RSIEGA^{88}) to interact with the polo-box domain of Cdc5[27]. Mutation of key residues at the pS/T-binding pocket does not affect Dbf4 binding, indicating that Dbf4 binds a different surface of the polo-box domain[23,30,31]. However, the surface of the polo-box domain where Dbf4 binds is not known. To understand how Dbf4 binds and modulates the function of Cdc5, we solved the crystal structures of the polo-box domain of Cdc5 on its own and in complex with peptides derived from the Cdc5-binding motifs of Dbf4 and the spindle pole-body protein Spc72 – a bona fide Cdc5 phosphorylated substrate. As expected, the Spc72 peptide bound to the groove at the interface of the two polo boxes. The structure in the presence of the Dbf4 peptide was obtained at lower resolution, but it clearly showed additional electron density on a surface of the polo-box domain opposite to the phosphopeptide binding site. Point mutations on this surface of Cdc5 abrogated binding to the Dbf4 peptide, confirming that the extra density did indeed correspond to the Dbf4 peptide. Mutations on this surface of the polo box domain also result in early anaphase arrest phenotypes distinct from the mitotic exit defect typically observed in *cdc5* mutants. Based on these results, we propose a model for how binding to Dbf4 may help release the auto-inhibition of the Cdc5 kinase.

3.3 RESULTS

Yeast Cdc5 recognizes phosphorylated substrates like human Plk1

We determined the crystal structure of the polo-box domain of Cdc5 using a fragment of Cdc5 that included residues 418–705 (Fig. 3.1a and Table 3.1). Similar to other previously determined structures of Plk1, the extension preceding the polo-box domain (residues 418–459) was disordered in the structure, while

residues Leu460-Asp705 adopted the characteristic polo-box domain fold with the first polo-box defined by the β1-β2-β3-β4-β5-β6-α2 structure elements, and the second by the β7-β8-β9-β10-β11-β12-α3-α4 structure elements (Fig. 3.1b). The loop connecting the two polo boxes (Ala594-Ser601) was disordered in this structure. The distance between the last ordered residue from the first polo-box (Lys593) and the first ordered residue in the second polo-box (Thr602), as well the conformation of residues Thr602-Asp611 folding back towards β5-β6 rather than reaching across the first polo-box towards α2, suggested that the loop may had been proteolytically cleaved during crystallization (Fig. 3.1b–c). The side chains of Glu553, His569 and His609, as well as that of His524 from a symmetry related molecule, coordinate a zinc metal ion from the crystallization solution (Fig. 3.1c). This metal ion further stabilizes the conformation of the ordered portion of the loop and places the phenyl group of Phe603 inside an exposed hydrophobic pocket delimited by Leu546, Trp555, Ile557 and Ala567 (Fig. 3.1b and Supplementary Fig. 3.1a).

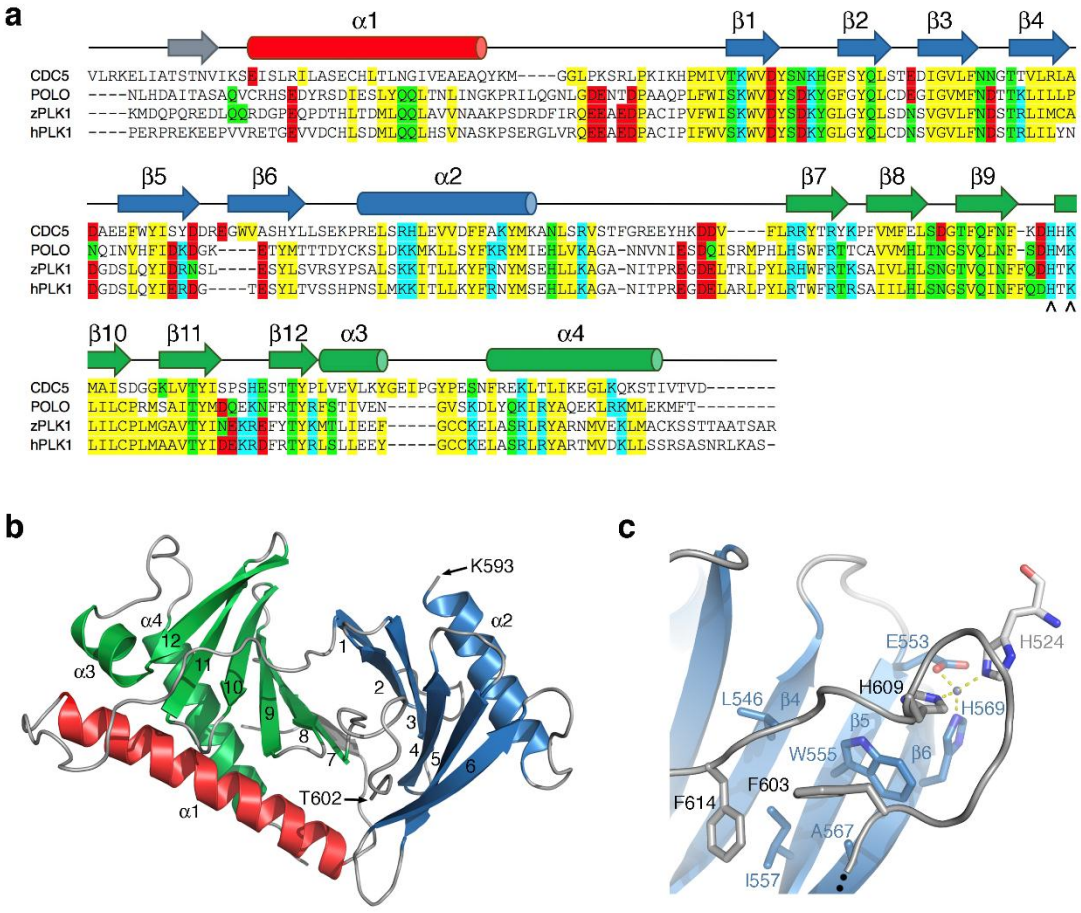

Figure 3.1. Cdc5 architecture and structure of its polo-box domain. (a) Sequence alignment of budding yeast Cdc5 (residues 451–705) and the drosophila (POLO, residues 333–576), zebrafish (zPLK1, residues 343–595) and human (hPLK1, residues 352–603) Plk1 homologs. Secondary structure elements are shown above the alignment and color red (polo cap), blue (first polo-box) and green (second polo box). Conserved hydrophobic (yellow), polar (green), positive charged (blue) and negative charged (red) residues are highlighted. Conserved residues involved in phosphopeptide binding are marked with (^). **(b)** Ribbon diagram of the structure of Cdc5 color coded as in **(a)**. **(c)** Detail of the interactions between the ordered region of the α2-β7 loop (residues Thr602-Phe614) and strands β5 and β6 from the first polo-box. The Zn^{2+} metal ion is shown as a grey sphere with hydrogen bonds drawn as dashed lines.

Table 3.1. Data collection and refinement statistics.

	Cdc5	Cdc5-Spc72	Cdc5-Dbf4
Data Collection			
Beamline	08B1; CLS	17-ID; APS	17-ID; APS
Wavelength (Å)	1.2834	0.9795	0.9762
Space group	P 2_1 2_1 2_1	P 2_1	P 6_5
Cell dimensions: a, b, c	51.1, 65.9, 96.1	51.5, 68.2, 86.2	134.6, 134.6, 75.3
α, β, γ	90, 90, 90	90, 102.6, 90	90, 90, 120
Resolution (Å)*	48.1–1.8 (1.86–1.8)	42.1–2.7 (2.78–2.7)	50–3.5 (3.63–3.5)
Reflections (unique/total)	58,352 / 235,329	16,158 / 93,716	10,923 / 205,926
Completeness (%)	100 (99.9)	99.9 (99.4)	100 (100)
$CC_{1/2}$ (%)	99.7 (35.8)	99 (31.7)	99.9 (36.7)
I/σ(I)	12.58 (0.96)	9.1 (1.56)	17.3 (0.8)
Redundancy	4 (3.8)	5.8 (4.9)	19.8 (20.9)
Refinement			
Resolution (Å)	48.1–1.8	42.1–2.7	35.8–3.6
Completeness (%)	100	99.9	99.5
R_{work}/R_{free} (%)	18.1 / 19.7	21.4 / 25.7	31.4 / 32.2
Ramachandran plot (%)			
Favoured /Outliers	98.3 / 0	93.6 / 0.2	87.4 / 0.9
rmsd in bonds (Å)	0.004	0.004	0.003
rmsd in angles (°)	0.885	0.805	0.504

*Data in the highest resolution shell is shown in parentheses.

To confirm how Cdc5 recognizes phosphorylated substrates, we then solved the structure of Cdc5 bound to a phosphorylated peptide derived from the spindle-pole body protein Spc72 (Spc72^{P}, ^{227}SLA**QSpSP**AGSQ^{237}). Spc72^{P} is a well-studied binding partner of Cdc5 that contains the conserved phosphorylated motif recognized by polo-box domains[34]. As expected, the Spc72^{P} phosphopeptide bound along the groove determined by the two polo-boxes and adopts an extended conformation that runs antiparallel to β1, effectively extending the β-sheet of the first polo-box by one strand (Fig. 3.2). The strictly conserved His641 and Lys643 cradle the phosphate group (Figs. 3.1a, 2), confirming that Cdc5 recognizes phosphorylated substrates the same way as other Plk1 homologs (Supplementary Fig. 3.2).

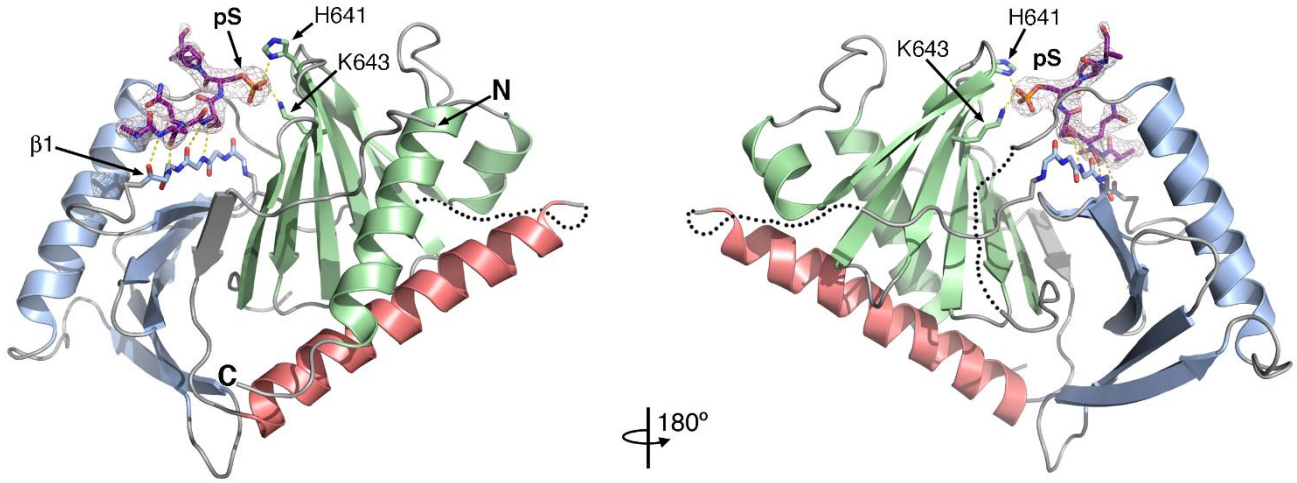

Figure 3.2. Structures of the polo-box domain (PBD) of Cdc5 bound to Spc72^{P}. Opposite views of the PBD of Cdc5 bound to the Spc72^{P} peptide (magenta). Spc72^{P} binds at the groove defined by the two polo boxes forming a short antiparallel β-sheet with the β1 strand. The 2Fo-Fc electron density map around the Spc72^{P} peptide contoured at 1.2σ is shown as a grey mesh.

Dbf4 binds a surface of the polo-box domain opposite to the pS/T-binding pocket

The ^{83}RSIEGA^{88} motif, located upstream of the BRCT domain of Dbf4, is necessary for the interaction with Cdc5[23,27]. This motif lacks the X-S-p(S/T)-(P/X) sequence recognized by polo-box domains and point

mutations on the pS/T-binding pocket of Cdc5 do not affect the interaction with Dbf4[27], suggesting that Dbf4 recognizes a different surface of Cdc5.

To elucidate how Dbf4 interacts with Cdc5, we next solved the structure of the polo-box domain in the presence of a Dbf4-derived peptide (^{76}RARIERA**RSIEGA**VQVSKGTG^{96}). Crystals of this complex diffracted X-rays to lower resolution (~3.5 Å), but the structure could be readily determined by molecular replacement using the polo-box domain of the Cdc5-Spc72^{P} structure (Table 3.1) The polo-box domain was virtually identical to the other two structures (r.m.s. deviations of 0.86 and 0.66 Å with the apo- and Spc72^{P}-bound structures, respectively). As expected, the pS/T-binding pocket was not occupied by Dbf4. However, there was not obvious electron density for the Dbf4 peptide anywhere around the polo-box domain. After a cycle of refinement, the surface opposite to the pS/T-binding pocket showed significant peaks (>2.5σ) on the Fo-Fc electron density maps (Fig. 3.3a). While these additional electron densities lacked connectivity and could not account for the entire Dbf4 peptide, they could accommodate few amino acids. Intriguingly, these electron density peaks were close to the hydrophobic pocket defined by residues Leu546, Trp555, Ile557 and Ala567 from Cdc5 and coincided with the position that Phe603 occupies in the structure of the polo-box domain of Cdc5 on its own (Fig. 3.1c).

The ^{83}RSIEGA^{88} motif defines the minimal region of Dbf4 necessary for binding to Cdc5. Mutation of Ile85 within this motif completely abrogates binding to Cdc5[27]. We speculated that Ile85 could occupy the hydrophobic pocket defined by Leu546, Trp555, Ile557 and Ala567 (Fig. 3.3a and Supplementary Fig. 3.1). If this was the case, Glu86 would bind close to Asp611 and Asp631 from polo-box domain, and this could explain why a Dbf4-E86K peptide binds to the polo-box domain of Cdc5 with higher affinity than the wild-type Dbf4 peptide (Supplementary Fig. 3.1)[27]. To test whether this surface of the polo-box domain was important for the interaction with Dbf4, we introduced a A567W mutation on the polo-box domain to occlude the hydrophobic pocket. This variant of the polo-box domain was stable and monodisperse in solution as judged by size-exclusion chromatography and dynamic light scattering. Using isothermal

calorimetry, we then compared the binding affinities of the Dbf4 peptide to the polo-box domain and the polo-box domain of Cdc5 carrying the A567W substitution. In good agreement with previously published data, the Dbf4 peptide bound to the polo-box domain of Cdc5 with a k_D ~ 0.5 µM (Fig. 3.3b and[27]). Conversely, the Dbf4 peptide failed to interact with the A567W variant of the polo-box domain (Fig. 3.3b), indicating that the integrity of the hydrophobic surface of the polo-box domain of Cdc5 opposite to the pS/T-binding pocket is important for Dbf4-peptide binding.

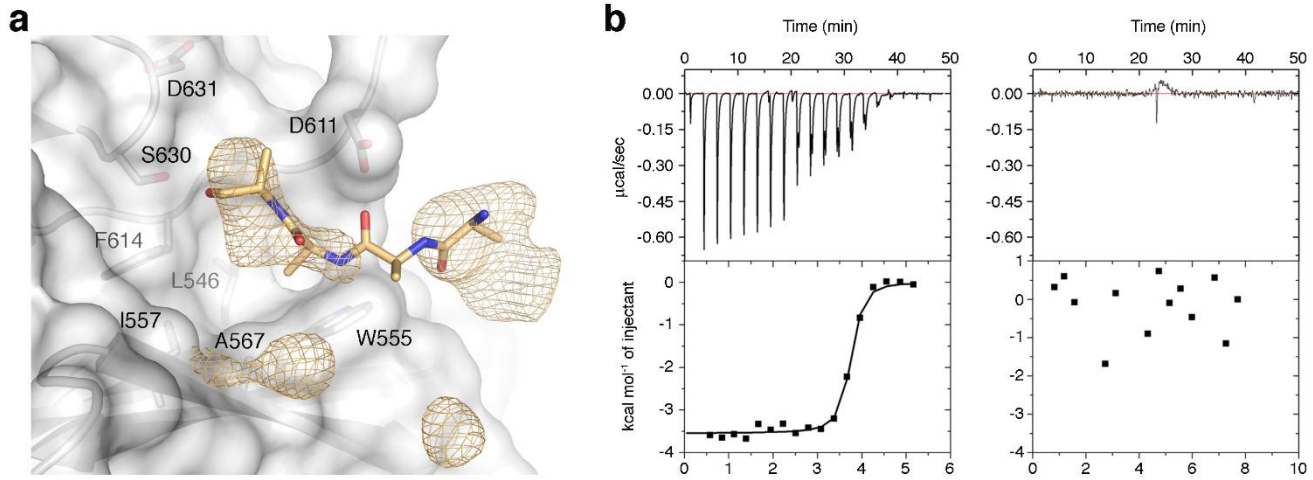

Figure 3.3. The Dbf4 peptide binds to the hydrophobic surface opposite to the pS/T-binding pocket. (a) Structure of the PBD of Cdc5 in the presence of the Dbf4 peptide. The Fo-Fc>0 electron density map around the hydrophobic pocket opposite to the phosphopeptide binding site is shown as a yellow mesh contoured at 2.5σ with a tetrapeptide modelled for reference (yellow sticks). **(b)** Isothermal calorimetry data and analysis for the titration of the Dbf4 into the polo-box domain of $Cdc5^{PBD}$ (left) or Cdc5 ^{PBD}-A567W (right).

Dbf4 and $Spc72^P$ bind simultaneously and non-competitively to Cdc5

Since Dbf4 and $Spc72^P$ bind to opposite faces of the polo-box domain, we next tested whether both peptides could simultaneously bind to Cdc5. Using isothermal calorimetry, we confirmed that the $Spc72^P$ peptide interacted with the polo-box domain of Cdc5, and the interaction was phosphorylation dependent (Fig. 3.4a,b). The binding isotherms for the Cdc5-Dbf4 and Cdc5-$Spc72^P$ complexes point to saturation

occurring at around 1:3 molar ratios of Cdc5:peptide (Figs. 3.4a,d). Therefore, we tested whether Spc72^{P} could bind with Cdc5 that had been pre-incubated with Dbf4 at 1:4 molar excess. We found that Spc72^{P} bound to Cdc5 and Cdc5-Dbf4 with similar affinities (Figs. 3.4a,c). The reciprocal experiment yielded similar results (Fig. 3.4d,e), confirming that Cdc5 can bind both substrates simultaneously *in vitro*.

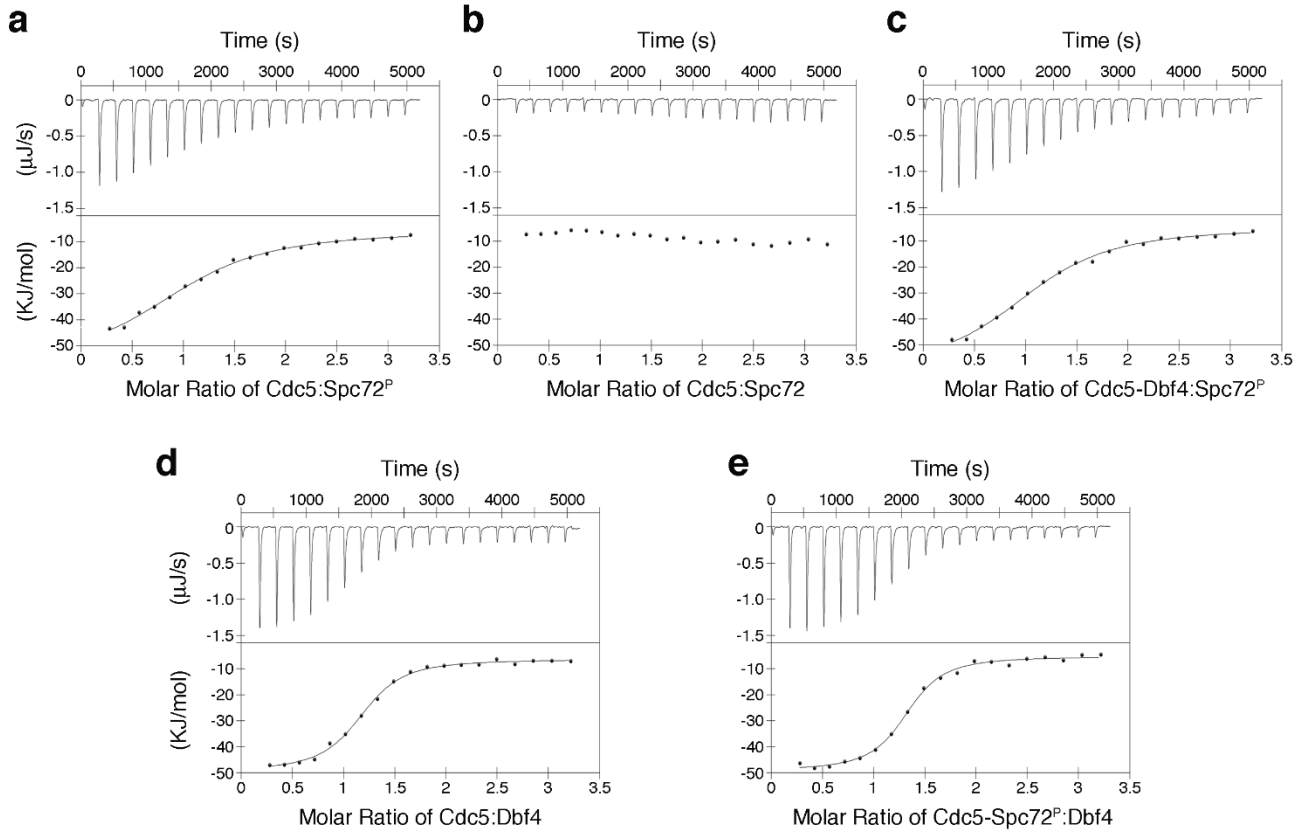

Figure 3.4. Cdc5 interacts simultaneously and non-competitively with Spc72^{P} and Dbf4. Isothermal calorimetry data and analysis for the titration of the Spc72^{P} peptide **(a)**, its non-phosphorylated version (Spc72) **(b)**, and the Dbf4 peptide **(d)** into the polo-box domain of Cdc5. **(c)** Titration of the Spc72^{P} peptide into the polo-box domain of Cdc5 pre-incubated with the Dbf4 at 1:4 molar excess. **(e)** Titration of the Dbf4 peptide into the polo-box domain of Cdc5 pre-incubated with the Spc72^{P} at 1:4 molar excess.

Although measurements at different excess molar ratios yielded consistent results, we could not exclude the possibility that the pre-bound peptide was displaced by the titrating peptide. To test whether both peptides were bound simultaneously to the polo-box domain, we used saturation-transfer difference (STD)

NMR. One dimensional ^{1}H NMR spectra were acquired for Cdc5, Dbf4 and Spc72^{P} to determine suitable saturation frequencies selective for Cdc5 (Fig. 3.5a). Since there was significant spectral overlap between the aliphatic region of Cdc5 and the two peptides, we selected a saturation frequency within the aromatic region of Cdc5 (6.604 ppm). The Dbf4 peptide lacks aromatic residues and had no signals in this region, thereby avoiding the risk of non-selective saturation. This was confirmed by acquiring control STD experiments in the absence of Cdc5, which resulted in no observable STD signals (Supplementary Fig. 3.3). Spc72^{P} had a minor peak at around 6.7 ppm and the STD spectra acquired in the absence of Cdc5 resulted in additional Spc72^{P} resonances (Supplementary Fig. 3.3). To account for this saturation leak through, STD spectra of Spc72^{P} observed in the presence of Cdc5 were compared to those in its absence.

We then acquired STD experiments for the Cdc5-Dbf4 and Cdc5-Spc72^{P} complexes. The saturation transfer for Spc72^{P} resulted in a signal significantly greater than the phosphorylated peptide alone (Fig. 3.5b, Supplementary Table 3.1). The results for the Dbf4 peptide were less clear due to signal broadening and spectral overlap with Cdc5 (Fig. 3.5c). However, comparing the STD spectra acquired in the absence and presence of Dbf4, we were able to identify several peaks with increased intensities that were consistent with the positions of Dbf4 resonances in the absence of Cdc5. The interaction between Cdc5 and Dbf4 was also evident from the chemical shift changes in the aromatic resonances of Cdc5 upon addition of Dbf4 (Fig. 3.5d). Since Dbf4 has no resonances in this region, the spectral differences could only be explained due to Cdc5 chemical shift changes caused by the binding of Dbf4.

When analyzing the ternary complex (Dbf4-Cdc5-Spc72^{P}), we observed STD signals for Spc72^{P} (Fig. 3.5e). The normalized STD intensities (i.e. the STD/STR ratios) indicated that the binding affinity was similar to that of the Cdc5-Spc72^{P} complex (Supplementary Table 3.1). As for Dbf4, the STD detection was impaired by spectral crowding and weak intensities, similar to the Cdc5-Dbf4 complex (Fig. 3.5f). However, chemical shift changes between the Cdc5-Spc72^{P} and Dbf4-Cdc5-Spc72^{P} complexes confirm that Dbf4 interacts with Cdc5 in the presence of Spc72^{P} (Fig. 3.5g). Overall, the STD/STR spectra confirmed that Cdc5 can bind the

Spc72^{P} phosphopeptide and Dbf4 simultaneously, and that binding to one peptide does not significantly affect binding of the other.

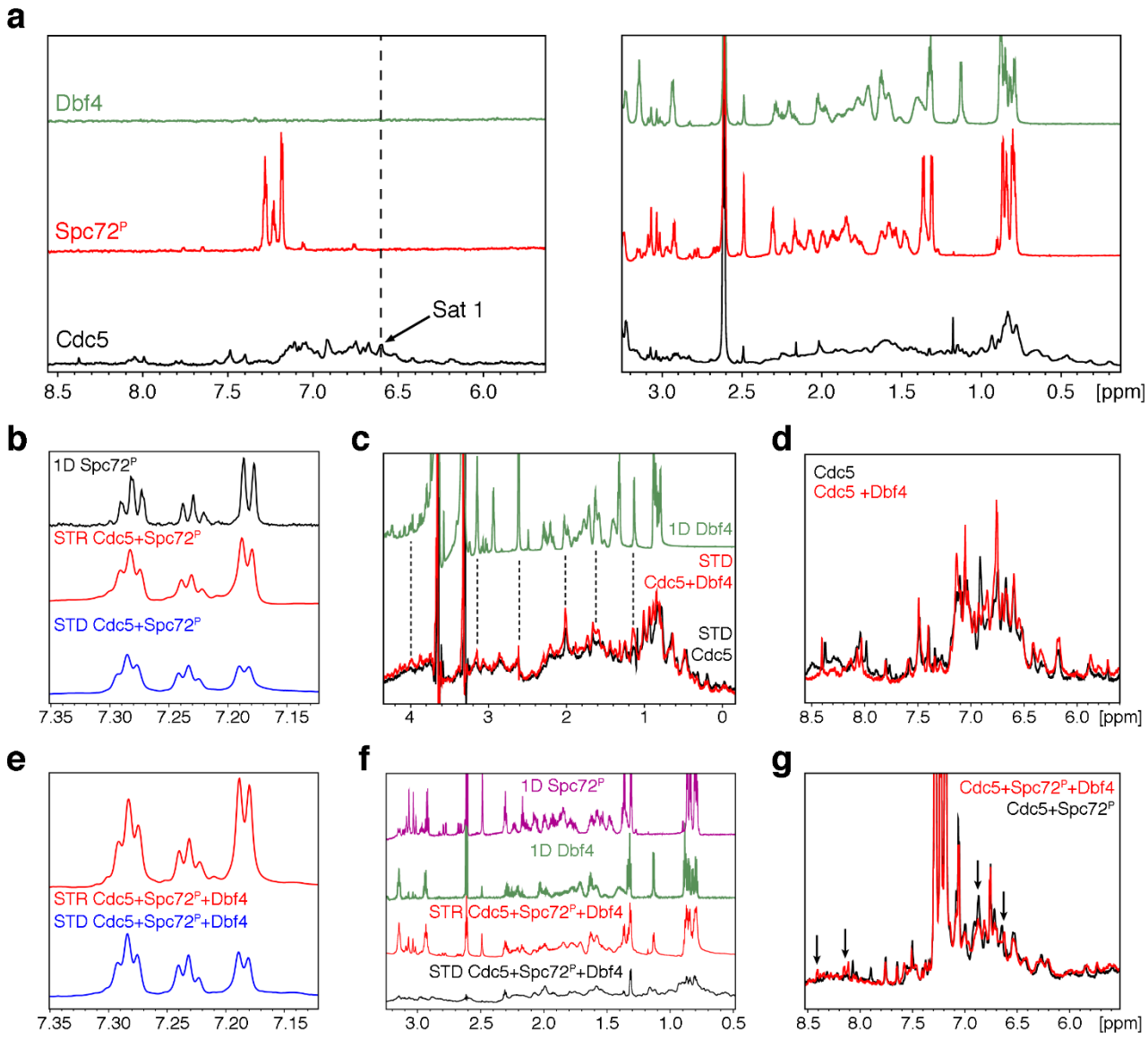

Figure 3.5. NMR spectra probing the Cdc5-substrate interactions. (a) One dimensional ^{1}H NMR spectra for Cdc5, Spc72^{P}, and Dbf4. The Cdc5-selective saturation frequency used in the STD experiments is indicated. **(b)** Aromatic expansion of the STD NMR spectrum of Scp72^{P} acquired in the presence of Cdc5. The 1D ^{1}H and STD reference (STR) spectra of Scp72^{P} in the absence and presence of Cdc5 are shown for comparison. **(c)** Overlay of the Cdc5 and Cdc5-Dbf4 STD spectra. Dashed lines highlight Cdc5-Dbf4 peaks with increased intensities that align with free Dbf4 (green) chemical shifts. **(d)** Overlay of the aromatic region of 1D ^{1}H NMR spectra for Cdc5 and Cdc5-Dbf4. **(e)** Aromatic expansion for the STD and STR spectra of the Cdc5-Scp72^{P}-Dbf4 complex. **(f)** Aliphatic region of the Cdc5-Spc72^{P}-Dbf4 STD spectra. 1D ^{1}H NMR spectra for each peptide and the STR spectrum of the Cdc5-Spc72^{P}- Dbf4 complex are shown for reference. **(g)** Overlay of the aromatic region of 1D proton spectra for the Cdc5-Spc72^{P} and Cdc5-Scp72^{P}-Dbf4 complexes. Arrows indicate chemical shift changes caused by Dbf4 binding.

Targeted mutations in Cdc5 generate conditional lethal alleles *in vivo*

We have previously shown that mutations affecting the phosphopeptide-binding activity of the polo-box domain of Cdc5 are tolerated in yeast as they generate viable alleles that are not associated with strong proliferation defects[35]. However, these alleles show a striking misregulation of cell cycle checkpoints and are defective in their maintenance of genome stability[35,36]. In light of these observations, we wanted to assess the physiological impact of mutations affecting the hydrophobic pocket of the polo-box.

We introduced point mutations at residues delimiting the surface where Dbf4 binds within one copy of the *CDC5* gene in diploid yeast, and the resulting heterozygous mutant strains were sporulated. After dissection of sporulated tetrads, all haploid segregants carrying *cdc5* mutations were viable and germinated normally. Interestingly, several hydrophobic pocket mutants showed reduced cell proliferation capacity at high temperature and in medium containing DNA damaging agents (Fig. 3.6). In particular, the *cdc5-S630Q* mutant showed a striking growth defect both at high temperatures (Fig. 3.6a) and in the presence of 4-nitroquinoline 1-oxide (4-NQO; Fig. 3.6b), with very little or no growth observed under these conditions. The 4-NQO hypersensitivity of the *cdc5-S630Q* mutant was comparable to that of cells defective in the Smc5-6 complex, a known DNA repair enzyme (Fig. 3.6b)[37]. Importantly, the proliferation of the *cdc5-S630Q* mutant was unaffected relative to wild-type controls at the permissive temperature of 23 °C, indicating that it is a *bona fide* conditional-lethal mutant *in vivo*. Ser630 is located at the end of the β8 strand and it is held in place by hydrogen bonds with Phe614 leading to β7. Ser630 defines the back wall of the Dbf4-binding pocket (Supplementary Fig. 3.1c). A point mutation introducing a larger side chain (S630Q) at this position would reduce the size of the hydrophobic pocket and, thus, it would be more deleterious than one introducing a smaller side-chain (S630A). Similarly, other interactions mediated by this surface of Cdc5 would also be affected by reducing the size of the pocket.

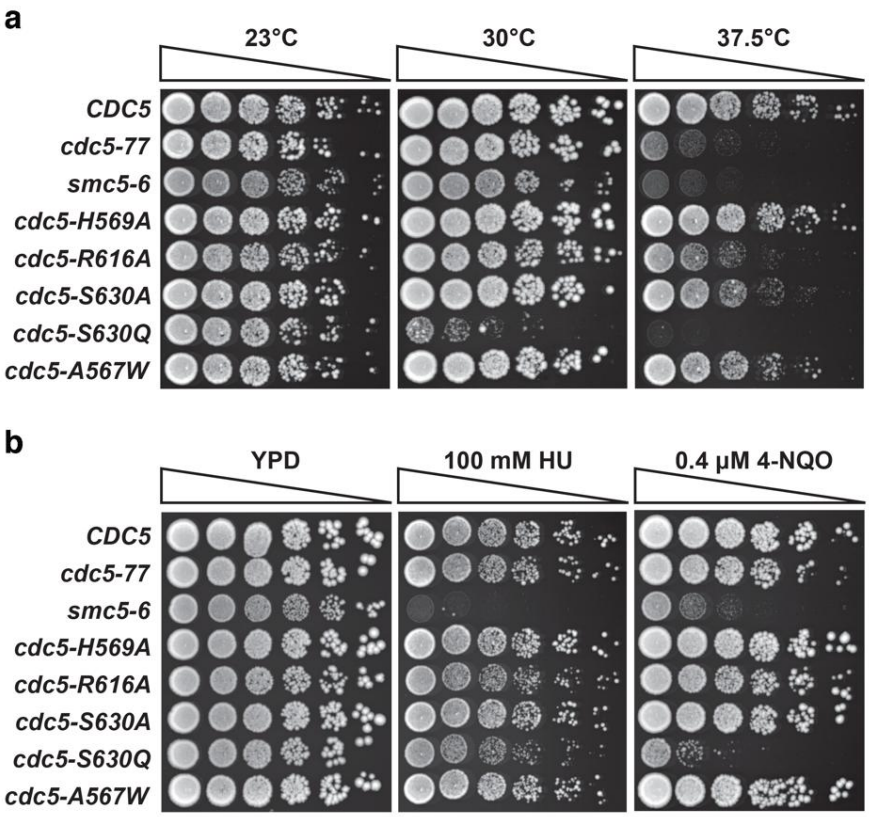

Figure 3.6. Disruption of Cdc5 hydrophobic pocket generates conditional lethal mutants. Exponential culture of the indicated mutant and control cells were spotted on solid medium as fivefold dilution series. Cells were allowed to grow in temperature-controlled incubators for 36–72 hours until individual colonies were visible. **(a)** Effect of temperature on the proliferation of *cdc5* mutants. Cells were incubated at 23 °C, 30 °C, and 37.5 °C on solid YPAD medium. **(b)** Sensitivity of *cdc5* mutants to DNA replication inhibitor and DNA damaging agent. Cells were grown at 23 °C, and mutants defective in DNA repair (*smc5-6*[37]) and Cdc5 kinase activity (*cdc5-77*[35]) were included as controls. (n = 5).

The *cdc5-S630Q* mutant fails to complete the segregation of its chromosomes and arrests in anaphase

Since Cdc5 function is essential for mitotic exit[35], we hypothesized that the conditional-lethal behavior of the *cdc5-S630Q* mutant might reflect a defect in mitotic progression. To test this hypothesis, we monitored cell cycle kinetics in synchronized populations of cells. Yeast strains were first arrested in G1 phase with α-factor at 23 °C and subsequently released from this arrest into a synchronous cell cycle at restrictive temperature for *cdc5-S630Q* and *cdc5-99* mutants. The latter mutant shows the typical mitotic exit defect associated with Cdc5 inactivation and was used as a positive control in the experiment[7]. Samples of cells

were taken at regular intervals during the time-course experiment to monitor the appearance of cellular landmarks by microscopy. The initial formation of buds on mother cells, which is concomitant with the early stages of DNA replication, followed similar kinetics in all strains (Fig. 3.7a). Whereas wild-type cells and a control mutant (*cdc5-S630A*) completed mitosis and returned to G1 normally, as evidenced by the formation of large-budded cells and their separation late in the time-course, both *cdc5-S630Q* and *cdc5-99* mutants accumulated large budded cells and failed to divide into two separate cells, even at the last time-point of the experiment (135 min; Fig. 3.7a). This phenotype is consistent with a late mitotic defect in *cdc5-S630Q* cells.

To more precisely pinpoint at which stage of mitosis the *cdc5-S630Q* mutant is defective, we monitored the separation of DAPI-stained nuclei in dividing cells. A synchronous time-course experiment was conducted as described above and the morphology of nuclei in mutant and control cells was classified according to three different categories reflecting the normal timeline of chromosome segregation in yeast: mononucleated, extended/irregular nucleus, and binucleated. *cdc5* mutants often show a minor delay in chromosome segregation during anaphase[8] and typically experience a terminal arrest in late telophase (*i.e.*, as binucleated cells that sometimes carry thin anaphase bridges between separated nuclei[35]). The negative (*CDC5* and *cdc5-S630A*) and positive (*cdc5-99*) control strains showed the expected behavior in their kinetics of nucleus segregation in the time-course experiment (Fig. 3.7a). Namely, segregation started 70 min after the release from the α-factor block for all strains and reached a maximum at 90 min post-release for *CDC5* and *cdc5-S630A* strains, whereas the *cdc5-99* mutant accumulated as a pure population of binucleated cells due to its defect in mitotic exit. In striking contrast, chromosome segregation was strongly delayed in the *cdc5-S630Q* mutant, and about 50% of the cells completely failed to segregate their DNA by the end of the experiment (see "extended nucleus" category in Fig. 3.7a). Examination of nucleus morphology in *cdc5-S630Q* mutants revealed cells blocked early or mid-way in the process of chromosome segregation (Fig. 3.7b). This phenotype was observed using a generic dye to label genomic DNA (DAPI,

Fig. 3.7b), as well as a fluorescent marker for chromatin (histone H2A fused to mCherry, Supplementary Fig. 3.4a,b). Consistent with this observation, the distance that separates spindle pole bodies (SPBs), which reflects nuclear spindle length[38], was remarkably shorter in *cdc5-S630Q* mutants relative to wild-type cells in early anaphase (*i.e.*, 2.67 ± 0.35 µm *vs* 4.51 ± 0.04 µm; Supplementary Fig. 3.4c). An early anaphase arrest phenotype has not been observed previously in other *cdc5* mutants and likely reflects the unique nature of the molecular defect in the *cdc5-S630Q* allele. The *cdc5-S630A* mutant did not show detectable defects in chromosome segregation, indicating that the phenotype of the *cdc5-S630Q* mutant is specific. Taken together, these results indicate that the integrity of the hydrophobic surface of the polo-box domain opposite to the pS/T-binding pocket is important for effective segregation of chromosomes in anaphase.

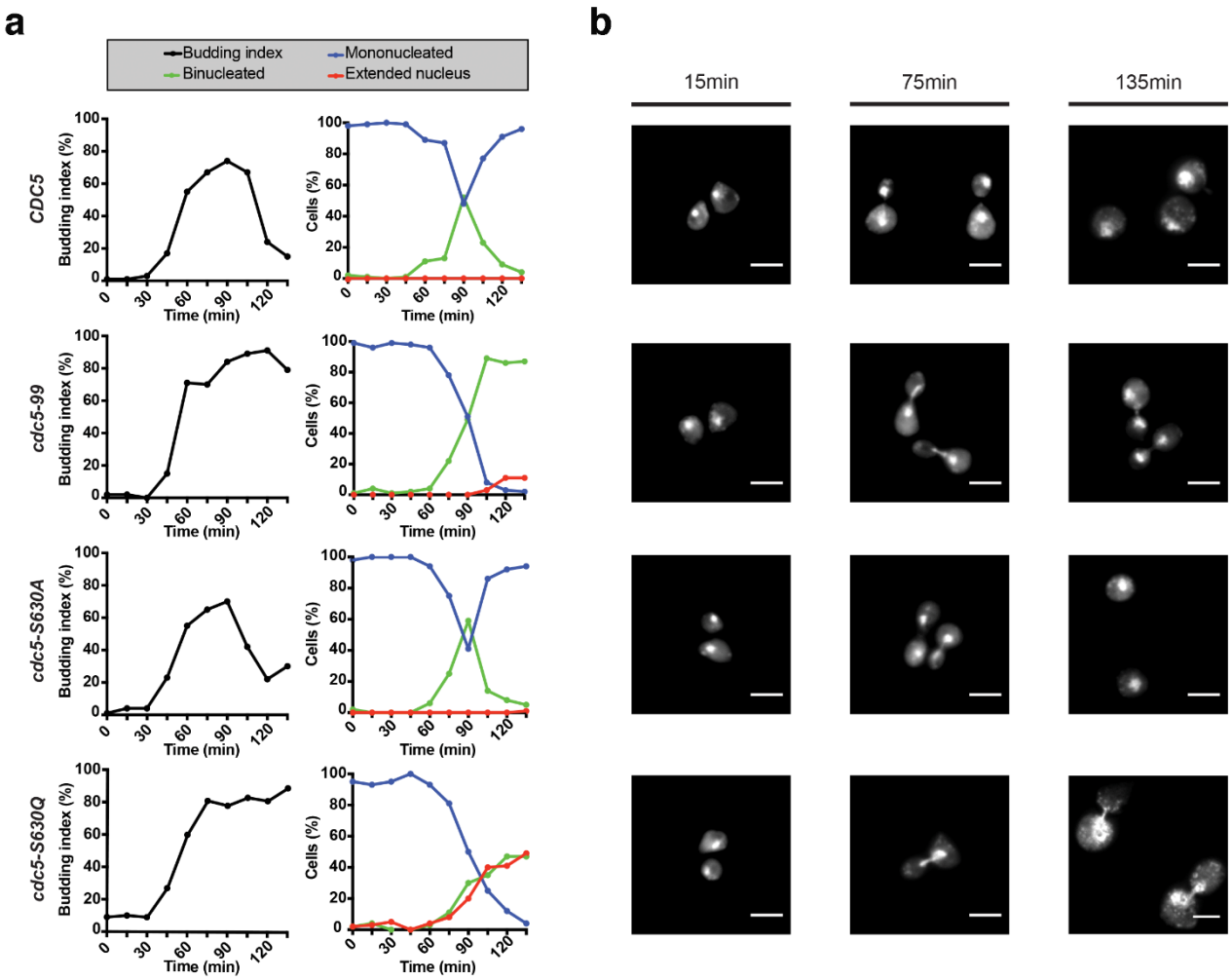

Figure 3.7. Chromosome segregation defect in *cdc5-S630Q* mutants. Exponential cultures of *cdc5-S630Q* mutant and control cells were synchronized in G1 at 23 °C using α-factor. After synchronous release of cells in fresh YPAD medium at 37 °C, samples of culture were collected at regular intervals and processed to monitor the appearance of cell cycle landmarks. (**a)** Kinetics of bud formation and nucleus separation during the cell cycle. 100 cells were counted at each time point. (**b)** Micrographs showing representative nucleus morphology at the indicated times during the time-course experiment. White bar is 5 µm. Note that the 135 min micrograph for the *cdc5-S630Q* mutant shows a slightly larger surface area than other micrographs to allow visualization of 2 cells, hence the slightly shorter scale bar. (n = 4).

3.4 DISCUSSION

Genetic and structural studies have described the hierarchy of interactions involving the polo-box domain of Plks, as well as the molecular determinants mediating recognition of phosphorylated targets[31]. Phosphorylation-independent interactions mediated by polo-box domains are also common but have received less attention[23,27,32]. Here we demonstrate that the polo-box domain of Cdc5 uses distinct surfaces to bind phosphorylated and non-phosphorylated substrates. *In vitro*, Dbf4 and phosphorylated peptides can bind simultaneously and not competitively to the polo-box domain of Cdc5. Dbf4-binding to Cdc5 during the spindle positioning checkpoint promotes DDK-mediated phosphorylation of the polo-box domain of Cdc5, and this presumably prevents Cdc5 from recognizing its phosphorylated binding substrates in the MEN pathway[23]. The structure of Cdc5 bound to a Dbf4 peptide cannot recapitulate the spatial organization of the complex formed by Cdc5 and DDK, but it is plausible that additional regions of Dbf4 or Cdc7 (the kinase subunit of the DDK complex) beyond the interacting motif partially block Cdc5 access to its phosphorylated substrates.

The Dbf4-Cdc5 interaction is also necessary to phosphorylate the Mms4-Mus81 nuclease[29]. If Dbf4-binding to Cdc5 enhances its kinase activity, it would explain how the Dbf4-Cdc5 interaction promotes Cdc7 and Cdc5 phosphorylation of Mms4-Mus81. The crystal structure of zebrafish Plk1 (zPlk1) is the only one containing both the kinase and polo-box domains (Fig. 3.8a). Association of the kinase and polo-box domains is responsible for the auto-inhibition of the kinase activity of zebrafish[33]. It has been proposed that binding

of phosphorylated substrates loosens the interaction, in turn releasing the inhibition[30,33,39,40]. Indeed, the kinase domain of Plk1 has higher kinase activity than the full-length protein. Although Cdc5 auto-inhibition has not been studied, the interface mediating the interaction between the polo-box and kinase domains is conserved. Therefore, the compact form visualized in the structure of zPlk1 likely provides a general description for the auto-inhibited state of Plks. Superimposition of the polo-box domain of Cdc5 onto that of zPlk1 reveals that the Dbf4-binding surface overlaps with the surface where the kinase domain binds (Fig. 3.8a,b). Although only the RSIEGA motif of Dbf4 is required for the interaction with the polo-box of Cdc5, binding of the DDK complex likely introduces additional contacts that may detach the polo-box domain from the kinase domain of Cdc5 (Fig. 3.8c).

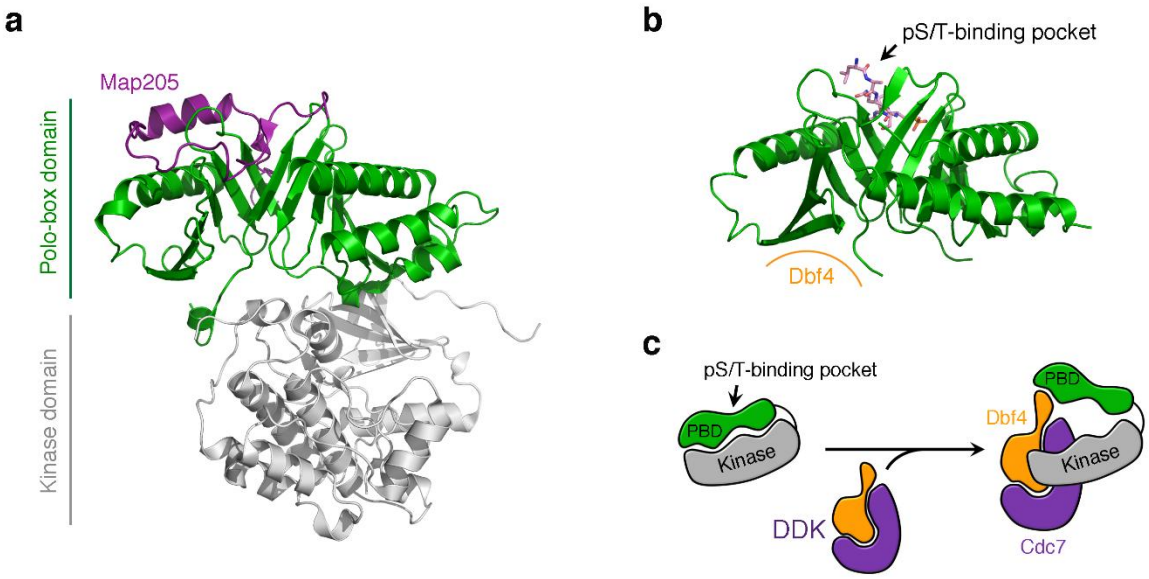

Figure 3.8. Comparison of yeast Cdc5 and zebrafish Plk1. (a) Ribbon diagram of the structure of zebrafish Plk1 in complex with Map205 (purple). The kinase domain (grey) and the polo-box domain (green) are labeled. **(b)** Ribbon diagram of the structure of the polo-box domain of Cdc5 in complex with the Spc72^{P} peptide shown in the same orientation as **(a)**. The Spc72^{P} is shown as color-coded sticks and the Dbf4-binding interface indicated in orange. **(c)** Model depicting how the DDK complex may bind to Cdc5. Dbf4 interacts with the polo-box domain (PBD) of Cdc5, but additional interactions mediated by the Dbf4 or Cdc7 subunits of the DDK complex may contribute to the interaction and potentially alter the kinase activity of Cdc5.

The Cdc5-Dbf4 structure is the first of a polo-box domain in complex with a binding partner that interacts through a surface beyond the conserved pS/T-binding pocket and, thus, provides new ways of modulating Plk activity. Importantly, the interaction between Dbf4 and Cdc5 has allowed us to identify a conserved residue (Ser630) that is critical for Cdc5 function. Mutation of this residue results in an unexpected defect in anaphase progression *in vivo*. Classic studies by Hartwell and colleagues have shown that *cdc5* mutants arrest in the cell cycle with fully segregated chromosomes in a telophase-like terminal state[41,42]. This phenotype reflects the mitotic exit defect of *cdc5* mutants, and more recent analyses of Cdc5-deficient cells have revealed that they are also defective in pre-telophase events, such as the release of Cdc14 from the nucleolus in early anaphase[43] and the phosphorylation of cohesin at the metaphase-anaphase transition[8]. Importantly, these defects were not previously associated with severe consequences on chromosome segregation, as we have observed in *cdc5-S630Q* mutant cells. This strongly suggests that the novel Dbf4-binding pocket we have characterized is promoting a *hitherto* unknown function of Cdc5 at the metaphase-anaphase transition and/or in early anaphase.

Although possible, it seems unlikely this function is related to Cdc5 phosphorylation of Scc1/Mcd1/Rad21, since complete abrogation of this regulatory event results in a mild delay in cohesin inactivation and does not lead to a strong anaphase arrest phenotype[8]. Rather, we favor the hypothesis that the polo-box domain of Cdc5 controls a currently unknown but essential process in mid-mitosis. This interpretation is consistent with our previous observation that inactivation of the canonical phosphopeptide-binding activity of Cdc5 PBD does not impact cell viability, whereas full inactivation/deletion of the polo-box domain is a lethal event *in vivo*[35]. It is also interesting to note that cells defective in Cdc7, the main binding partner of Dbf4, were originally characterized as defective in the medial stage of cell division[42], similar to the phenotype we observed in *cdc5-S630Q* mutants. It is thus possible that the regulatory interplay between Cdc5 and Dbf4 goes beyond these two proteins and requires the Cdc7 kinase to take effect. Indeed, the Dbf4 peptide does not affect the kinase activity of Cdc5 (Supplementary

Fig. 3.5), reinforcing the idea that additional interactions may be required. Apart from this scenario, it is important to consider the fact that the Dbf4-binding pocket on Cdc5 might mediate interactions with other binding partners, including the Cdc5 kinase domain itself, thus leading to a complex network of combinatorial interactions for the regulation of mitosis by Cdc5/PLKs. It will be interesting to test these possibilities and their physiological implications in future studies.

Acknowledgements

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Author contributions

A.W.A., L.L.-L., S.B., J.R.G., G.M., D.D., A.G. designed the experiments and analyzed data. A.W.A., L.L.-L., S.B., J.R.G. performed experiments and prepared figures. A.G. and D.D. wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

3.5 MATERIALS AND METHODS

Production of the Cdc5 polo-box domain

The plasmid encoding *Saccharomyces cerevisiae* Cdc5 (pMW537) was a kind gift from Prof. Michael Weinreich. For the crystallographic analysis, we subcloned the polo-box domain of Cdc5 (residues 418–705) in the pPROEX HTa vector (pAG 8531) (Invitrogen Life Technologies). The Cdc5-A567W variant of the polo-box domain was generated by site-directed mutagenesis and clones were confirmed by DNA sequencing (Nanuq, McGill University and Génome Québec Innovation Centre). Expression and solubility of the Cdc5 polo-box domain was optimized as described earlier[44]. For large scale expression, Cdc5 plasmids were transformed in BL21 (DE3) cells containing a plasmid encoding for rare tRNAs. Cultures were grown in Luria-Bertani media to OD_{600} ~ 0.6, induced by addition of 2 mM isopropyl ß-D-1-thiogalactopyranoside, and incubated overnight at 16 °C with orbital agitation.

Cell pellets were resuspended in 50 mM TRIS-HCl pH 7.0, 500 mM NaCl, 1.4 mM 2-mercaptoethanol, 5% glycerol and lysed by sonication. Lysates were cleared by centrifugation at 39,000 *g*, and the supernatants loaded onto a HiTrap nickel-chelating HP column (GE Healthcare). His_6-tagged Cdc5 was eluted at 240 mM imidazole. The histidine tag was removed with tobacco etch virus (TEV) protease, and tagless Cdc5 further purified by affinity and size-exclusion (Superdex 75 (10/300) GL, GE Healthcare) chromatography. Purified Cdc5 was kept in storage buffer (50 mM TRIS-HCl pH 7.0, 200 mM NaCl, 1.4 mM 2-mercaptoethanol, 5% glycerol). Protein concentration was determined using the Beer-Lambert equation with an extinction coefficient of 38,850 $M^{-1}cm^{-1}$ for Cdc5, and 44,350 $M^{-1}cm^{-1}$ for Cdc5-A567W. Purified proteins were monodisperse in solution as judged by dynamic light scattering.

For the circular dichroism measurements, Cdc5 and Cdc5-A567W (7 µM) were buffer exchanged to 50 mM TRIS-HCl pH 7.0, 150 mM NaF, 1.4 mM 2-mercaptoethanol, 5% glycerol. Data was collected at room temperature using a Chirascan instrument from Applied Photophysics. Twenty iterations were done for each run, and measurements were recorded between 190–250 nm wavelength. Data was analyzed using the software provided with the Chirascan system.

Crystallization and structure determination

Crystals of Cdc5 grew in 100 mM Bis-TRIS Propane pH 8.5, 20% PEG 3350 (v/v), 200 mM sodium formate, and cryo-protected with 8% glycerol. A complete data set was collected at the 08B1 beam line at the Canadian Light Source, and data was processed and scaled using XDS[45] (Table 3.1). The Cdc5 structure was determined by molecular replacement using a fragment of the polo-box domain of human Plk1 (PDB ID: 1Q4O) as the search model. An initial model was built using auto-build in PHENIX and improved by iterative cycles of manual model building in Coot and refinement in PHENIX[46,47] (Table 3.1).

Peptides derived from Spc72 (^{227}SLAQSpSPAGSQ^{237}) and the polo-interacting region of Dbf4 (^{76}RARIERARSIEGAVQVSKGTG^{96}) were purchased from GenScript and resuspended in storage buffer. The polo-box domain of Cdc5 (5 mg/mL) was mixed with Spc72^{P} at a 1:15 (protein:peptide) molar ratio and incubated overnight at 4 °C prior to crystallization trials. Crystals of Cdc5-Spc72^{P} grew in 100 mM Bis-TRIS pH 6.5, 25% PEG3350 (v/v), 200 mM $MgCl_2$, 100 mM CsCl, and were cryoprotected with 10% glycerol. Cdc5 (5 mg/mL) was mixed with Dbf4 at a 1:10 (protein:peptide) molar ratio and incubated overnight at 4 °C prior to crystallization trials. Crystals of Cdc5-Dbf4 grew in 20% PEG 3350, 200 mM sodium potassium tartrate, 10 mM trimethylamine hydrochloride, and were cryo-protected with 14% glycerol. Complete data sets of the Cdc5-Spc72^{P} and Cdc5-Dbf4 complexes were collected at the 17-ID beam line at Advanced Photon Source (at Argonne National Laboratory), and processed using MOSFLM[48] (Table 3.1). The structures were determined by molecular replacement using the polo-box domain of Cdc5 as the search model. Initial models of the Cdc5-Spc72^{P} and Cdc5-Dbf4 structures were further improved by iterative cycles of manual model building in Coot and refinement in PHENIX[46,47] (Table 3.1). Figures showing molecular structures were generated using PyMOL[49]. Crystal structures have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID: 6MF4, 6MF5, and 6MF6).

Isothermal calorimetry experiments

The Cdc5-Spc72^{P} and Cdc5-Dbf4 interactions were analyzed using the Nano ITC instrument (TA Instruments), while the interaction between Cdc5-A567W and Dbf4 was analyzed using the MicroCaliTC$_{200}$ instrument (Malvern Instruments Inc.). Peptides derived from Dbf4 (^{73}EKKRARIERARSIEGAVQVSKGTG^{96}), and Spc72^{P} (^{222}DKEEFLSLAQSpSPAGSQ^{237}), as well as a non-phosphorylated variant of the Spc72 peptide were purchased from GenScript and resuspended in storage buffer supplemented with 20 mM EDTA pH 8.0. The Cdc5-peptide interactions analyzed using the Nano ITC instrument were designed by filling the chamber cell with 30 µM protein and the injection syringe with 280 µM peptide. The ternary complex (Dbf4-Cdc5-Spc72^{P}) was analyzed by filling the chamber cell with either Cdc5-Dbf4 or Cdc5-Spc72^{P} (at a 1:4 molar ratio of protein:peptide), and the injection syringe with 280 µM peptide. Data was processed using the NanoAnalyze program (TA Instruments). The interaction between Cdc5-A567W and Dbf4 was analyzed with 45 µM protein in the chamber cell and 1.2 mM peptide in the injection syringe. Data was processed using the MicroCaliTC$_{200}$ program (Malvern Instruments Inc.).

Saturation Transfer Difference (STD) Analysis

All samples were either dissolved or buffer exchanged into 20 mM sodium phosphate buffer pH 7.0, 200 mM NaCl, 20 mM EDTA pH 8.0, 1.4 mM 2-mercaptoethanol, >99% D_2O. The concentration of Cdc5 was kept at 20 µM, while the Dbf4 (^{73}EKKRARIERARSIEGAVQVSKGTG^{96}) and Spc72^{P} (^{222}DKEEFLSLAQSpSPAGSQ^{237}) peptides were at 500 µM, unless otherwise specified. STD experiments were acquired at 298 K on a Bruker Avance 850 MHz spectrometer with a TXI probe[50,51,52]. The spectra were acquired with 32 K points, a spectral width of 16 ppm and a carrier frequency of 4.7 ppm. STD NMR spectra were acquired with 2 K scans, while saturation transfer reference (STR) spectra were acquired with 256 scans. Spectra were processed in Topspin 3.5 using an exponential multiplication window function

with a 3 Hz line broadening. STD/STR ratios were then compiled for the Spc72^{P} peptide in the absence and presence of Cdc5 or Cdc5 bound to Dbf4.

Yeast strains

All yeast strains used in this study were constructed in the W303 background and their relevant genotypes are summarized in (Supplemental Table 3.2). Standard conditions and procedures were used for yeast growth, sporulation and tetrad dissection. Growth of temperature-sensitive (ts) mutants in liquid media was performed at 23 °C (permissive temperature) and 37 °C (non-permissive temperature).

Construction of *cdc5* mutant strains

Site-directed mutagenesis of plasmid p409 (YCplac22-*CDC5*-T_{ADH1}-*HIS3MX6*) was used to insert mutations in *CDC5* gene[35]. Mutant alleles were inserted at their endogenous locus by plasmid digestion and subsequent transformation into a diploid wild-type (WT) strain. Plasmid digestion generated a DNA fragment encompassing *CDC5* ORF along with its *HIS3MX6* marker. The backbone of p409 was lost during the yeast transformation and integration of selected mutations was confirmed through sequencing of the *CDC5* locus. Diploid transformants were then sporulated and dissected. Haploid segregants were isolated and the presence of the required mutation (and absence of any secondary mutation) was established by whole gene sequencing.

Proliferation assays

The temperature-sensitive (ts) growth behavior of *cdc5* mutants was monitored on solid YPAD medium at the following temperatures: 23, 30 and 37.5 °C. Their sensitivity to DNA replication inhibitors and DNA damaging agents was assessed at 23 °C on YPAD medium containing either 100 mM hydroxyurea (HU) or 0.4 µM 4-nitroquinoline N-oxide (4-NQO). In all cases, cultures of yeast strains were diluted fivefold and

subsequently spotted on solid medium, using a starting OD_{600} of 0.2 in the first dilution[36]. Yeast were allowed to grow for approximately 36–72 hours at the indicated temperatures prior to scanning plates. The experiment was repeated 5 times (n = 5).

Budding index assay

Conditions for growth and cell synchronization/release are as described earlier[53]. To compare strains that arrest in mitosis with those that do not, α-factor was re-added to all cultures 75 minutes after the initial G1 release. This was necessary to prevent yeast strains from re-starting a new cell cycle after completion of the first cycle. After the initial α-factor release, 1 mL aliquots of yeast cultures were taken every 15 minutes, for a total of 135 minutes. Samples of cells were centrifuged and then suspended in 1 mL of a 70% ethanol solution for 12–24 hours. Cells were subsequently resuspended in 500 µL 0.1 M potassium phosphate buffer pH 6.4 and briefly sonicated before performing bud morphology assessment by light microscopy, as previously published[53]. This experiment was repeated 4 times (n = 4).

Fluorescence microscopy

Visualization of DNA morphology was performed on a Nikon Eclipse T*i*2 inverted microscope. The microscope was equipped with a 100×/NA 1.45 objective. Staining of the nuclei was performed with 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 2 µg/ml in cells suspended in 0.1 M potassium phosphate pH 6.4 buffer. Aliquots of cells containing DAPI were incubated at room temperature (~22 °C) in the dark, for a total duration of 20 minutes before being washed with a 0.1 M potassium phosphate solution at pH 6.4. Final samples were resuspended in 100 µl 0.1 M potassium phosphate buffer pH 6.4 and 5 µl of each sample were used for observation. The criteria used to classify yeast nucleus morphology were as follow: (1) mononucleated, (2) binucleated with or without minor anaphase bridges, and (3) extended/undivided nucleus. To be classified in category 2, nuclei must be free of anaphase bridges or be

connected by a small bridge only (*i.e.*, the width of the bridge being smaller than the radius of the nucleus). 100 cells were scored at each time point. Pictures were taken 15, 75, and 135 min after α-factor release. This experiment was repeated 4 times (n = 4). Imaging of Spc42-GFP position in cells and calculation of distances that separate Spc42-GFP foci was performed as previously described[35,38] using the 3D measurement module of the NIS-Elements software (Nikon Instruments Inc.). This experiment was repeated 3 times (n = 3).

In vitro kinase assays

Full-length Cdc5 was produced and purified as previously described[7]. The *in vitro* kinase assays were done as described earlier[35] with minor modifications. In brief, 0.125 pmol of Cdc5 were incubated for 30 min at 30 °C in kinase reaction buffer containing 5 µg of dephospho-casein (Sigma), 25 mM Tris-HCl pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 100 µM ATP, 1 µCi $ATP_{\gamma}32^{P}$, 0.5 mM EDTA, 25 µM bromolevamisole oxalate, 5 mM β-glycerophosphate, 1 µM benzamidine, 10 µM pepstatin A, 5 µg/mL leupeptin, 0.2 mM tungstate, and 0.1 mM Na_3VO_4. Reaction mixtures were resolved in 13% SDS-polyacrylamide gels. Phosphorylated casein was detected using a Typhoon Trio (GE Healthcare) and quantified using Image J[54]. The effect of Dbf4 was assessed by addition of 4 molar excess of a Dbf4-derived peptide (^{73}EKKRARIERARSIEGAVQVSKGTG^{96}) to the kinase reactions. Experiments were performed in triplicates (n = 3).

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3.7 SUPPLEMENTARY FIGURES

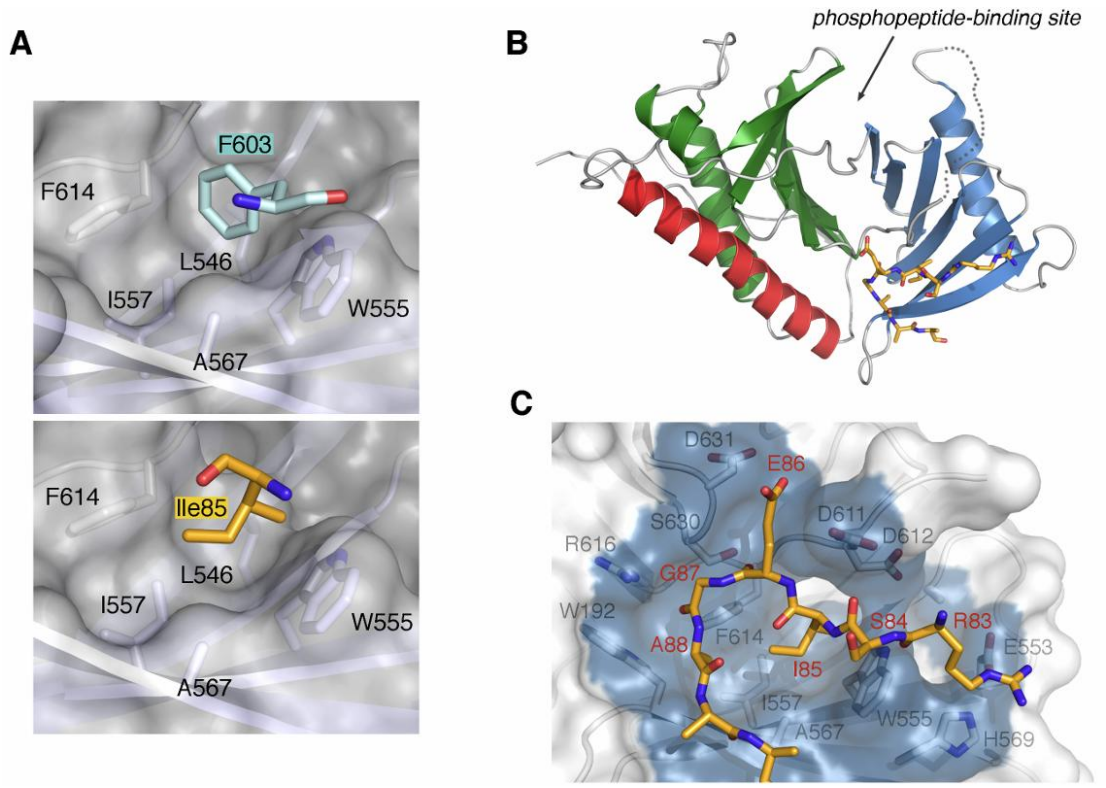


Figure 3.S1. Conserved hydrophobic pocket on polo box 1. a) Detail of the Cdc5 structure with Phe603 residue from the cleaved a2-b7 loop sitting in the hydrophobic pocket defined by Leu546, Trp555, Ile557 and Ala567 (top). Detail of the Cdc5 structure crystallized in the presence of the Dbf4 peptide with Ile85 (from the Dbf4 peptide) modelled based on the difference electron density maps (see Fig. 3a). Note that the extra electron density is found at a similar position to that of Phe603 on the crystal of the polo-box domain of Cdc5 on its own (see Fig.1c). **b)** Model of the Dbf4 peptide in the structure of Cdc5 bound to Dbf4. **c)** Detail view of the Dbf4-binding pocket with Dbf4 residues (red labels) shown as color coded sticks and Cdc5 residues indicated with grey labels.

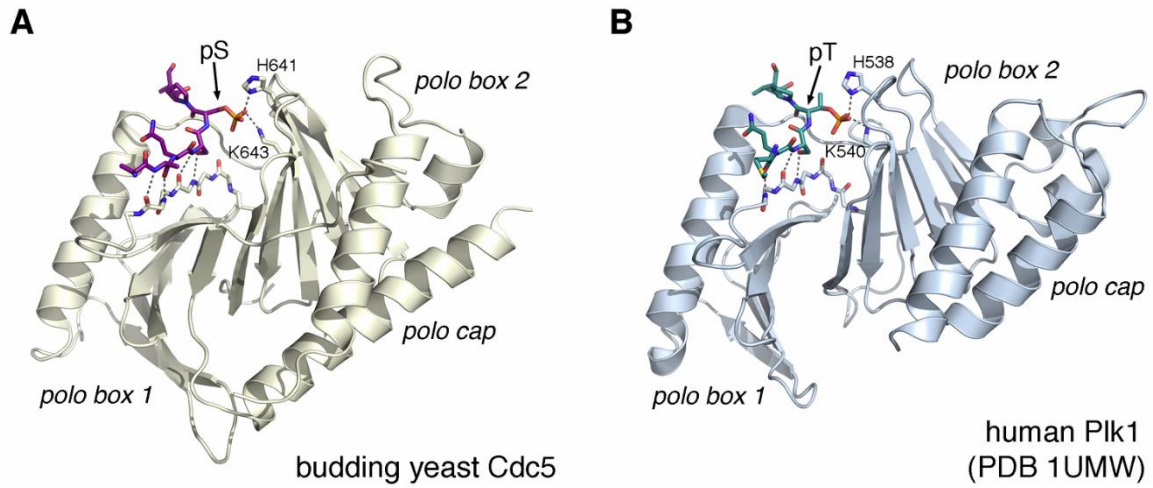


Figure 3.S2. Polo-box domains have a conserved phosphopeptide binding mode. The polo box domain of S. cerevisiae Cdc5 **(a)** and human Plk1 **(b)** bind target phosphopeptides at the groove defined by the two polo boxes and use conserved His and Lys residues to stabilize the phosphate group.

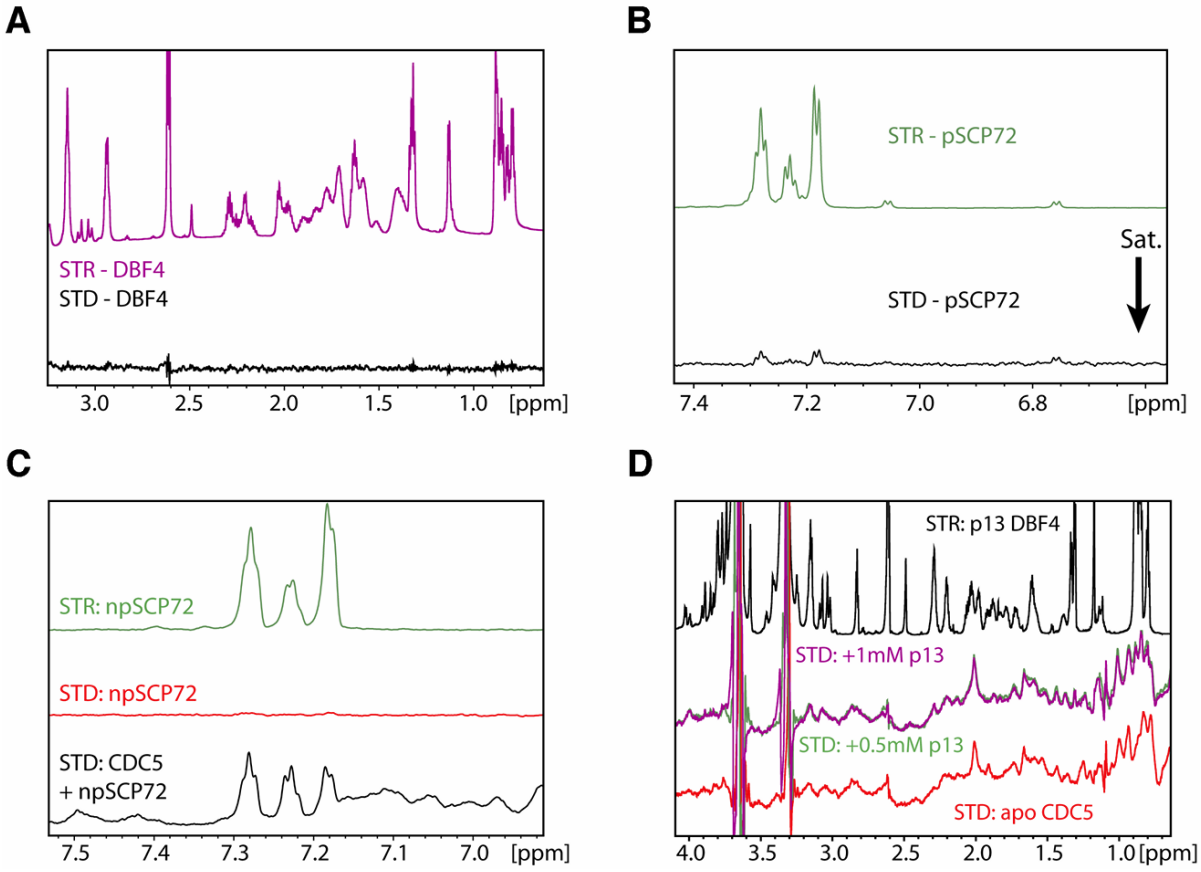
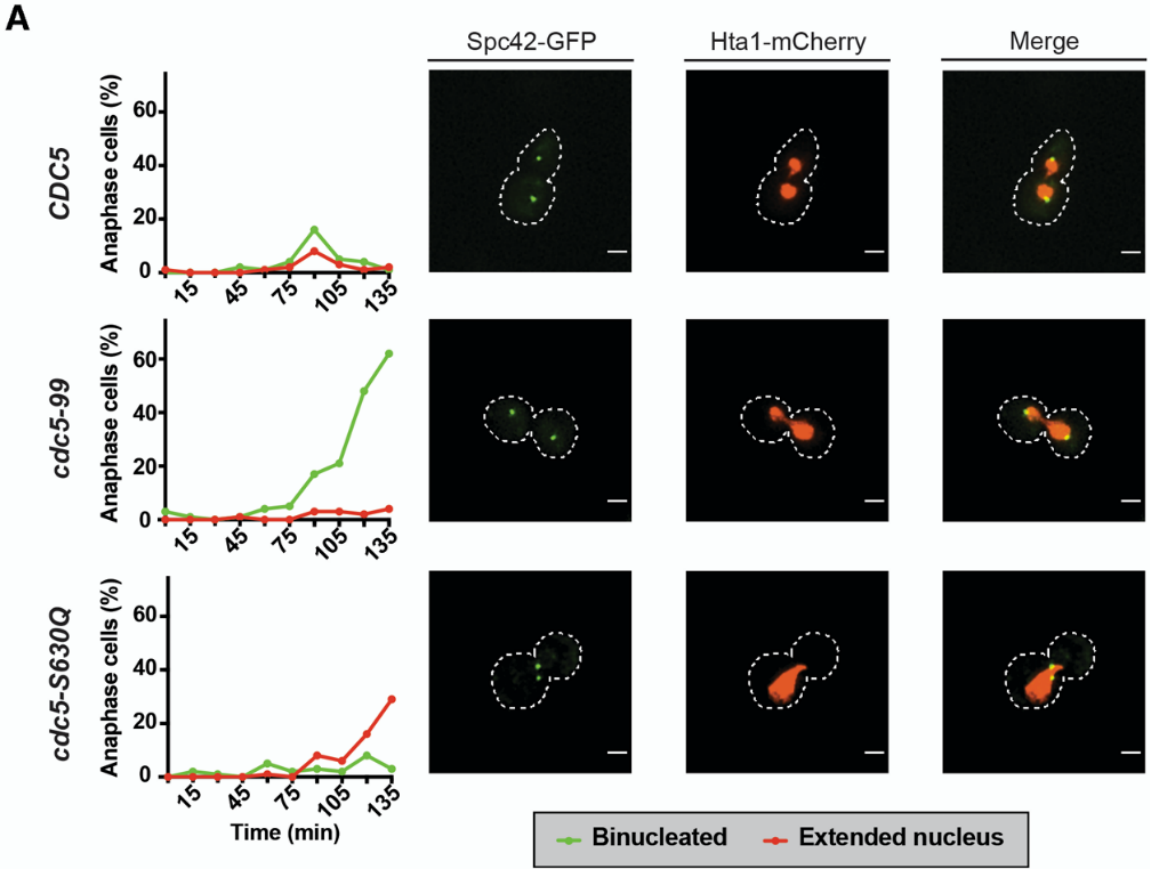
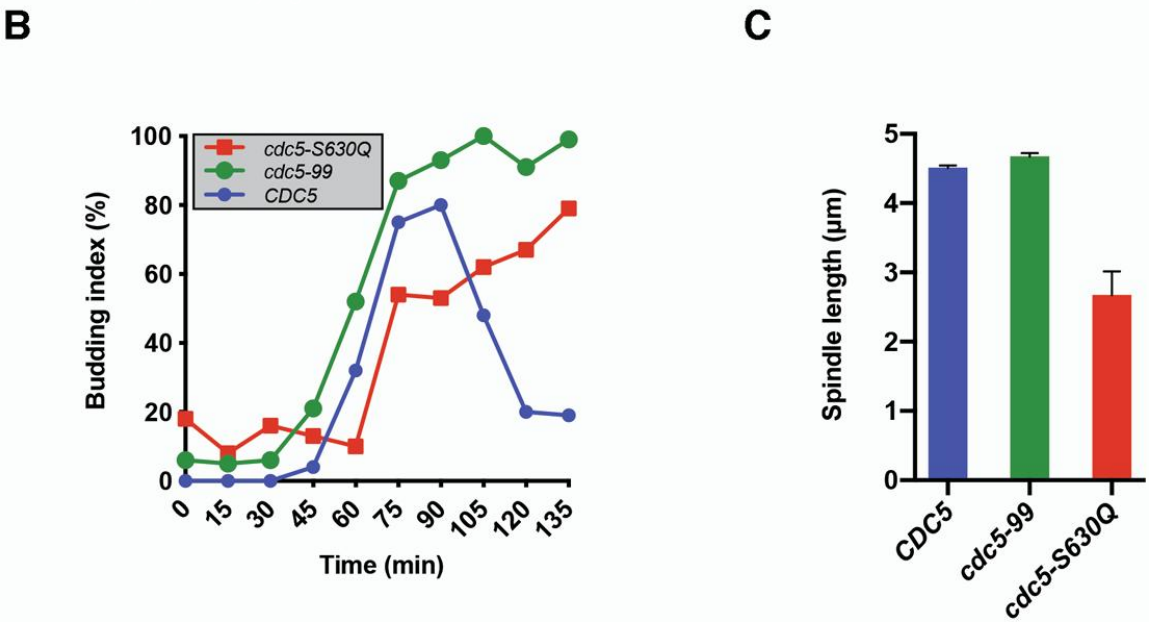


Figure 3.S3. Control STD NMR spectra of the individual substrates. STD spectra of Dbf4 **(a)**, Scp72^{P} **(b)**, and Spc72 **(c)**. **d)** STD spectra of a short Dbf4 (13 amino acids: p13) peptide. The STR spectra and STD spectra of Cdc5 are provided for comparison.


A
Spc42-GFP
Hta1-mCherry
Merge
CDC5
cdc5-99
cdc5-S630Q
Anaphase cells (%)
0
20
40
60
15
45
75
105
135
Time (min)
Binucleated
Extended nucleus


B
Budding index (%)
cdc5-S630Q
cdc5-99
CDC5
0
20
40
60
80
100
0
15
30
45
60
75
90
105
120
135
Time (min)

C
Spindle length (µm)
0
1
2
3
4
5
CDC5
cdc5-99
cdc5-S630Q

Figure 3.S4. Nucleus and spindle pole body separation phenotypes of the *cdc5-S630Q* mutant. Exponential cultures of *CDC5, cdc5-99* and *cdc5-S630Q* strains expressing Spc42-GFP and Hta1 mCherry were synchronized in G1 using α-factor at 23 °C. After synchronous release from the G1 arrest, cells were grown at 37 °C and samples collected at regular intervals to monitor the morphology of nuclei and distance separating SPBs by fluorescent microscopy. **(A)** The graphs on the left show a quantification of nuclear morphology for each strain during the time-course experiment, whereas the micrographs on the right show the morphology and position of nuclei/SPBs in mid-anaphase cells. The quantification of nuclear morphology focused on two classes of Hta1-mCherry signal observed during anaphase: binucleated cells with little or no chromatin connecting the nuclear masses or mitotic cells with extended/stretched nuclei in the early stages of chromosome separation. Cell outlines were marked with dotted lines. Bar is 2 μm. **(B)** Kinetics of bud formation (i.e., budding index) in cultures of yeast described in panel (A). At least 100 cells were counted at each time point. Note that we observed *cdc5-S630Q* cells expressing Spc42-GFP and Hta1 mCherry markers progressed in the cell cycle with a slight delay relative to mutants carrying wild-type *SPC42* and *HTA1*, possibly reflecting a synthetic interaction between the CDC5 allele and fluorescent markers. **(C)** Analysis of spindle length in mid-anaphase cells expressing *CDC5, cdc5-99*, and *cdc5-S630Q* mutants. To measure the length of the nuclear spindle in yeast, the distance separating Spc42-GFP foci was determined in at least 30 anaphase cells, as previously described (Maddox et al (2012) Methods Enzymol. 505:81-103). Error bars represent SEM (n=3).

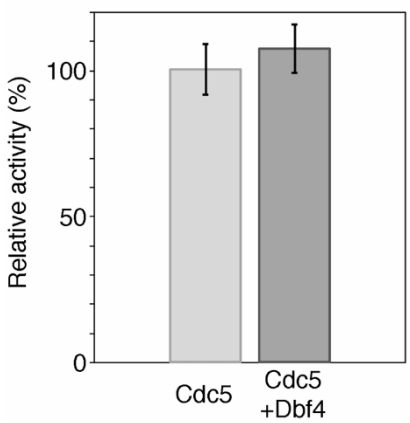


Figure 3.S5. Effect of the Dbf4 peptide on the kinase activity of Cdc5. (A) The kinase activity of Cdc5 (0.125 pmol) was tested as previously described (Ratsima et al (2011) PNAS 108(43), E914-E923) in the presence/absence of the Dbf4 peptide (^{73}EKKRARIERARSIEGAVQVSKGTG^{96}). Error bars represent SEM over 3 measurements.

3.8 SUPPLEMENTARY TABLES

Table 3.S1. STD/STR ratios of the Spc72 peptides.

Sample	STD/STR (1)* ($x10^{-3}$)	STR/STR (2) ($x10^{-3}$)	STD/STR (3) ($x10^{-3}$)
Spc72^{P}	9±1	7±2	7±1
Spc72	9±3	N/D	8±2
Cdc5-Spc72^{P}	97.0±0.5	125±1	50.4±0.4
Cdc5-Spc72	70±2	107±3	38±1
Dbf4-Cdc5-Spc72^{P}	89.8±0.5	114±1	50.5±0.4

**The STD/STR ratios were calculated for the three peaks in Figure 5B*

Table 3.S2. Yeast strains used in this study.

	Strain #	Relevant genotype
Figure 6	D4107	*MAT**a** CDC5*
	D1224	*MAT**a** cdc5-77::HIS3MX6*
	D224	*MAT**a** smc5-6::HIS3*
	D6502	*MAT**a** cdc5-H569A::HIS3MX6*
	D6460	*MAT**a** cdc5-R616A::HIS3MX6*
	D6462	*MAT**a** cdc5-S630A::HIS3MX6*
	D6505	*MAT**a** cdc5-S630Q::HIS3MX6*
	D6507	*MAT**a** cdc5-A567W::HIS3MX6*
Figure 7	D4107	*MAT**a** CDC5*
	D777	*MAT**a** cdc5-99::HIS3MX6*
	D6462	*MAT**a** cdc5-S630A::HIS3MX6*
	D6505	*MAT**a** cdc5-S630Q::HIS3MX6*

4 CHAPTER IV: GENERAL DISCUSSION

4.1 Centrosomes and docking codes: A two-tier logic for modulating PLK activity

This thesis explored how centrosome docking and combinatorial PBD binding to receptors and substrates broadens the functional repertoire of the budding yeast Polo-like kinase Cdc5 during mitosis, both under normal and genotoxic conditions. Chapter II crucially establishes that Cdc5, enriched at SPBs, spatially orchestrates DNA damage adaptation –the decision to resume division while irreparable chromosomal lesions persist– by converting MTOCs into signal-integration hubs that support checkpoint adaptation. This allowed me to identify the putative stepwise sequence of events and interactors that dynamically facilitate Cdc5-driven DNA damage adaptation at SPBs in cells carrying persistent chromosomal lesions. In Chapter III, I shifted focus to the molecular basis of Cdc5 substrate interactions. We uncovered that Cdc5 PBD can engage in simultaneous and non-competitive dual substrate binding, broadening the kinase's reach and providing a conceptual framework for fine-tuning its activity across key cellular pathways. We revealed, using structural and biochemical studies, a novel unexpected function for a second substrate-binding PBD interface in Cdc5-driven mitotic processes. Disrupting this non-canonical interface by point mutation causes a unique early anaphase phenotype, dissimilar to the classical mitotic exit defects typically associated with *cdc5* mutants. By exploring the intricacies of Cdc5 regulation from two different angles, these findings highlight a previously unappreciated two-tiered control system that equips PLKs to meet shifting mitotic demands. The sections below revisits SPB-directed checkpoint adaptation (section 4.2), dissects the logic of dual-site PBD targeting (section 4.3), and integrate both threads into a unified model that explores how PLK function undergoes careful regulation to strike a seamless balance between cell proliferation and genome stability (section 4.4).

4.2 Living with lesions: Exploring the adaptation response to unrepairable DNA damage

Although DNA damage adaptation was once believed to depend primarily on the passive deactivation of core DDR effectors (Donnianni et al. 2010; Vidanes et al. 2010; Pellicioli et al. 2001), rising evidence now shows that Cdc5 PLK enrichment at SPBs is equally pivotal for checkpoint silencing (Ratsima et al. 2016). *How (and why) does Cdc5 exploit yeast centrosomes during adaptation?* Under normal cycling conditions, the peak of Cdc5 activity in G2/M correlates with the timing of adaptation (Botchkarev et al. 2017). Albeit functionally restrained during DDR activation in a Rad53-dependent manner, Cdc5 nonetheless accumulates at both nuclear SPB plaques, raising questions about how this spatial targeting facilitates adaptation (Sanchez et al. 1999; T. Zhang et al. 2009; Edenberg et al. 2014; Coutelier et al. 2023; Botchkarev et al. 2017). Previous studies have delineated three pivotal regulatory layers governing Cdc5-driven checkpoint adaptation: 1) Protein dosage – overexpression of Cdc5 accelerates adaptation kinetics, whereas *CDC5/cdc5Δ* diploids reveal that *CDC5* is haplo-insufficient for adaptation, underlining a dose-dependent threshold for this response (Donnianni et al. 2010; Vidanes et al. 2010); 2) Catalytic activation state – the *cdc5-T238A* T-loop phosphorylation mutant impairs timely Cdc5 localization to SPBs and specifically abolishes adaptation, despite supporting normal cell cycle progression under unperturbed cycling conditions (Rawal et al. 2016); 3) Spatial targeting – a *cdc5-16* PBD mutant that fails to drive adaptation can be functionally rescued by artificial tethering at SPBs (Ratsima et al. 2016).

Here, we provide the first comprehensive evidence that yeast centrosomes actively contribute to DNA damage checkpoint override by functioning as modulatory signaling hubs co-opted by Cdc5 PLK to drive adaptation. Consistent with the emerging concept of centrosomes/SPBs as signal transduction organizing centers (STOCs) (Langlois-Lemay and D'Amours 2022), we show that cells bearing persistent DNA lesions endow SPBs with additional signaling functions that dynamically coordinate DNA damage adaptation. Our findings suggest that PLK activity is spatially organized to integrate and respond to a convergence of signals at the interface of nuclear, chromosomal, and centrosomal remodeling. As DNA damage adaptation relies

on multiple interdependent regulatory nodes, cells need a versatile, spatially focused kinase (i.e., Cdc5) that can concurrently engage diverse cellular modules to dismantle checkpoint signaling and restore cell cycle progression under genotoxic stress. The following section contextualizes our findings within the broader mechanistic framework of checkpoint adaptation and PLK regulation upon exposure to DNA damage, and discusses how these novel insights address previously unresolved questions while informing potential therapeutic interventions.

4.2.1 Diversifying the poles: Yeast centrosomes in checkpoint adaptation

Our identification of several structural SPB components as previously unrecognized members of Cdc5 interactome during DNA damage adaptation suggests that Cdc5 *actively* engages SPBs as dynamic signaling hubs in this context. From a systems-level point-of-view, the transient exploitation of core SPB proteins to integrate and transmit adaptation signals represents an evolutionarily economical strategy: by modulating its canonical interactome, Cdc5 is able to trigger a coordinated, multi-pathway response – an energy-efficient way to reinstate cell cycle progression under fluctuating environmental or genomic conditions.

The observation that Cdc5 transiently engages Nud1, Spc110 and Spc72 as SPB scaffolds, while targeting Cnm67 and Mps3 as phospho-substrates during adaptation, aligns well with current models of Cdc5 enrichment dynamics at SPBs (Figure 4.1) (Botchkarev and Haber 2018). Upon DNA damage, Cdc5 is known to localize to the nuclear face of both SPBs, a process that our findings attribute to the PBD-compatible scaffolding properties of Spc110 (Botchkarev and Haber 2018; Snead et al. 2007). This raises the question: *What functional purpose might this transient inner SPB plaque sequestration serve in modulating Cdc5 spatiotemporal dynamics during adaptation?* This phase could plausibly function as a provisional restraining phase, where premature Cdc5 activity at critical sites is prevented to ensure checkpoint disengagement prior to full mitotic reactivation. By binding to Spc110 during the early stages of DDR signaling, Cdc5 becomes strategically positioned at the nuclear face of SPBs, in close proximity to 1) actively

processed DNA lesions and associated chromatin remodeling complexes, 2) spindle stability effectors – including Spc110 itself, and 3) key regulators of checkpoint signaling and mitotic progression. Given that Cdc28-mediated phosphorylation of Spc110 partially governs spindle assembly dynamics and timely mitotic exit, this site could serve as a strategic docking platform for Cdc5 to both sense and propagate signals related to cell cycle re-entry (Abbasi et al. 2022). Importantly, the active enrichment of Cdc5 at Spc110 brings the kinase into spatial proximity with the inner nuclear membrane protein Mps3, which we have identified as a decisive Cdc5 substrate during adaptation in Chapter II. The well-established role of Mps3 in anchoring persistent DSBs and eroded telomeres at the nuclear periphery may directly contribute to adaptation by spatially constraining checkpoint signaling (Oza et al. 2009; Nagai et al. 2008). Such sequestration would concentrate checkpoint effectors within a confined nuclear subdomain, thereby facilitating checkpoint silencing and global DDR dismantling at sites of damage. This DNA damage repositioning event, which relies on the momentary Cdc14-mediated dephosphorylation of Spc110 to transiently stabilize the spindle during chromosomal movements, coincides with the early phases of checkpoint-induced cell cycle arrest –typically within two hours of DDR activation– and must therefore be completed prior to cell cycle re-entry (Villoria et al. 2017; Oza et al. 2009). This timeline would ideally position Cdc5 at Spc110 to support adaptation. In this role, Cdc5 could 1) reinstate spindle flexibility once DNA damage is repositioned, and 2) attenuate perinuclear tension around chromosomal lesions, thereby accommodating SPB migration and spindle elongation prior to its relocation to the cytoplasmic plaque for MEN coordination (Chen 2019). This model is supported by the observation that, even under Cdc5 overexpression, *mps3-3A* mutant cells exposed to persistent DNA damage remain delayed in their escape from G2/M arrest (Figure 4.2) – perhaps due to a failure to synchronize spindle-NE remodeling with MEN activation. Moreover, the insight that Cdc5 dynamically phosphorylates Spc110 in late mitosis under unperturbed cycling conditions (Figure 4.3) further supports this model, which hinges on the canonical timing of Cdc5-Spc110 interaction at this stage of the cell cycle.

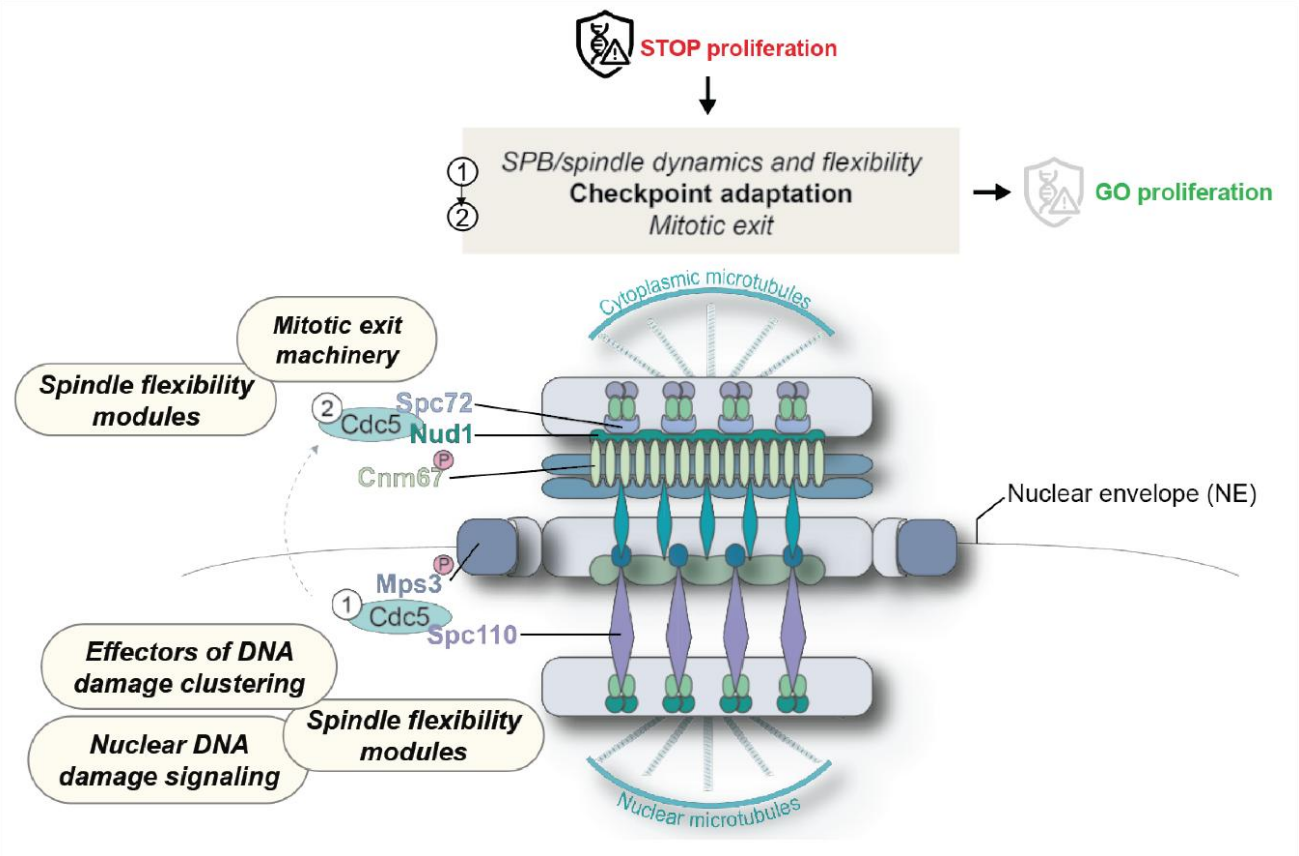


Figure 4.1. Working model of Cdc5 sequential enrichment at SPBs during the adaptation response to persistent DNA damage. Upon DNA damage-induced arrest (STOP proliferation), Cdc5 is first anchored at the nuclear SPB plaque via Spc110 (Step 1). This initial nuclear SPB pool may promote adaptation by integrating multiple pathways –DNA damage signaling and clustering, regulation of spindle dynamics, chromatin remodeling– to collectively attenuate the DNA damage response (DDR). Phosphorylation of Mps3 by Cdc5 at the nuclear SPB plaque may further coordinate DNA damage adaptation with DDR attenuation by supporting the clustering of DNA lesions at the nuclear periphery. Once DDR checkpoint signaling is silenced, Cdc5 repositions to the cytoplasmic SPB plaque by engaging the Nud1-Spc72 receptor module and phosphorylating Cnm67 (Step 2). This stepwise relocalization places Cdc5 at the crossroads of spindle flexibility modules and the mitotic exit network (MEN), thereby coupling spindle remodeling to mitotic exit and permitting cell cycle re-entry in the presence of persistent DNA damage (GO proliferation).

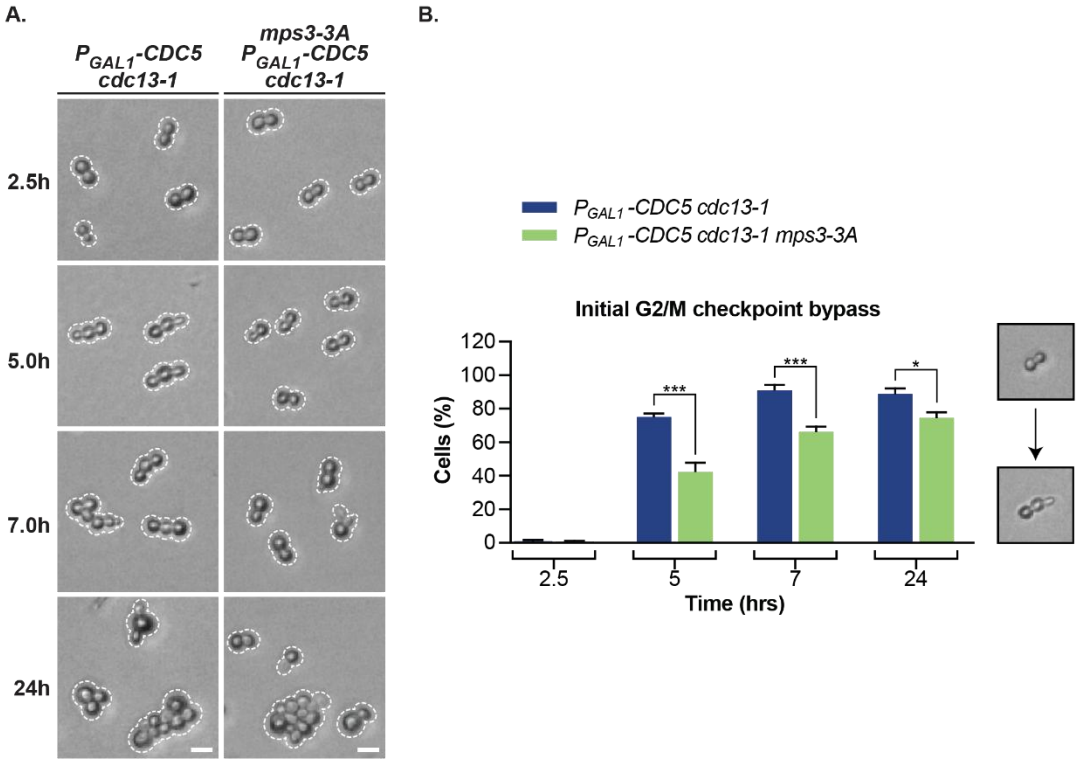


Figure 4.2. Mps3 is required for cell cycle re-entry in Cdc5-driven DNA damage checkpoint adaptation. **(A)** A *mps3-3A* mutant strain overexpressing *CDC5* (*PGAL1-CDC5*) was tested for microcolony formation in a *cdc13*-based adaptation assay at 32.5 °C. Cells were plated on solid YEP medium containing 2% galactose (YEPG). Overexpression of *CDC5* in the *mps3-3A* mutant failed to accelerate adaptation to kinetics comparable to those of the control strain, with a significant fraction of cells exhibiting delayed initiation of adaptation-dependent cell cycle re-entry. **(B)** Quantification of the fraction of cells escaping arrest for each strain in panel A. At least 100 cells per strain were counted at each time point. Data represented as mean values and SEM of at least 3 independent experiments. Statistical analysis performed using an unpaired *t* test on normally distributed values. Statistical significance is shown on graph with asterisks (*P ≤ 0.05 and ***P ≤ 0.001, respectively).

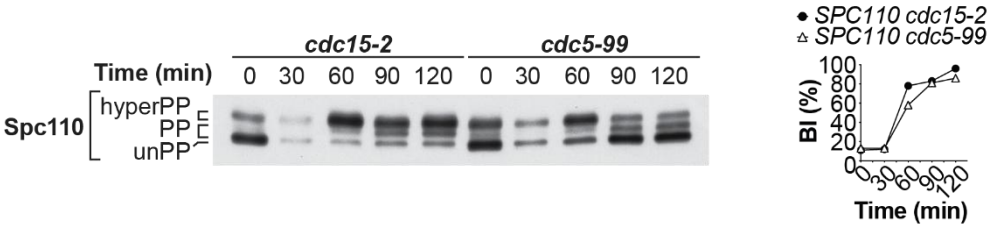


Figure 4.3. Cdc5 modifies Spc110 in vivo in a time-dependent manner. Cells synchronized in G1 with α-factor were released into a synchronous cell cycle at 37 °C, a restrictive temperature for *cdc15-2* and *cdc5-99,* and sampled at regular intervals to monitor the electrophoretic behavior of HA-tagged Spc110. The *cdc15-2* control strain induces a telophase arrest comparable to that of *cdc5-99* mutants (St-Pierre et al., 2009). Reduced Spc110 hyperphosphorylation was observed in absence of functional Cdc5 kinase activity. Other details are as described in Figure 2.5. (n=3)

By remaining anchored to the nuclear side of SPBs until cell cycle re-entry, Cdc5 is strategically positioned to progressively deactivate nuclear checkpoint signals, indirectly influence gene expression, engage in cross-talk with chromatin remodelers that process damaged DNA, and coordinate DDR deactivation with reactivation of the cell cycle machinery. For this process to unfold in a temporally controlled manner, Cdc5 activity must be transiently downregulated upon DDR activation. But *how, then, is this potent kinase held in check for hours before adaptation proceeds*? A decisive step is a transient surge in nuclear Cdc14 concentration, which triggers Cdc5 relocalization from the nuclear face to the outer SPB plaque (Botchkarev et al. 2014; 2017). A plausible model is that, upon DDR activation, both Cdc5 and nuclear export processes are transiently inhibited in a Rad53-dependent manner (Sanchez et al. 1999; T. Zhang et al. 2009; Edenberg et al. 2014; Coutelier et al. 2023; Smolka et al. 2007). As adaptation progresses, the reciprocal feedback loop linking Rad53/Chk1 deactivation to Cdc5 reactivation eventually triggers FEAR network activation and facilitates the Cdc14-mediated translocation of Cdc5 to the cytoplasmic face of the SPB, thereby sustaining mitotic exit (Liang and Wang 2007). In this context, the local accumulation of Cdc5 at nuclear-facing SPB plaques may play a pivotal role in maintaining controlled Rad53 dephosphorylation dynamics during adaptation. Prior studies, along with our own findings, demonstrate that Cdc5 strains lacking proper SPB anchoring –such as the *cdc5-16* PBD mutant and the *nud1-92 spc110-7 spc72-8* triple SPB mutant– exhibit diffuse cellular Cdc5 distribution rather than typical SPB-associated foci (Ratsima et al. 2016). Such mutants trigger an abrupt and poorly regulated wave of Rad53 dephosphorylation –mirroring the indiscriminate dephosphorylation kinetics observed when Cdc5 is overexpressed and abundantly distributed– while failing to mount an efficient DNA damage adaptation response (Ratsima et al. 2016; Vidanes et al. 2010). These findings support a potentially novel role for sustained Cdc5 anchoring at nuclear SPB plaques following DDR activation: the gradual dismantling of the Rad53 branch of the DDR signaling cascade. This localized retention may help prevent abrupt Rad53 dephosphorylation that could result from unregulated, diffuse Cdc5-Rad53 interactions. Such a model would explain why mutants with impaired

Cdc5-SPB interactions exhibit a rapid dismantling of the Rad53 signaling branch yet still display persistent defects in DNA damage adaptation. Within this framework, SPBs may act as molecular sinks that sequester Cdc5, preventing it from prematurely accessing nuclear targets –including Rad53– and thereby dictating the timing of checkpoint adaptation. Such spatial control by SPBs would echo other cell cycle regulatory strategies – for example, the phosphatase Cdc14 remains tethered in the nucleolus away from its substrates until late anaphase to prevent premature mitotic exit (Visintin et al. 1999). The duration of Cdc5 sequestration at SPBs could be governed by the kinetics of its accumulation, suggesting that a threshold level of Cdc5 at SPBs –integrated with other cell cycle cues– may have to be reached to trigger adaptation and allow mitotic re-entry under DNA damage conditions. This model aligns with the evidence that Cdc5's role in adaptation is dose-dependent, with elevated Cdc5 levels accelerating checkpoint override (Vidanes et al. 2010; Donnianni et al. 2010).

Upon its preferential enrichment at the daughter-directed cytoplasmic SPB plaque, Cdc5 becomes locally positioned to trigger mitotic exit (Botchkarev et al. 2017). As both Nud1 and Spc72 directly contribute to spindle pole asymmetry and age-biased SPB inheritance, the adaptation defects associated with the *nud1-92 spc110-7 spc72-8* SPB triple mutant could partially stem from a disruption of SPB functional asymmetry, leading to mislocalization of key adaptation factors –including Cdc5– and a failure to coordinate checkpoint relief with mitotic progression (Geymonat et al. 2020; Maekawa et al. 2007; Pereira and Schiebel 2005; Vannini et al. 2021; Hotz et al. 2012). Spatial cues at SPBs play a critical role in orchestrating multiple cell cycle processes, including checkpoint signaling. For instance, during SPoC activation, Spc72 symmetrically recruits the Kin4 kinase to both SPBs. This bilateral Kin4 signal inhibits MEN activity specifically at the daughter-facing SPB, preventing asymmetric, bud-directed mitotic exit until the spindle is correctly aligned (Maekawa et al. 2007; Pereira and Schiebel 2005). It is therefore plausible that the triple SPB mutant not only 1) lacks critical Cdc5 docking sites, thereby impairing full Cdc5 accumulation at SPBs, but also 2) disrupts spindle pole asymmetry, further contributing to the global adaptation defects observed

in this background. Given that DNA damage adaptation is dose-dependent for Cdc5, disruption of its normal asymmetric enrichment at SPBs –along with that of its cofactors– may lead to diluted kinase distribution across both poles, and compromised protein-protein interactions (Toczyski et al. 1997; Rawal et al. 2016; Vidanes et al. 2010; Donnianni et al. 2010). This, in turn, could elevate the local accumulation threshold required to effectively trigger adaptation. By contrast, routine mitotic exit –reinforced by redundant feedback loops– remains largely unaffected under unperturbed cycling conditions, as supported by our data and other studies, likely owing to a reduced dependency on Cdc5 concentration thresholds (Ratsima et al. 2016; Rawal et al. 2016). In line with the requirement to coordinate spindle flexibility with mitotic exit during adaptation, Cnm67 may fulfill a dual role as a Cdc5 substrate by contributing to both SPB/spindle architectural remodeling and local enrichment of Cdc5 at SPBs (Park et al. 2004; Snead et al. 2007). Mechanistically, the simultaneous use of SPB components as docking platforms and phosphorylation targets for Cdc5 would reflect a spatially efficient strategy to propagate signals with temporal precision. Supporting this model, Nud1 not only anchors Cdc5 at SPBs but is also dynamically modified in a Cdc5-dependent manner during mitosis (Figure 4.4), highlighting the putative multifunctionality of centrosomal scaffolds in adaptation.

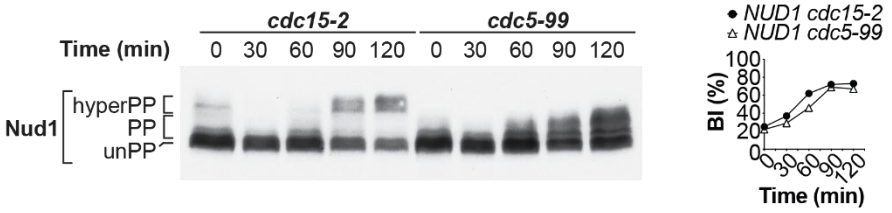


Figure 4.4. Nud1 is a substrate for Cdc5 in vivo. Cells synchronized in G1 with α-factor were released into a synchronous cell cycle at 37 °C, a restrictive temperature for *cdc15-2* and *cdc5-99*. Samples were collected at regular intervals to assess the electrophoretic mobility of HA-tagged Nud1. Loss of functional Cdc5 kinase activity resulted in reduced Nud1 hyperphosphorylation, evident from faster migration of the protein after SDS-PAGE. Other details are as described in Figure 2.5. (n=3)

An intriguing aspect of Cdc5-driven adaptation is the absolute requirement for RSC^{Rsc2} activity to achieve full DNA damage adaptation (Ratsima et al. 2016). With its reported function in both the DNA

damage- and SAC checkpoint pathways, the RSC chromatin-remodeling complex emerges as a multipurpose effector compulsory in coupling chromatin state to adaptation. Accordingly, a *rsc2Δ* mutant in a *cdc13-1* background exhibits adaptation defects that persist even upon Cdc5 overexpression, suggesting a role for RSC downstream or in parallel to Cdc5 in chromatin remodeling events critical for checkpoint silencing (Ratsima et al. 2016). In the context of SAC adaptation, RSC^{Rsc2} supports Net1 phosphorylation and subsequent release of nucleolar Cdc14 –via the FEAR pathway– by directly interacting with Cdc5 (Rossio et al. 2010). It is therefore plausible that RSC^{Rsc2} contributes to DNA damage adaptation through a similar chromatin-mediated mechanism, and could perhaps even facilitate the loosening of DSB-NE tethers prior to spindle elongation. Notably, RSC^{Rsc2} binds Cdc5 PBD in a phospho-independent manner, with its interaction markedly enhanced following DNA damage, suggesting an alternative binding mode that may endow Cdc5 with expanded functional versatility under conditions of cell cycle stress (Rossio et al. 2010; Ratsima et al. 2016).

Taken together, this multi-layered process of checkpoint shutdown –from DDR silencing to structural NE/SPB remodeling and cell cycle reactivation– positions Cdc5 as a central orchestrator of DNA damage adaptation at SPBs, leveraging spatial and temporal control to choreograph a precise and dynamic escape from checkpoint-imposed arrest. Apart from its role in DNA damage adaptation, Cdc5 has also been shown to directly regulate SAC signaling by phosphorylating Mad3 at kinetochores (Rancati et al. 2005). Given the synergistic interaction between the DDR and the SAC during the G2/M checkpoint, it would be plausible for Cdc5 to act as a coordinator of simultaneous DDR and SAC checkpoint adaptation during override, positioning this kinase as a central integrator of cell cycle progression and global checkpoint control (Dotiwala et al. 2010; Zhou et al. 2024).

4.2.2 Targeting PLK1: Therapeutic inhibitors and the consequences of DNA damage adaptation in cancer.

What places PLK1 at the forefront of carcinogenesis and cancer progression? Aberrantly elevated PLK1 expression promotes epithelial-to-mesenchymal transition (EMT), activates pro-proliferative pathways, enhances immune cell infiltration and inflammatory signaling, and facilitates immune checkpoint evasion (Wang et al. 2025). Misregulation of PLK1's diverse cellular functions can drive tumor evolution, prompting the development of urgently needed therapeutic strategies that now encompass five distinct mechanistic classes: 1) First, ATP-competitive inhibitors such as BI 2536, Volasertib and Onvansertib act by binding the catalytic cleft of the kinase domain, thereby blocking PLK1 activity. While effective in preclinical models, these inhibitors often exhibit limited selectivity among PLK family members and induce dose-limiting myelosuppression, restricting their clinical utility (Chapagai et al. 2025); 2) Second, PBD inhibitors –notably Rigosertib and Poloxin– interfere with PLK1 substrate docking and subcellular localization. However, these compounds show poor stability and limited efficacy *in vivo*, restraining their translational potential (Archambault and Normandin 2017; Yuan et al. 2011; Stafford et al. 2023; Chapagai et al. 2025); 3) Third, combination therapies aim to enhance efficacy and prevent resistance by co-targeting complementary pathways (Timme et al. 2020; Su et al. 2022). For example, ARAC (Antigen Release Agent and Checkpoint inhibitor) nanoparticles co-deliver a PLK1 inhibitor and a PD-L1 antibody, thereby integrating mitotic disruption with immune checkpoint blockade to improve tumor specificity and immune engagement (Reda et al. 2022; Wang et al. 2024); 4) Fourth, allosteric modulators –such as RK-10, which binds a region near PLK1 T-loop– target non-catalytic pockets to induce conformational shifts that impair PLK1 function while avoiding resistance mechanisms commonly associated with ATP-competitive inhibition (Raab et al. 2022; Patel et al. 2022; Raab et al. 2024); 5) Finally, cancer vaccine strategies that leverage PLK1 mRNA-loaded dendritic cells aim to elicit PLK1 antigen-specific $CD4^+$/$CD8^+$ T cells responses, offering an immunogenic route to tumor control (Park et al. 2011). Other emerging strategies, such as proteolytic targeting chimeras

(PROTAC)-based PLK1 degraders or gene-silencing approaches –such as CRISPR/Cas9 genome editing– are also under investigation (Song et al. 2025; Gunasekaran et al. 2024; McCarroll et al. 2015; R. Wang et al. 2023). While these approaches show considerable promise in targeting PLK1-overexpressing cancers, a common limitation lies in their global and indiscriminate suppression of PLK1 activity, which often results in dose-limiting toxicities and adaptive resistance mechanisms (Chiappa et al. 2022). Taken together, these therapeutic drawbacks warrant the exploration of alternative approaches –such as spatially restricted strategies designed to selectively target the centrosomal pool of PLK1– which could, in principle, preserve anti-tumour efficacy while minimizing systemic toxicity.

In multicellular contexts, the decision to undergo DNA damage adaptation carries significant consequences for genome stability. The unrestrained transmission of damaged DNA is a potent driver of oncogenesis in higher eukaryotes; accordingly, the process of adaptation is tightly regulated by robust p53-dependent control mechanisms (Dalton et al. 2010; Orth et al. 2012). However, oncogenic transformation is frequently accompanied by the simultaneous activation of growth-promoting pathways and deactivation of critical surveillance mechanisms, facilitating aberrant proliferation despite chromosomal instability (Eischen et al. 1999; Ries et al. 2000; Ogawara et al. 2002). In transformed cells, PLK1 is a well-established driver of genomic instability and its frequent overexpression across a broad spectrum of tumors correlates with radioresistance and poor chemotherapy outcome (Gheghiani and Fu 2023; Gheghiani et al. 2021; Syljuåsen et al. 2006; Rödel et al. 2010; Poyil et al. 2024). Elevated PLK1 activity in cancer cells may be especially consequential in solid tumours, where intratumoural heterogeneity intersects with spatial drug- and oxygen-delivery gradients: therapies exert maximal pressure at well-perfused, oxygenated tumor rims while poorly vascularized, fibrotic, hypoxic cores are relatively shielded – fostering uneven selection and CIN-linked diversification across cellular subpopulations (Iliaki et al. 2021; Chiappa et al. 2022; Li et al. 2021; Marusyk et al. 2020; Chen et al. 2023; Hosea et al. 2024). This spatial ecology is well-documented: aberrant vasculature, high solid/interstitial stress, and dense ECM can obstruct penetration and generate

exposure/oxygenation gradients that PLK1 hyperactivity could exploit (Stylianopoulos et al. 2018; Matuszewska et al. 2021; Stylianopoulos et al. 2012; Martin et al. 2019). Submitting cancer cells to sub-lethal genotoxic stress has the potential to clonally select aggressive, invasive, therapy-tolerant subpopulations in these protected cores, yielding survivors with enhanced radio-/chemo-resistance and setting the stage for relapse – an expected consequence of clonal evolution under hypoxia and uneven drug exposure (Marusyk et al. 2020; Boulefour et al. 2021). Aberrant PLK1 can directly enhance damage tolerance in such settings: PLK1 attenuates checkpoint signalling, fosters lesion processing/repair via RAD51, and supports mitotic-phase salvage pathways, reinforcing a feedback between error-prone mitoses, DNA-damage handling, and clonal outgrowth of fitter core populations (Mamely et al. 2006; Mailand et al. 2006; Peschiaroli et al. 2006; Yata et al. 2012; Gelot et al. 2023; Wu et al. 2023). The development of more integrative treatment plans –combining delivery barrier bypass (i.e., vascular normalization or stromal stress alleviation, and tumor-penetrating or acidity-targeted carriers) with spatially targeted PLK1 inhibition– may therefore yield substantial therapeutic gains (Chauhan et al. 2013; Stylianopoulos and Jain 2013; Järveläinen et al. 2023; Nikitovic et al. 2024; Lorusso et al. 2024). In line with evidence for a centrosome-specific p-RXRα-PLK1 pathway in cancer cells, in which Cdk1-phosphorylated RXRα accumulates at centrosomes to enhance PLK1 activation, our findings suggest that elevated centrosomal PLK1 activity may promote mitotic progression under checkpoint-engaging conditions that would otherwise trigger arrest or apoptosis (Xie et al. 2020). To that end, centrosomal targeting of PLK1 represents a rational avenue to improve therapeutic index.

Centrosomes and their yeast SPB counterparts function as conserved signaling hubs: both attenuate checkpoint activity under cell cycle stress and, through analogous scaffolds, not only recruit PLKs but also undergo PLK-dependent phosphorylation (Ito and Bettencourt-Dias 2018). Nud1, the outer SPB plaque scaffold that recruits Cdc5 and acts as a platform for MEN effectors during mitotic exit, has a mammalian counterpart –centriolin– which localizes to the distal appendages of the centrosome and dynamically

interacts with PLK1 (Gromley et al. 2003; Le Roux-Bourdieu et al. 2022). Similarly, the nuclear plaque γ-tubulin tether Spc110 parallels mammalian pericentrin (PCNT), a PLK1 substrate involved in PCM expansion, centrosome maturation, and, importantly, DDR signaling (Lee and Rhee 2011; Griffith et al. 2008). The outer plaque γ-TuRC organizer Spc72 functionally corresponds to CDK5RAP2 (CEP215), which requires indirect PLK1 regulation to promote γ-TuRC activation and regulate spindle orientation (Hanafusa and Matsumoto 2015; Lin et al. 2015). Finally, the NE protein Mps3 has a functional counterpart in the inner-nuclear-membrane SUN-domain proteins SUN1/2, which dynamically fine-tune nuclear-lamina tension in a PLK1-dependent manner, reshaping chromatin interactions and accommodating spindle dynamics during mitosis and under DNA damage stress (Patel et al. 2014; Lei et al. 2012). Mammalian work shows that RXRα –a ligand-regulated nuclear receptor that relocates to centrosomes during mitosis– accelerates PLK1 activation in cancer cells (Xie et al. 2020). Accordingly, administering the RXRα inhibitor XS060 to trigger mitotic catastrophe in a tumour-selective fashion could represent a pivotal strategy for enhancing therapeutic efficacy (Xie et al. 2020). In the same vein, engineering allosteric PBD ligands refined for PLK1 scaffold recognition or designing centrosome-biased, PCM-targeted PROTACs could provide compartment-restricted inhibition, sparing cytosolic PLK1 and improving therapeutic selectivity (Wang et al. 2021; Song et al. 2025).

4.3 Combinatorial targeting as a regulatory strategy: Insights from Cdc5 PBD

The dual kinase-PBD architecture of PLKs provides them with exceptional versatility, coupling dynamic PBD-mediated substrate recognition to adaptable spatiotemporal localization. The tandem Polo boxes (PB1 and PB2) fold into a β-sandwich PBD whose interface contains a pronounced ligand-binding cleft (Cheng et al. 2003). This phospho-dependent mode of engagement underpins many of Cdc5 functions throughout the cell cycle, ensuring that kinase activity is deployed with precise spatial and temporal control by following Cdc28 priming directions (Elia, Rellos, et al. 2003). Although the canonical, phospho-dependent mode

predominates, early studies suggested that some Cdc5 partners are engaged in a phospho-independent manner, nestling into a hydrophobic pocket on the face opposite to the classical phosphopeptide-binding PBD cleft (Chen and Weinreich 2010). These findings indicate that Cdc5 PBD harbours additional recognition surfaces, broadening its functional versatility by engaging a wider and more diverse set of targets than previously anticipated. While this cryptic hydrophobic pocket is remarkably conserved across eukaryotes, how its engagement –alone or together with the canonical phosphopeptide-binding cleft– modulates Cdc5 substrate selectivity and catalytic activity remains unclear (Sharma et al. 2019).

Through our work, we demonstrate for the first time that Cdc5 PBD functions as a bivalent docking domain. We solved high-resolution crystal structures of Cdc5 PBD in four states: 1) unbound, 2) bound to Spc72 –a canonical phosphopeptide ligand– 3) bound to Dbf4, a non-phosphorylated peptide that forms the DDK complex with Cdc7, and 4) simultaneously bound to both peptides. Structural analysis revealed that Cdc5 PBD harbors distinct ligand-binding surfaces, enabling the recognition of both canonical and non-canonical partners. This structural versatility significantly broadens the spectrum of potential Cdc5 interactors and suggests that an expanded ligand repertoire is strategically deployed to diversify PLK signaling outputs. Strikingly, saturation-transfer difference (STD) NMR confirmed that Cdc5 can bind both peptides simultaneously and in a non-competitive manner, as the ligand sites do not sterically overlap, supporting a bivalent binding mode that may enhance PBD-driven signal integration and transduction. The section below revisits our findings in the context of cell cycle regulation, and examines the significance of combinatorial substrate engagement in enabling Cdc5 multifunctionality.

4.3.1 Expanding Polo function: Dual substrate tethering by Cdc5 PBD

A central question emerging from our findings is: *How does PBD-driven combinatorial substrate engagement modulate Cdc5 activity during cell division?* We investigated the functional relevance of the non-canonical binding pocket by introducing point mutations into residues lining the Dbf4-binding interface in *cdc5*

mutants. The *cdc5-S630Q* allele abolished the non-canonical interaction while preserving phosphopeptide recognition, indicating that the two docking surfaces within Cdc5 PBD operate as functionally independent modules.

Testing the *cdc5-S630Q* mutant for cell cycle progression revealed a specific early anaphase arrest, characterized by shortened spindles and incomplete nuclear mass separation. These findings suggest a previously unrecognized role for Cdc5 PBD-mediated, phospho-independent target engagement in mid-mitosis. This phenotype is intriguingly reminiscent of that observed in FEAR pathway mutants, where duplicated but unsegregated nuclear masses persist at the bud neck alongside shortened spindles (Queralt and Uhlmann 2008). It also parallels the phenotype of *cdc7* and *dbf4* mutant cells, which accumulate at an earlier stage of cell division with a 1N DNA content (Hartwell et al. 1973; Toyn et al. 1995). Given the reported contribution of Dbf4 to the SPoC –where it transiently interacts with Cdc5 PBD to prevent premature MEN activation upon spindle misalignment– it is conceivable that Dbf4 would also play a previously unknown role in coordinating the completion of DNA replication with mitotic entry (Miller et al. 2009). Alternatively, the possibility of a FEAR-related segregation defect is particularly compelling, especially given the established role of the RSC chromatin remodeling complex in promoting early nucleolar release of Cdc14 in cooperation with Cdc5 (Rossio et al. 2010). RSC^{Rsc2} has been shown to interact with Cdc5 PBD in a phospho-independent manner, further supporting this model and implicating the non-canonical hydrophobic pocket as a potential mediator of this interaction (Rossio et al. 2010). Measuring DNA content in the *cdc5-S630Q* mutant would help determine whether the early anaphase defect arises from defective chromosome replication or, alternatively, from improper FEAR network function leading to incomplete nucleolar Cdc14 release and consequent rDNA missegregation (Pereira and Schiebel 2004).

4.3.2 Latching the kinase: Allosteric stabilization via dual PBD engagement

During cell cycle progression, spatial and temporal regulation of PLK1 function is crucial to ensure its potent catalytic activity is appropriately deployed (Archambault et al. 2015). A central mechanism regulating PLK1 activity involves intramolecular interactions occurring between its KD and PBD, wherein the PBD folds back onto the KD hinge region to restrict activation loop mobility and sterically occlude ATP and substrate-binding sites (Chapagai et al. 2025; Xu et al. 2013). To transition from the clamped, autoinhibitory state to an open and catalytically active conformation, PLK1 must undergo two complementary regulatory events: 1) phosphorylation of its activation loop at T210 by the Aurora A-Bora complex –a function fulfilled by Cdc28 at T242 in its budding yeast homolog Cdc5– (Seki et al. 2008; Mortensen et al. 2005), and 2) high-affinity engagement of a primed phosphopeptide within the canonical PBD binding cleft (Elia, Rellos, et al. 2003). Collectively, these signals unlock PLK1 autoinhibitory clamp, shifting the kinase into an open, catalytically competent conformation.

Within the framework of PLK allosteric regulation, the identification of a non-canonical hydrophobic pocket on the back of PB2 broadens the kinase's conformational repertoire, adding a previously overlooked layer of structural control. The docking of the *Drosophila* MT-binding protein Map205 to the canonical PBD pocket further locks the kinase in an autoinhibited conformation and spatially stabilizes Polo on MTs, supporting the idea that PBD interactions can directly modulate kinase function and flexibility (Xu et al. 2013; Kachaner et al. 2014; Archambault et al. 2008). The presence of dual, spatially restricted binding sites on Cdc5 PBD may plausibly confer heightened spatiotemporal sensitivity, wherein engagement of the canonical site reflects local phospho-priming while docking at the opposite hydrophobic pocket conveys cell cycle-restricted regulatory cues. This proposed mechanism dovetails nicely with our current understanding of the role of Dbf4-mediated non-canonical DDK binding to Cdc5 PBD during SPoC activation, where a cell cycle-restricted regulatory cue –namely, spindle misalignment– triggers immediate inhibition of Cdc5 PBD-mediated phospho-substrate targeting, thereby transiently suppressing MEN activation (Miller et al. 2009). Abrogation of this alternative PBD interaction in the *cdc5-S630Q* allele produces an early-anaphase cycling

arrest (Chapter III), demonstrating that the non-canonical pocket is not a mere unidirectional inhibitory clamp, but rather a versatile regulatory interface that can potentiate Cdc5 activity and mediate context-dependent protein-protein interactions. Because the alternative pocket sits at the KD-PBD hinge that secures PLK's closed conformation, its occupation –together with canonical site engagement– could serve as a molecular latch, transiently modulating Cdc5 activity within confined subcellular locales with enhanced flexibility (Archambault et al. 2015).

4.3.3 From docking to hubs: PBD-mediated multivalent complex formation

The ability of Cdc5 PBD to bind two mechanistically distinct ligands at once supports a model in which dual-site occupancy converts the kinase into a context-specific multivalent scaffold able to coordinate an enhanced range of signaling events. In such a system, Cdc5 tethers to a phospho-independent anchor or mediator, while its canonical groove captures primed substrates as they appear. This dual engagement concentrates Cdc5 locally, speeds multisite phosphorylation, and permits rapid dissolution of the multivalent assembly – perhaps through Cdc28-driven phosphorylation of the anchoring partner, analogous to disruption of the Map205-PLK1 interaction (Archambault et al. 2008).

Extending this idea, the non-canonical docking of other kinases –such as the DDK through a PBD-Dbf4 interaction– suggests that Cdc5 can assemble transient multi-kinase modules (Miller et al. 2009). The formation of reversible two-ligands platforms can broaden Cdc5 on-site modification spectra, while providing an additional layer of regulatory precision necessary to coordinate mitotic events with exquisite temporal control. This mechanism parallels classical MAPK cascades, wherein sequential kinases are co-localized within higher-order signaling complexes to facilitate rapid and specific signal transduction. In this configuration, Cdc5-centred hubs could potentiate intra-complex cross-activation and sharpen coincidence detection, thereby fortifying mitotic surveillance pathways. Although the proposed model lacks the classic

three-tier MAPK hierarchy, a dual-site scaffold still integrates discrete inputs and bridges key cell cycle transitions through coordinated kinase activation.

Evidence supporting this model comes from the Mus81-Mms4 endonuclease, whose full activation requires phosphorylation by both Cdc5 and DDK at partially overlapping sites (Princz et al. 2017). Cdc7 interacts with Mus81-Mms4 via the Rtt107 scaffold, suggesting that non-canonical engagement of Cdc5 with DDK could transiently position Cdc5 in the same vicinity, enabling sequential/cooperative phosphorylation (Princz et al. 2017). A similar DDK-mediated strategy may also place Cdc5 near the Ulp2 SUMO peptidase, and the cohesin subunit Scc1/Mcd1/Rad21 (Princz et al. 2017; Alexandru et al. 2001). After these DDK-dependent steps, Cdc5 might transiently partner with other chromatin regulators –such as the RSC complex– to access mitotic targets (Rossio et al. 2010; Ratsima et al. 2016).

4.4 Integrated model and future directions

Our results support a model in which SPBs are dynamically exploited to couple checkpoint down-regulation with cell cycle re-entry once the perceived DNA damage burden falls below an adaptation threshold. In budding yeast, the active relocalization of damaged DNA (including DSBs and eroded telomeres) to the nuclear periphery is contingent on Mps3 activity along with chromatin remodelers, which help determine the perinuclear anchorage site – a process plausibly linked to adaptation kinetics (Oza et al. 2009; Horigome et al. 2014). Cdc14-mediated dephosphorylation of Spc110, a core SPB component that we identified as a contributor to DNA damage adaptation, stabilizes the spindle to facilitate DNA damage relocation (Villoria et al. 2017). Testing whether Mps3 and Spc110 functions converge within a common signaling branch – potentially one that attenuates damage signaling before checkpoint adaptation– would clarify their relative importance in this process. To this end, analyzing whether a *mps3-3A spc110-7* double mutant displays 1) enhanced sensitivity to DNA damaging agents and 2) reduced adaptation kinetics in the context of *cdc13-1* or HO-induced DNA damage will help determine whether perinuclear relocalization and SPB signaling

cooperate to modulate checkpoint intensity. Complementary live-cell imaging of DNA damage relocation in *mps3-3A* using peripheral tethering reporters would further test whether defective perinuclear anchoring correlates with slower adaptation. Given established roles for Swr1 and Ino80 in perinuclear DSB relocation and for RSC in regulating nuclear envelope structure and NPC localization (Horigome et al. 2014; Titus et al. 2010), probing RSC involvement in this relocation –especially RSC^{Rsc2}, which is implicated in DNA damage checkpoint adaptation (Ratsima et al. 2016)– could clarify how chromatin remodeling interfaces with the adaptation pathway. Finally, because Cdc14-mediated dephosphorylation of Spc110 stabilizes the spindle and supports DNA damage relocation (Villoria et al. 2017), examining whether Spc110 phosphorylation kinetics after DNA damage induction correlate with checkpoint attenuation would help assess the relevance of this regulation during adaptation.

Because Cdc5 coordinates numerous mitotic events, its activity must be governed with meticulous spatiotemporal precision to safeguard genome integrity. This thesis poses a unifying question: *Which molecular features enable Cdc5 PLK to remain functionally versatile as cellular conditions change?* Chapter II and III converge on the same answer: Cdc5 reconfigures its local interactions –and leverages context-dependent partner selection– to assemble transient, localized assemblies that maintain the timing and order of cell cycle transitions across unperturbed and genotoxic conditions. Chapter III may shed new light on DNA damage adaptation by elucidating how Cdc5 consistently orchestrates a broad spectrum of spatially distinct, functionally diverse tasks not only throughout an unperturbed cell cycle, but also under genotoxic conditions. *Could Cdc5 deploy combinatorial binding to broaden its functional scope during DNA damage adaptation?* Cdc5 may engage in non-classical interactions under atypical cycling conditions, increasing its mechanistic versatility. The fact that Cdc5 acquires multiple T-loop modifications to broaden its repertoire –illustrated by the *cdc5-T238A* allele, which impairs DNA damage adaptation yet leaves the cell cycle untouched– supports a model in which the kinase is dynamically fine-tuned for its own activity and substrate range to meet cellular demands with pinpoint precision (Rawal et al. 2016). During genotoxic stress, dual

tethering may lock the adaptation program into the correct sequence and timing. As a PBD-organized multivalent complex, Cdc5 may locally concentrate key effectors and –through non-canonical ligand binding– establish a permissive configuration for checkpoint silencing under conditions that would otherwise impose checkpoint-mediated arrest. Multiple lines of evidence converge on a model in which Cdc5 engages the RSC complex dynamically and non-canonically during adaptation (Ratsima et al. 2016; Rossio et al. 2010). Consistent with the view that Cdc5 reconfigures its canonical interactome under genotoxic stress, recruiting RSC to override checkpoint constraints would align with the timing of their usual collaboration during FEAR-related events (Rossio et al. 2010). Future work could test whether the non-canonical PBD-binding mutant *cdc5-S630Q* 1) displays persistent DNA damage adaptation defects, and 2) loses the Cdc5-RSC^{Rsc2} interaction, thereby clarifying the role of RSC in Cdc5-mediated adaptation. Another intriguing avenue would be to investigate whether Cdc5 transiently docks CKII –another key adaptation kinase– via the same phospho-independent interface during adaptation (Toczyski et al. 1997). This catalytic synapse could provide a direct route for Rad53 dephosphorylation (Guillemain et al. 2007; Toczyski et al. 1997).

Together, Chapters II and III outline an integrated model that casts Cdc5 as a versatile PBD-guided multivalent scaffold that actively exploits SPBs through context-dependent partner selection and extends its functional reach via combinatorial binding. Three broad questions now emerge: 1) First, *what is the full range of non-canonical PBD ligands that extend Cdc5 functional reach, and does mammalian PLK1 exploit the same combinatorial, non-competitive binding strategy?*; 2) Second, *how do Cdc5 and RSC^{Rsc2} collaborate during adaptation – do they deploy as a coupled module, or as sequential, complementary players?*; 3) Third, *can this dual-occupancy logic be translated into therapeutic interventions through inhibitors that target both the canonical and cryptic PBD pockets?* Tackling these questions will clarify the molecular logic underlying Cdc5 multifunctionality and pave the way for meaningful cross-species comparisons of PLK regulation.

How to translate these insights into mammalian models? PLK1 is a key driver of checkpoint adaptation in human cells, and our findings suggest that a centrosome-localized PLK1 subpopulation may be especially important for adaptation-like mitotic entry after DNA damage (Syljuåsen et al. 2006; Xie et al. 2020). Quantifying PLK1 recruitment and activity at centrosomes during adaptation –using HeLa, U2OS, or HCT116 cells standard for PLK1 work– by IF/FRAP and complementary live imaging should reveal how PLK1 behaves at MTOCs under genotoxic stress (Kishi et al. 2009; Liu et al. 2012; Xie et al. 2020). Comparing centrosomal PLK1 accumulation and adaptation kinetics in isogenic HCT116 $p53^{+/+}$ vs $p53^{-/-}$ pairs, while tracking persistent DNA damage foci, would clarify how p53-dependent safeguards repress PLK1 expression and/or restrict its centrosomal enrichment during damage (Zhou et al. 2013; Sur et al. 2009; McKenzie et al. 2010). To test whether centrosome-localized PLK1 is necessary for DNA damage adaptation, it would be worthwhile to perturb its recruitment by 1) PBD-targeting docking disruption (Park et al. 2015), 2) disrupting the AKAP12/Gravin scaffold to impede docking at MTOCs (Colicino et al. 2018), or 3) uncoupling RXRα-PLK1 at centrosomes to blunt local activity (Xie et al. 2020), and use adaptation frequency, clonogenic survival, and anchorage-independent growth as readouts. Another interesting avenue would be to test, given the evolutionary conservation of yeast Mps3 and mammalian SUN-domain proteins (Chang et al. 2015; Schober et al. 2009), whether SUN1/2 depletion in U2OS or SUN1/2 DKO MEFs –conditions that impair motion of damage foci (Lottersberger et al. 2015)– also yields adaptation defects. Finally, human tumor organoids provide a powerful system to interrogate the spatial aspects of PLK1 regulation in a tissue-like context, where defined oncogenic combinations –including *TP53*-null and *TP53*-proficient states– can more faithfully mirror clinical scenarios (Drost et al. 2015). Visualization and quantification of centrosomal PLK1 enrichment via endogenous tagging (Bollen et al. 2022) –especially before and after irradiation/chemotherapeutics, both in *TP53*-null and *TP53*-proficient organoid models– (Riffle and Hegde 2017; Gunti et al. 2021) would help capture core-vs-rim differences across spheroids, along with the impact of *TP53* status on PLK1-dependent DNA damage adaptation and oncogenic potential in such tumors (Däster et al. 2016). Together,

these avenues will help define the conserved principles that operate at the interface of PLK biology, genome maintenance, and disease, thereby deepening our understanding of cancer progression and informing more effective therapeutic strategies.

4.5 Conclusion

Overall, this thesis sheds light on how Cdc5 expands its functional reach during unperturbed mitosis and under genotoxic stress. Our results reveal a previously unrecognized role for Cdc5 as a flexible multivalent scaffold that integrates spatial cues and combinatorial substrate binding to rewire its interactome and meet changing cell cycle demands. We show that SPBs are dynamically engaged by Cdc5 as signal-integration hubs during DNA damage adaptation: through Nud1, Spc110, and Spc72, Cdc5 docks at SPBs, integrates multiple pathways, and couples checkpoint silencing to cell cycle re-entry – notably through the dynamic modification of key SPB components that include Cnm67 and Mps3. The discovery that Cdc5 can bind two distinct ligands simultaneously –using a non-canonical hydrophobic pocket opposite the classical phosphopeptide cleft– extends prior PLK1 findings and raises the possibility that PLK1 may likewise support dual-interface co-occupancy. Importantly, this model provides a conceptual template for targeting PLK1 in cancer by pointing to two complementary therapeutic avenues –spatially restricting PLK1 activity at centrosomes, and allosterically blocking its combinatorial PBD interactions– to disable oncogenic signaling nodes while preserving PLK essential functions. Future studies in mammalian models will be essential to confirm these regulatory mechanisms in a physiological setting and to determine how well the proposed PLK1-targeted strategies can curb cancer progression and counter multimodal therapy resistance.

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