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## Mitotic functions of poly(ADP-ribose) polymerases

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### Abstract

Mitosis ensures accurate segregation of duplicated DNA through tight regulation of chromosome condensation, bipolar spindle assembly, chromosome alignment in the metaphase plate, chromosome segregation and cytokinesis. Poly(ADP-ribose) polymerases (PARPs), in particular PARP1, PARP2, PARP3, PARP5a (TNKS1), as well as poly(ADP-ribose) glycohydrolase (PARG), regulate different mitotic functions, including centrosome function, mitotic spindle assembly, mitotic checkpoints, telomere length and telomere cohesion. PARP depletion or inhibition give rise to various mitotic defects such as centrosome amplification, multipolar spindles, chromosome misalignment, premature loss of cohesion, metaphase arrest, anaphase DNA bridges, lagging chromosomes, and micronuclei. As the mechanisms of PARP1/2 inhibitor-mediated cell death are being progressively elucidated, it is becoming clear that mitotic defects caused by PARP1/2 inhibition arise due to replication stress and DNA damage in S phase. As it stands, entrapment of inactive PARP1/2 on DNA phenocopies replication stress through accumulation of unresolved replication intermediates, double-stranded DNA breaks (DSBs) and incorrectly repaired DSBs, which can be transmitted from S phase to mitosis and instigate various mitotic defects, giving rise to both numerical and structural chromosomal aberrations. Cancer cells have increased levels of replication stress, which makes them particularly susceptible to a combination of agents that compromise replication fork stability. Indeed, combining PARP1/2 inhibitors with genetic deficiencies in DNA repair pathways, DNA-damaging agents, ATR and other cell cycle checkpoint inhibitors has yielded synergistic effects in killing cancer cells. Here I provide a comprehensive overview of the mitotic functions of PARPs and PARG, mitotic phenotypes induced by their depletion or inhibition, as well as the therapeutic relevance of targeting mitotic cells by directly interfering with mitotic functions or indirectly through replication stress.

### Keywords

Poly(ADP-ribose) polymerases; Poly(ADP-ribose) glycohydrolase; Mitosis; PARP inhibitor; Replication stress

## 1 Nuclear functions of poly(ADP-ribose) polymerases

Poly(ADP-ribose) polymerases (PARPs) catalyse reversible ADP-ribosylation on serine, glutamate, aspartate, arginine, lysine and cysteine residues of histone and non-histone proteins using NAD as a cofactor [1]. Of the 17 known PARP members in humans, PARP1, PARP2, PARP3, PARP5a (TNKS1) and PARP5b (TNKS2) are localized in the nucleus and can synthesize poly(ADP-ribose) (PAR), with the exception of PARP3 [1]. Other PARPs act as monoenzymes to attach mono(ADP-ribose), whereas PARP13 has no reported enzymatic activity [1]. Poly (ADP-ribose) (PAR) is a highly negatively charged homopolymer of repeating ADP-ribose units linked by unique glycosidic ribose-ribose bonds [1]. It is rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG) to ensure that the PAR levels are tightly regulated [2].

Nuclear PARPs and PARG regulate various nuclear functions such as DNA repair, DNA replication, gene expression, chromatin dynamics, cell death and mitotic progression [1,3–5]. PARP1 is strongly activated in response to DNA damage and is responsible for the synthesis of the majority of PAR following genotoxic stress [4,6]. PARP1 regulates various DNA repair pathways, replication, mitosis, gene expression and cell death [3,6–16]. PARP2 is important for DNA repair, mitosis, meiosis I and haploid gamete differentiation [17,18]. PARP3 synergizes with PARP1 in the repair of double-strand DNA breaks (DSBs) and regulates mitosis and telomere integrity [19]. PARP5a, also called tankyrase 1 (TNKS1), regulates telomere integrity, mitosis and DNA repair [20,21]. PARG is necessary for replication and recovery from prolonged replication stress, DNA repair and mitosis [22–25].

PARP1 deficiency in mice leads to DNA damage-induced genomic instability and embryonic lethality in combination with PARP2 deficiency [18]. PARP2-deficient mice also exhibit impaired spermatogenesis and mitotic chromosomal aberrations [17,18]. PARP1<sup>-/-</sup> PARP3<sup>-/-</sup> double knock-out mice exhibit increased sensitivity to ionizing radiation compared to PARP1<sup>-/-</sup> animals [19]. TNKS1<sup>-/-</sup> TNKS2<sup>-/-</sup> double knock-out is embryonically lethal [26]. TNKS1-depleted cells arrest in metaphase and suffer from telomere shortening and telomere fusions [27–29]. PARG knock-out leads to early embryonic lethality, while PARG-deficient cells are sensitive to ionizing radiation and exhibit mitotic abnormalities after genotoxic exposure [22,30].

In this review I will focus on the role of nuclear PARPs (PARP1-3 and TNKS1) and PARG in mitosis. I will begin with an overview of key events during mitotic progression, followed by a detailed description of the mitotic functions of PARPs and PARG, as well as mitotic phenotypes caused by their depletion or inhibition.

## 2 Mitosis

During mitosis, cells divide in such a way that genetic information, which was duplicated during S phase, is equally split between two daughter cells [31]. Mitosis starts after G2, ends with cytokinesis, and consists of five distinct stages: prophase, prometaphase, metaphase, anaphase and telophase (Fig. 1A). Before mitosis, duplication of the genetic material in S

phase generates two sister chromatids that are intertwined (catenated) and held together by the cohesin complex [32].

At the onset of prophase, sister chromatids are decatenated by topoisomerase II and subsequently condense through the action of condensins [33,34]. Furthermore, Haspin phosphorylates histone H3 at Thr-3 as a platform for the recruitment of the chromosomal passenger complex (CPC) components Survivin and the Aurora B kinase, which phosphorylates Ser-10 and Ser-28 [35]. Histone phosphorylation regulates the phospho/methyl switch, whereby H3T3ph adjacent to H3K4me3 elicits dissociation of transcription factors from promoters [36], H3S10ph adjacent to H3K9me3 leads to dissociation of HP1 (**h**eterochromatin **p**rotein 1) [37,38], while H3S28ph adjacent to H3K27me3 causes expulsion of the Polycomb repressive complexes [39]. H3 phosphorylation by Aurora B also promotes condensin I deposition [35].

During prophase and prometaphase, the cohesin complex is removed from chromosome arms through phosphorylation of the cohesin subunit STAG2 (**s**tromal **a**ntigen 2) by PLK1 (**P**olo-like **k**inase 1) [40]. In addition, Aurora B and CDK1 (**c**yclin-**d**ependent **k**inase 1) phosphorylate sororin and thereby abolish its ability to antagonize the cohesin release factor WAPL (**w**ings **a**part-like protein homologue) [41]. Centromeric cohesion is protected by SGO1 (Shugoshin 1) and phosphatase PP2A, which antagonize Aurora B and CDK1 by dephosphorylating sororin [42].

Another important event during prophase is the formation of kinetochores around centromeric regions, which enable attachment of centromeres to spindle microtubules [43,44]. During prometaphase, the nuclear envelope breaks down and the mitotic spindle is formed from dimers of  $\alpha$  and  $\beta$  tubulin that nucleate from  $\gamma$ TuRCs ( **$\gamma$ -tubulin ring complexes**) [45]. While centrosomes are the major site of microtubule nucleation at the spindle poles, microtubule nucleation is additionally mediated by chromatin or by microtubules themselves [45]. The mitotic spindle is assembled from kinetochore microtubules, which attach the chromosomes to spindle poles via the kinetochore; astral microtubules, which radiate from spindle poles and position the spindle; and non-kinetochore microtubules, which separate the poles and provide stability to the spindle [45]. Chromosome assembly along the spindle equator and alignment in the metaphase plate marks the transition to metaphase [46]. The CPC monitors and corrects erroneous kinetochore-microtubule attachments by phosphorylating kinetochore components and also activates the spindle assembly checkpoint (SAC) [47,48]. SAC prevents progression into anaphase until all kinetochores are properly attached to the spindle [49]. If SAC is satisfied, cyclin B and securin are degraded by the APC/C (**a**naphase-**p**romoting **c**omplex/cyclosome) ubiquitin ligase and securin can thus no longer inhibit separase [50].

At the end of metaphase, centromeric cohesion is removed by separase, which cleaves phosphorylated SCC1 (RAD21) to break the cohesin ring [51]. This enables separation of sister chromatids to opposite poles during anaphase, followed by the formation of two nuclei during telophase.

Post-translational protein modifications (PTMs) such as phosphorylation, ubiquitination and SUMOylation were shown to regulate different functions across all stages of mitosis [52]. In this review, I will discuss the importance of poly(ADP-ribosyl)ation (PARylation) for mitosis.

### 3 PARPs in mitosis

PARylation in mitosis was visualized nearly 40 years ago and was shown to be more pronounced in mitotic than in interphase cells [53–55]. Research efforts over the past 20 years have identified different functions of PARylation and PARPs in mitosis. PARP1, TNKS1 and PARG were found to regulate centrosome function [13,14,22,56,57]; PARP3 and TNKS1 regulate mitotic spindle assembly [19,27,58–60]; PARP1 and PARG regulate the anaphase and the spindle assembly checkpoints [12,22,61–64]; PARP1, PARP3, TNKS1 and PARG regulate telomere length, t-loop integrity and sister telomere cohesion [22,28,65–70] (Fig. 1). PARylation of mitotic chromatin may also serve as an epigenetic bookmark at transcription start sites required for transcription reactivation after mitosis [71].

A plethora of mitotic phenotypes has been observed upon PARP depletion or inhibition (Fig. 2). PARP1-depleted cells exhibit centrosome amplification, ultrafine anaphase DNA bridges and loss of spindle assembly checkpoint integrity [12–14,72]. PARP2 depletion causes anaphase DNA bridges, lagging chromosomes and chromosome missegregation [18], while PARP3 depletion results in multipolar spindles, chromosome misalignment and metaphase arrest, as well as telomere fusions [19]. TNKS1-depleted cells show centrosome amplification, multipolar spindles, metaphase arrest, telomere shortening and telomere fusions [28,29,56]. PARG depletion protects from telomere fusions but induces multiple mitotic defects after exposure to ionizing radiation, including centrosome amplification, centrosome fragmentation, multipolar spindles, chromosome misalignment and missegregation [22].

PARP1/2 and PARG inhibitors recapitulate the aforementioned mitotic phenotypes and generate additional defects through replication stress and DNA damage [15,73–77] (Fig. 2). PARP1/2 inhibitors inactivate and entrap PARP on DNA; the resulting PARP-DNA complexes interfere with DNA replication causing S-phase stalling, G2 delay, replication stress and DSB formation [15,73–76,78,79]. PARP1/2 entrapment and replication blockage also weaken cohesion between sister chromatids, resulting in premature loss of cohesion ('cohesion fatigue') and metaphase arrest [74] (Fig. 2). Unresolved replication intermediates, underreplicated DNA and DNA damage are known to cause chromosome misalignment and missegregation that lead to cell death by mitotic catastrophe [80–82]. Replication stress-induced mitotic failure thus seems to be the major mechanism of PARP1/2 inhibitor-induced cytotoxicity [76].

The following sections provide details on PARP localization to different mitotic structures, as well as the structural and regulatory components of the mitotic machinery that PARPs interact with and modify by PARylation.

## 4 Regulation of the centrosome function by PARPs

Centrosomes are the main site of microtubule nucleation (*de novo* microtubule formation) required for the assembly of the mitotic spindle [45]. Centrosomes consist of two centrioles surrounded by the pericentriolar material, which contains sites of microtubule nucleation and expands at the onset of mitosis [45]. Centrosomes are duplicated prior to mitosis and separate to two opposite poles during chromosome condensation in prophase in order to form a bipolar spindle. In vertebrates, both centrosomes and microtubules are localized in the cytoplasm, which is why the nuclear envelope needs to break down for microtubules to access the chromosomes. The minus ends of microtubules that form the mitotic spindle are anchored at the centrosomes. The plus ends of microtubules serve as an attachment site for kinetochores – a protein complex assembled on centromeres [43,44]. Centrosome dysfunction impairs faithful chromosome segregation, promoting aneuploidy and chromosome instability as hallmarks of cancer [83].

PARP1 associates with centrosomes throughout the cell cycle and regulates centrosome copy number, as PARP inhibition with 3-AB or depletion in primary and immortalized PARP1<sup>-/-</sup> mouse embryonic fibroblasts or primary PARP1<sup>-/-</sup> mammary epithelial cells result in centrosome dysfunction and amplification [13,14] (Fig. 2). PARG-depleted HeLa cells also exhibit centrosome amplification, which is exacerbated after irradiation [22]. Centrosome dysfunction may stem from aberrant PARylation of the tumour suppressor p53, which is one of the PARP1 substrates among centrosomal proteins known to regulate centrosome duplication [13,84]. PARP3 also localizes at the centrosomes throughout the cell cycle through its N-terminal domain [85]. Overexpression of the PARP3 N-terminal domain does not affect centrosome copy number but results in G1/S cell cycle arrest [85]. PARP3 depletion results in multipolar spindles [19]. Overall, the substrates of PARP1 and PARP3 relevant for their regulation of centrosome function have yet to be characterized.

Centrosome function in human cells is further regulated by TNKS1. TNKS1 localizes to centrosomes exclusively in the G1 phase of the cell cycle and PARylates CPAP (centrosomal P4.1-associated protein) [56] (Fig. 1A). CPAP is essential for centriole maturation in humans through regulation of centriole duplication and elongation [86–88]. TNKS1-mediated PARylation targets CPAP for proteosomal degradation in G1 [56]. Overexpression of TNKS1 phenocopies siRNA-mediated depletion of CPAP by preventing centriole duplication, while TNKS1 silencing phenocopies CPAP overexpression by giving rise to abnormally elongated centrioles, centrosome amplification and multipolar spindles [56]. Therefore, TNKS1 regulates centrosome function through regulation of CPAP levels.

Moreover, TNKS1 PARylates MIKI (**mitotic kinetics** regulator) at the transition into mitosis and leads to its relocalization from the Golgi apparatus to centrosomes [57]. On centrosomes, PARylated MIKI anchors a scaffold protein, CG-NAP, which is part of  $\gamma$ TuRC [57].  $\gamma$ TuRCs initiate microtubule assembly from dimers of  $\alpha$ - and  $\beta$ -tubulin [45]. MIKI PARylation thus promotes microtubule nucleation at centrosomes and initiation of prometaphase [57]. MIKI depletion in human cancer cells—but not in primary cells or mice—impairs chromosome alignment in metaphase causing metaphase arrest and apoptosis on the

one hand, and leads to mitotic exit without chromosome segregation yielding multinucleated cells on the other [57].

## 5 Regulation of the mitotic spindle assembly by PARPs

The mitotic spindle is a bipolar, dynamic macromolecular structure built by the self-organized assembly of microtubules (dimers of  $\alpha$ - and  $\beta$ -tubulin), microtubule-associated proteins and motor proteins [45]. Timely, efficient and accurate assembly of the bipolar mitotic spindle is a prerequisite for accurate chromosome segregation [45]. Multipolar spindles cause chromosome missegregation, which leads to aneuploidy [89]. Although centrosomes serve as main sites of microtubule nucleation (discussed in the previous section), microtubule nucleation is additionally mediated by chromatin or by the microtubules themselves [45]. Microtubule-dependent nucleation occurs within the spindle, whereby  $\gamma$ TuRC initiates microtubule nucleation from pre-existing microtubules [90].  $\gamma$ TuRC is loaded onto the pre-existing microtubules by the Augmin complex, initially identified in *Drosophila* [91]. The mammalian HAUS (**h**omologous to **A**ugmin subunits) complex consists of eight subunits, of which HAUS8 binds directly to microtubules through Aurora A and PLK1 phosphoregulation [45]. Moreover, Augmin together with NUMA1 (**n**uclear **m**itotic **a**pparatus protein 1) regulates spindle polarity factors and pole clustering of the microtubule minus ends [92]. NUMA1 binds the microtubule minus ends and dynactin – a cofactor of the motor protein dynein [93]. NUMA1-mediated dynactin recruitment localizes dynein activity at the microtubule minus ends, which drives the clustering of microtubules into poles as a prerequisite for bipolar spindle organization [93].

NUMA1 recruits TNKS1 to the mitotic spindle, which in turn PARylates NUMA1 [27,60]. NUMA1 also binds PAR non-covalently [59]. PARP3 can also PARylate NUMA1 directly or by enhancing TNKS1 activity [19]. TNKS1 activity is higher in mitosis and PAR chains generated by TNKS1 are required for the proper assembly and function of bipolar spindles [27,58,59] (Fig. 1A). TNKS1 depletion results in activation of the mitotic checkpoint and metaphase arrest due to defective spindle assembly and loss of spindle bipolarity [27] (Fig. 2). TNKS1 inhibition also causes aberrant spindle formation, but without metaphase arrest [77]. TNKS1 depletion does not perturb NUMA1 localization at spindle poles or microtubule clustering at the poles, but rather affects NUMA1-dependent regulation of spindle polarity [27]. PARP3 depletion also results in metaphase arrest with mild spindle defects, including supernumerary poles and chromosome misalignment, without affecting NUMA1 or TNKS1 localization at the spindle poles [19]. NUMA1 is a multimeric protein and its PARylation and PAR-binding property may promote accurate assembly of bipolar spindles by crosslinking NUMA1 molecules between two spindle poles [59,94].

Furthermore, TNKS1 PARylates  $\alpha$ -tubulin, which is recognized by the guanine nucleotide exchange factor ECT2 (**e**pithelial **c**ell **t**ransforming sequence 2 oncogene) [95]. ECT2 is required for control of cytokinesis and its interaction with PARylated  $\alpha$ -tubulin is required for its recruitment to the spindle during metaphase as a prerequisite for functional cytokinesis and completion of mitosis [95,96] (Fig. 1A).

## 6 Regulation of mitotic checkpoints by PARPs

Following bipolar spindle assembly, chromosomes attach to the spindle microtubules through kinetochores. The kinetochore is a large protein complex that connects centromeric DNA with spindle microtubules [43,44]. The kinetochores of sister chromatids are connected to microtubules that emanate from opposite spindle poles. Such chromosome bi-orientation is essential for accurate segregation of sister chromatids to opposite spindle poles. The error correction (EC) pathway, governed by the chromosomal passenger complex and the Aurora B kinase, stabilizes interactions with microtubules that drive chromosome bi-orientation (sensed as tension by the kinetochore-centromere system) and weakens erroneous interactions (sensed as lack of tension) [48]. Aurora B also promotes recruitment of the components of the spindle assembly checkpoint to the kinetochore [48]. The spindle assembly checkpoint (SAC), also called the mitotic checkpoint (MC), monitors the attachment of kinetochores to spindle microtubules, and prevents chromosome segregation in the presence of unattached or incorrectly attached chromosomes [49]. Persistent incorrect kinetochore-microtubule attachments may lead to chromosome missegregation resulting in aneuploidy [89]. Therefore, timely activation of the SAC is crucial for accurate progression through mitosis.

The SAC is regulated by the mitotic checkpoint complex (MCC), which consists of MAD1, BUBR1, BUB3 and APC/C co-activator CDC20 [49,97]. The MCC stably associates with APC/C and delays anaphase by preventing degradation of APC/C<sup>Cdc20</sup> metaphase substrates, such as cyclin B and securin [98]. Once all kinetochores are correctly attached to the spindle microtubules and the chromosomes are aligned in the metaphase plate, MCC-mediated inhibition of APC/C<sup>Cdc20</sup> is released, resulting in polyubiquitination and degradation of securin and cyclin B [50]. Degradation of cyclin B inactivates the mitotic kinase CDK1 and promotes mitotic exit [50]. Degradation of securin activates separase, which cleaves cohesin to allow sister chromatid separation [33,50,51].

PARP1 and PARP2 accumulate on centromeric chromatin until metaphase, interact with the centromeric proteins CENPA, CENPB and BUB3, and dissociate in anaphase [62,99,100]. PARP1 was also found to interact with Aurora B and inhibit its activity upon DNA damage, resulting in reduced H3S10 phosphorylation [101]. The functional significance of this is, however, unclear. Depletion of PARP1 in mouse oocytes leads to incomplete synapsis of homologous chromosomes, deficient sister chromatid cohesion in metaphase II and failure to maintain metaphase II arrest due to mislocalization of the MCC component BUB3 [64] (Fig. 1A). PARP1-deficient cells escape the SAC after spindle disruption with microtubule depolymerizing agents (e.g., nocodazole, colcemid) [12]. PARP1 deficiency was proposed to weaken SAC due to cyclin B1 degradation and reduction of CDK1 kinase activity [12], while PARP inhibition may weaken SAC by reducing BUBR1 levels [102]. Conversely, PARG deficiency prolongs SAC and metaphase duration, emphasizing the importance of appropriate PAR levels for regulating the kinetochore function and SAC [22]. Tetraploid PARP-deficient cells also bypass the post-mitotic G1 checkpoint by undergoing DNA endoreduplication and resisting apoptotic death [12]. How exactly PAR regulates mitotic and post-mitotic checkpoints remains to be elucidated.

Another mitotic checkpoint called the antephasic checkpoint precedes the SAC and occurs in early prophase. The antephasic checkpoint responds to microtubule poisons or DNA damage and elicits chromosome decondensation and mitotic delay [103]. The main antephasic checkpoint protein CHFR (**check**point with **F**HA and RING finger domains) is an E3 ubiquitin ligase that ubiquitinates Aurora A and PLK1 to halt mitotic progression [104,105]. PAR binding stabilizes CHFR and ensures an intact antephasic checkpoint [63,106] (Fig. 1A). Mitotic stress induces PARP1 auto-PARylation, which increases its interaction with CHFR [61]. CHFR polyubiquitinates auto-PARylated PARP1 and targets it for degradation, resulting in cell cycle arrest in prophase [61]. CHFR thus regulates the antephasic checkpoint by regulating, among others, PARP1 levels. PARP1-CHFR interaction is of clinical relevance, as PARP inhibitors or inhibitors of the CHFR-PARP1 interaction can sensitize CHFR-expressing cancer cells to microtubule poisons through attenuation of the antephasic checkpoint [61,106].

## 7 Telomere regulation by PARPs

Telomeres are end points of chromosomes, which consist of short tandem repeats (TTAGGG in humans) and associated protective proteins called the Shelterin complex [107,108]. TRF1 and TRF2 (telomere repeat binding factors) are the two main telomere-specific DNA-binding proteins. TRF1 negatively regulates telomere length by inhibiting telomerase, an enzyme that adds the telomeric sequences at the end of the chromosomes and thereby prevents their shortening due to the 'end-replication problem' [109]. TRF2 protects chromosomes ends from end-to-end fusions by generating and protecting t-loops, whereby the telomeric 3' overhang invades the double-stranded telomeric DNA [108,110,111] (Fig. 1B).

TNKS1 localizes at telomeres upon binding to TRF1 [65]. TNKS1 PARylates TRF1, resulting in its decreased binding to telomeric DNA and ubiquitination followed by proteosomal degradation [65,112]. The release of PARylated TRF1 from telomeric DNA may open the telomeric complex and allow access to the telomerase [66]. Indeed, persistent TNKS1 overexpression was shown to increase telomere length, whereas TNKS1 depletion or inhibition results in telomere shortening [28,66,113]. TNKS1 thus seems to act as a positive regulator of telomere length by antagonizing TRF1 [65,66]. TNKS1 interaction with TRF1 is modulated by the Shelterin protein TIN2 (TRF1-interacting nuclear protein 2), which blocks TNKS1 from PARylating TRF1 [114]. TNKS1 stability and activity at the telomeres and the mitotic spindle are positively regulated by the mitotic kinase PLK1 [67]. PLK1 inhibition leads to telomeric fusion and reduced localization of TNKS1 at spindle poles [67].

Furthermore, TNKS1 negatively regulates sister telomere cohesion and thus facilitates sister chromatid separation during anaphase [68]. Timely resolution of sister telomere cohesion is regulated by (de)ubiquitination of TNKS1. TNKS1 is ubiquitinated by RNF8 (**R**ing finger protein **8**) in the S/G2 phase, and deubiquitinated by ABRO1 (**A**braxas **b**rother **1**) in the G1 phase of the cell cycle [69]. TNKS1 deubiquitination prevents premature resolution of sister telomere cohesion, while TNKS1 ubiquitination promotes the resolution of sister telomere cohesion by increasing TNKS1 stability and association with telomeres [69]. Persistent

telomere cohesion in mitosis due to TNKS1 or RNF8 depletion results in deprotection of chromosome ends and end-to-end fusions between sister chromatids by non-homologous end-joining [29,69] (Fig. 2). Conversely, premature resolution of sister telomere cohesion in S phase due to ABRO1 depletion leads to telomere loss and fragile telomeres [69]. PARP3 depletion also leads to sister telomere fusions and sister telomere loss, most likely due to the activation of alternative NHEJ (alt-NHEJ) [19,115,116]. PARG depletion was shown to protect telomeres from sister telomere fusions and telomere loss [22], which is consistent with the positive effect of PAR (generated by TNKS1 or PARP3) on telomere integrity and stability.

While TNKS1 interacts with TRF1, PARP1 and PARP2 interact with TRF2 [70,117]. Consistent with the distinct functions of their telomeric partners TRF1 and TRF2, TNKS1 regulates telomere length whereas PARP1 may pose a threat to the t-loop integrity [66,117]. T-loops contain a PARP1 activation site (a 5' ds-ssDNA transition), which is masked by TRF2 [117]. In the absence of TRF2, activated PARP1 promotes t-loop cleavage and alt-NHEJ [108]. PARP1 promotes the recruitment of Holliday junction resolvases that cleave t-loops, resulting in telomere loss and telomere-free chromosome fusions [117,118]. In the absence of both TRF2 and the canonical NHEJ proteins Ku70/80, PARP1 was shown to initiate alt-NHEJ at telomeres, resulting in telomere fusions [119].

## 8 PARPs at the interface between DNA damage, mitosis and chromosomal instability

PARPs play an essential role in the maintenance of chromosomal stability. Chromosomal instability comprises numerical or structural chromosomal aberrations. Numerical aberrations, also called aneuploidy, denote an abnormal number of chromosomes. Aneuploidy may arise due to defects in the centrosome number, aberrant spindle assembly, defects in chromosome attachment to the spindle, dysfunctional spindle assembly checkpoint (SAC), impaired chromosome cohesion, telomere fusion or replication stress [89,120]. Structural chromosomal aberrations in the form of translocations, deletions or insertions arise due to DNA repair defects or due to mitotic defects in chromosome segregation.

A higher frequency of chromatid breaks, sister chromatid exchange, anaphase bridges, lagging chromosomes and micronuclei are found following PARP1, PARP2 or PARG depletion or inhibition, particularly in response to genotoxic stress induced with different DNA-damaging agents [18,72,77,102,121–128]. PARP depletion or inhibition also lead to aneuploidy [12,14,18,127]. Chromosomal instability due to PARP loss or inhibition might be caused by any or all the mitotic defects in which PARPs have been implicated (chapters 4–7), as well as by PARP-mediated regulation of DNA replication and DNA damage response. In fact, recent work has shown that merely inhibiting PARP1/2 in mitosis does not yield mitotic phenotypes and that mitotic phenotypes arise due to PARP1/2 inhibition during DNA replication in S phase [74,76]. This was shown for various cell lines, including cervical cell lines (HeLa, C-33), breast cancer (MDA-MB-468, BT-549), osteosarcoma (U2OS), neuroblastoma (Kelly, LAN-5, SHEP Tet21/N) and glioblastoma (U87MG) [74–

76,102]. Why does PARP1/2 inhibition during DNA replication result in mitotic phenotypes? I will address this by considering PARP functions during DNA replication and replication stress, and by linking the reported mitotic outcomes of replication stress and DNA damage with mitotic phenotypes arising from PARP depletion or inhibition.

PARP1 and PARG have important functions during DNA replication and DNA repair. PARP1 was shown to mediate replication fork reversal in response to replication stress [10]. PARP1/2-inhibited or -depleted cells show faster replication and accumulation of DNA damage in S phase cells [15,16,73,79,128]. PARP1 also protects stalled replication forks from excessive degradation by MRE11 and promotes fork restart [7,129]. Moreover, PAR is required for the intra S-phase checkpoint by promoting fork retention and activation of CHK1 (**checkpoint kinase 1**) upon replication stress [130]. PAR levels need to be regulated by PARG during DNA replication, as PARG deficiency or inhibition reduces cell proliferation rate due to the slowing down of replication forks and accumulation of abnormal DNA replication intermediates [23,25]. PARG is also necessary for recovery from prolonged replication stress [24].

Stalled replication forks may undergo fork reversal or require homologous recombination for lesion bypass. HR is also required for the repair of DSBs that occur due to fork collapse. HR repairs DSBs by using a sister chromatid as a template, resulting in sister chromatid exchange (SCE). A higher frequency of SCE was observed due to PARP1/2 depletion or inhibition, especially after DNA damage, indicating that PARP1/2 maintain replication fork integrity and protect replication forks from DSBs [18,121–125]. Deficiency in HR proteins, as in the case of genetic mutations in BRCA1/2, leads to accumulation of unrepaired DSBs or incorrectly repaired DSBs, and was shown to sensitize cells to PARP1/2 inhibitors [78,126,131–133] (see also Targeting mitotic cells in cancer therapy with PARP inhibitors).

In the absence of HR, NHEJ or alternative NHEJ (alt-NHEJ, also called microhomology-mediated end-joining or MMEJ) can repair DSBs, but often with errors that result in chromatid fusions [126,134,135]. Fusion of two broken sister chromatids, two broken chromosomes or telomere fusion by end joining pathways during interphase can generate dicentric chromosomes visible as radial chromosome formations in metaphase and as anaphase DNA bridges (also called chromatin bridges) [80] (Fig. 2). Although anaphase DNA bridges are particularly pronounced in homologous recombination-deficient cancer cells, they also occur in wild-type cells exposed to replication stress [76,136]. PARP1/2 inhibition in HR-deficient cells during S phase increases the frequency of radial chromosomes and anaphase DNA bridges by compromising replication fork stability and amassing lesions that cannot be repaired by HR and are therefore incorrectly repaired by NHEJ [76,126].

Abnormal replication intermediates are generated in response to replication stress by genomic regions called common fragile sites (CFS). CFS remain underreplicated at the G2/M transition [137]. Under-replicated CFS undergo unscheduled DNA synthesis at G2/M, and give rise to DNA damage in prometaphase, ultrafine anaphase DNA bridges and 53BP1-positive nuclear bodies in G1 cells [120,138–142]. Ultrafine anaphase DNA bridges originate from under-replicated CFSs that remain connected through thin threads of DNA

bound by FANCD2 (**F**anconi **a**nemia **c**omplementation group **D2**), BLM (**B**loom syndrome protein) helicase, PICH (**P**lk1-**i**nteracting **c**heckpoint **h**elicase), the BTRR complex (TOPOIIIa, RMI1 and RMI2), RIF1 (**R**ap1-**i**nteracting **f**actor 1 **h**omolog) and MUS81-EME1 endonuclease [81,143] (Fig. 2). Resolution of replication intermediates in mitosis through cleavage by MUS81-EME1 nuclease favours the formation of DNA breaks, which allows faithful segregation of sister chromatids [144–146]. Replication stress-induced DNA breaks in mitosis may thus originate from cleaved unresolved replication intermediates and may be necessary to forestall missegregation caused by ultrafine anaphase DNA bridges. Mitotic cells are hypersensitive to DNA damage as DNA damage response in mitotic cells is only partially activated; DNA damage is sensed, ATM kinase is activated and  $\gamma$ H2AX foci are formed, but signalling downstream of  $\gamma$ H2AX is impaired in a CDK or PLK1-dependent manner [81,147–149]. Partial activation of DNA damage response is thought to mark DNA damage sites for subsequent repair in G1, where replication stress-induced DNA lesions accumulate within 53BP1-positive nuclear bodies [138,139,150]. Ultrafine anaphase DNA bridges and 53BP1-positive nuclear bodies in G1 were observed upon PARP1 depletion or PARP1/2 inhibition [72,79]. Moreover, inhibition of the checkpoint kinase ATR exacerbates PARP1/2 inhibition by allowing mitotic entry in the presence of unrepaired DNA damage from interphase, leading to accumulation of 53BP1-positive nuclear bodies in G1 [79].

Replication stress may also cause weakening of sister chromatid cohesion in interphase and result in premature loss of cohesion ('cohesion fatigue') and chromosome alignment problems in metaphase [74]. PARP1/2 inhibition during S-phase replication was similarly shown to cause cohesion weakening and premature loss of cohesion visualized as chromatid scattering in metaphase cells [74] (Fig. 2). Premature loss of cohesion and chromatid scattering are more pronounced in cells with higher basal levels of replication stress and higher PARP1/2 protein levels [74].

Another feature of replication stress is micronuclei that can be observed in daughter cells following cytokinesis (Fig. 2). Micronuclei contain acentric chromosomes, chromatid fragments or lagging chromosomes that have their own nuclear envelope [151]. Acentric chromosomes and chromatid fragments are caused by unrepaired or incorrectly repaired DNA lesions, as explained above. Lagging chromosomes are caused by mitotic errors in kinetochore attachment to the spindle, whereby a single kinetochore from one chromosome is attached to microtubules from more than one spindle pole (merotelic attachment), resulting in lagging chromosomes that are simultaneously pulled towards opposite poles [152]. Merotelic attachment may also be induced by DNA damage signalling through Aurora A and PLK1, which, by increasing the stability of kinetochore-microtubule attachments, may increase the probability of erroneous attachments [153]. During anaphase, acentric chromosomes, chromatid fragments and whole lagging chromosomes all appear as 'lagging' due to the inability to segregate accurately. Chromosomes within micronuclei undergo reduced and asynchronous DNA replication resulting in DNA damage and chromosome fragmentation, known as chromothripsis [154]. Lagging chromosomes and micronuclei are elevated upon PARP1/2 depletion or inhibition, in accordance with the accumulation of unrepaired or inappropriately repaired DNA lesions during S phase that are transmitted to mitosis [102,121,123,127,128].

DNA damage and unresolved replication intermediates trigger S-phase checkpoint activation as judged by CHK1 phosphorylation in PARP inhibitor-treated cells [15,75,155]. However, despite checkpoint activation, these cells progress into mitosis. Minor DNA damage in mitosis allows progression to G1 when DNA damage is repaired [156]. If DNA damage occurs in early (but not late) prophase, cells undergo chromosome decondensation and return to interphase [157]. Extensive DNA damage in mitosis causes metaphase arrest due to defective kinetochore attachment and activation of the SAC [156]. Metaphase arrest due to DNA damage leads to mitotic catastrophe, whereby cells die by apoptosis or slip out of mitosis through multinucleation or macronucleation due to chromosome missegregation, as well as micronucleation that results from lagging or acentric chromosomes [158]. PARP1- or PARP2-depleted cells proliferate normally, whereas PARP1/2 inhibitor-treated cells die by mitotic catastrophe [15,72–76,79,102].

The reason behind the stronger phenotype of PARP1/2 inhibition compared to protein depletion is the entrapment of PARP on DNA by PARP inhibitors, which destabilizes replication fork progression [78]. Replication phenotypes of PARP inhibition can be attributed to both inactivation of PARP catalytic activity and its entrapment on DNA, whereas mitotic phenotypes seem to be generally caused by PARP entrapment. If both PARP depletion and PARP inhibition cause a specific phenotype and if PARP depletion cannot (completely) rescue PARP inhibition phenotypes, the phenotype is likely caused by PARP inactivation or by both PARP inactivation and PARP entrapment. Conversely, if PARP depletion can rescue PARP inhibition phenotypes, the phenotype is due to PARP entrapment. Based on this reasoning, PARP1 depletion increases replication fork speed and impairs replication fork reversal and replication fork restart following replication stress [7,10,15], indicating that PARP1 activity is required for maintaining the stability and integrity of replication forks under normal conditions and after replication stress. However, PARP1/2 depletion does not induce and can rescue mitotic phenotypes of metaphase chromatid scattering or anaphase DNA bridges induced by PARP inhibitors, suggesting that entrapment of PARP-DNA complexes by PARP1/2 inhibitors causes mitotic defects by compromising replication fork stability [74,76,78,159]. PARP1/2 inhibitors therefore exert cytotoxicity by relying on mitotic dysfunctions such as metaphase arrest, premature loss of cohesion and anaphase DNA bridges to elicit mitotic catastrophe [74,76].

Contrary to PARP1/2, both PARG depletion and inhibition result in severe mitotic phenotypes in cells exposed to DNA damage (ionizing radiation), which are more pronounced compared to PARP1/2 inhibition [22,77]. PARG activity thus seems to be important not only during DNA replication but also mitosis, where PARG-mediated removal of PAR synthesized by TNKS1 may be required for accurate mitotic spindle assembly [27,77] (see Regulation of the mitotic spindle assembly by PARPs).

## 9 Targeting mitotic cells in cancer therapy with PARP inhibitors

Cancer cells proliferate faster and have a higher mitotic index compared to normal cells. Cancer cells spend longer time in mitosis due to extra centrosomes and extra chromosomes, which delay satisfaction of the mitotic checkpoint [160]. Furthermore, mitotic cells are hypersensitive to DNA damage; activation of DNA damage response during mitosis may

result in missegregation, thus propagating genomic instability of cancer cells [149,153]. Targeting mitosis is therefore a promising avenue in cancer therapy [161].

To date, different models of targeting mitosis in anticancer therapy have been described. Inhibitors targeting mitotic entry, mitotic spindle, spindle assembly checkpoint and mitotic exit have been undergoing clinical trials [162]. The TNKS1 inhibitor XAV-939 combined with an inhibitor of the mitotic kinase PLK1 was shown to induce cell death of triple-negative breast cancer cells (TNBC) [163]. TNKS1 depletion also shows synthetic lethality with BRCA1/2 depletion by exacerbating centrosome amplification [164]. Furthermore, TNKS1 inhibitors have shown synergistic effects with telomerase inhibitors based on the importance of TNKS1 in regulating telomerase access to telomeres [21,66].

Rather than inhibiting specific components of the mitotic machinery, PARP1/2 inhibitors cause death by mitotic failure due to replication stress and DNA damage in S phase [74–76]. PARG inhibitors may also have the same mechanism of cytotoxicity, considering the replication phenotypes and mitotic defects caused by PARG depletion or inhibition [22–25,77]. Genomic instability caused by intrinsic deficiencies in DNA repair pathways or higher levels of replication stress was shown to sensitize cells to PARP1/2 and PARG inhibitors [25,78,126,131–133,165]. As the first example of synthetic lethality due to PARP inhibition, homologous recombination-deficient cells mutated in BRCA1/2 were shown to be specifically killed by PARP inhibitors [132,133]. Since these breakthrough studies, deficiency of different proteins required for recovery from replication stress and DNA repair was shown to sensitize the cells to PARP inhibitors resulting in chromosomal instability, cell cycle arrest and cell death [78,131–133,166–169]. Among HR proteins that show synthetic lethality with PARP1/2 inhibitors, BRCA1/2, PALB2, ABRAXAS and BARD1 have also shown synthetic lethal interactions with PARG inhibitors [25,165].

PARP expression levels were found to positively correlate with the cytotoxic effects of PARP inhibition in agreement with PARP entrapment on DNA being the main cause of cytotoxicity [74,75,78,159]. PARP1 levels are increased in different cancer types, which may sensitize them to PARP inhibitors [170–173]. In addition to fostering PARP entrapment-induced cytotoxicity, PARP1 overexpression may also deregulate the antepause checkpoint and allow mitotic entry in the presence of mitotic stress [61]. TNKS1 expression levels are also increased in different tumour types, but this has a counter effect on cancer therapy as it confers resistance to telomerase inhibitors [21,113].

Combination therapy is often used in cancer treatment and is beneficial also in the case of PARP and PARG inhibitors. Given that PARP/PARG inhibitors compromise replication fork stability and impair DNA repair, DNA-damaging agents (e.g., ionizing radiation, camptothecin, temozolomide) as well as inhibitors of proteins involved in replication stress response, DNA damage response and cell cycle checkpoints (e.g., ATR, CHK1, WEE1 inhibitors) have shown synergistic effects with PARP1/2 or PARG inhibitors in killing cancer cells [77,79,155,166,174–177]. Combination treatment with PARP1/2 and ATR inhibitors results in accumulation of DNA damage in mitosis, yielding severe mitotic defects in the form of lagging chromosomes, anaphase bridges and micronuclei, and resulting in 53BP1 nuclear bodies in G1 phase or death by mitotic catastrophe [79,155]. Rather than

directly inhibiting DDR and checkpoint proteins, the BET domain inhibitor JQ1 synergizes with PARP1/2 inhibitors by reducing the expression levels of DDR factors BRCA1, RAD51 and TOPBP1, as well as the G2/M checkpoint regulator WEE1 [178,179]. As a result, unrepaired DNA damage is carried over into mitosis causing mitotic catastrophe [178]. Finally, PARP1/2 inhibitors have shown stronger anticancer effects when combined with TNKS1 inhibitors through targeting at once DNA replication, telomere stability and mitotic progression [113,166].

Overall, combination therapy with PARP1/2 inhibitors capitalizes on compromised replication fork stability, which is exacerbated in the case of (i) genetic mutations in DNA repair pathways that rescue stalled or collapsed forks, (ii) increased DNA damage burden through combination with DNA-damaging agents, and (iii) inhibitors of cell cycle checkpoints that allow compromised cells to progress to mitosis. In light of numerous connections between genomic instability acquired during S phase and chromosomal instability ensuing from mitotic progression, it is critical to analyse in detail mitotic phenotypes linked with the administration of PARP and other inhibitors that compromise genomic stability.

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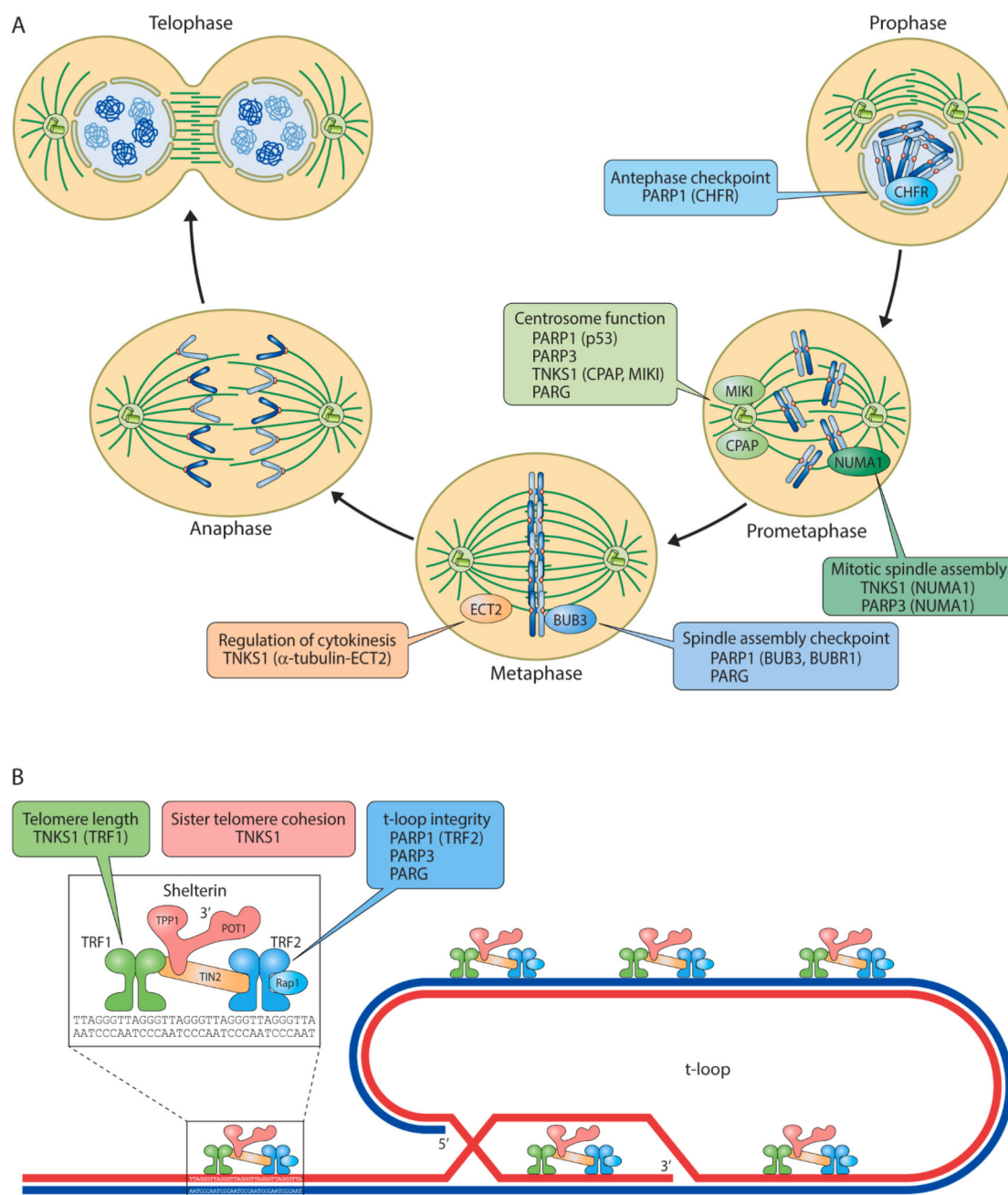
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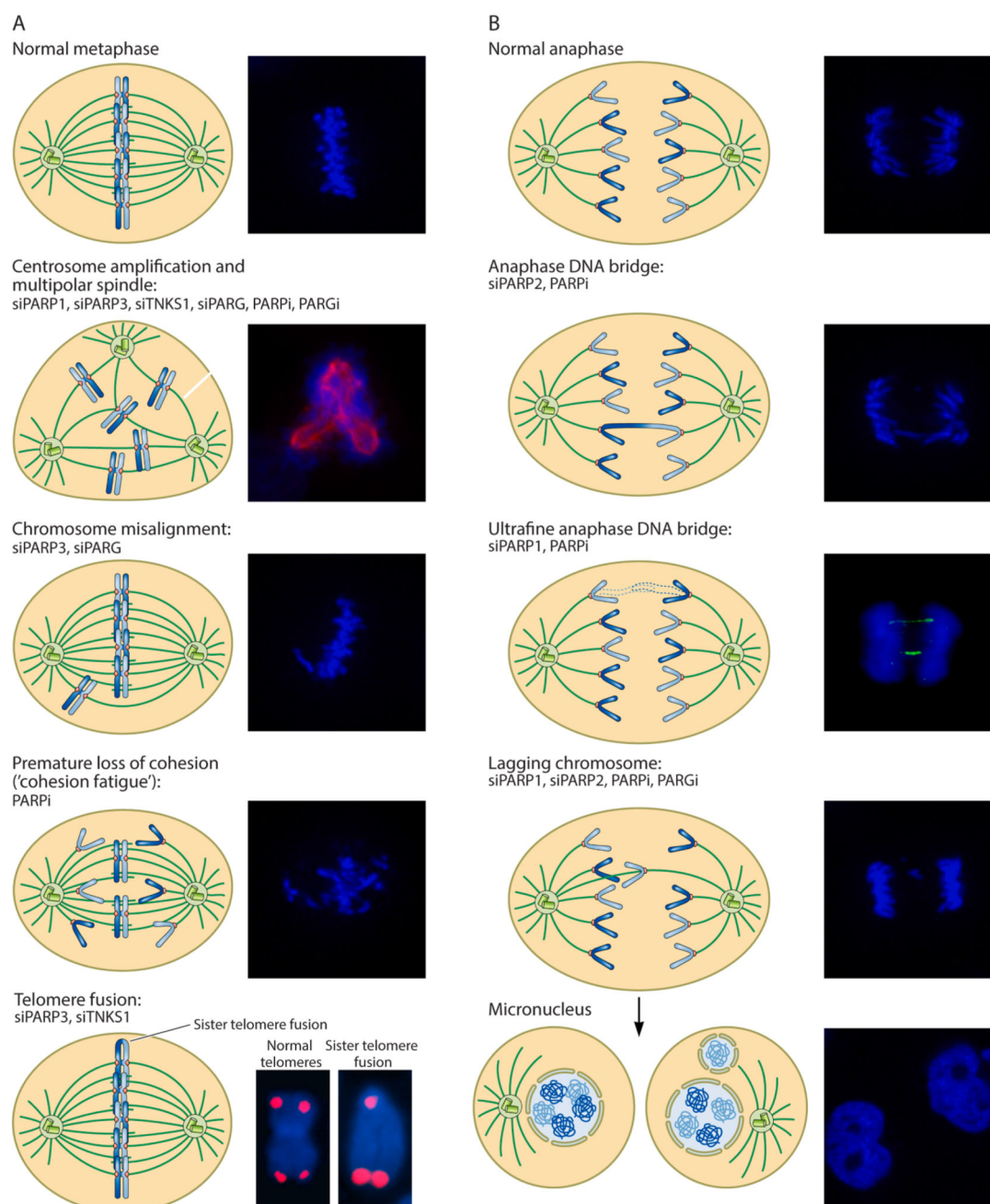
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**Fig. 1.** Regulation of mitotic functions and telomeres by PARPs and PARG. A) Mitotic stages. B) Telomere and the Shelterin complex. PARP/PARG functions are indicated in bubbles and known substrates are indicated in brackets within the bubbles and as blobs within schematics. The schematics are based on Cheeseman and Desai, 2008 and de Lange, 2018 [44,108].



**Fig. 2.** Mitotic phenotypes resulting from depletion or inhibition of PARPs or PARG. Schematics of the phenotypes observed in (A) metaphase and (B) anaphase, and the corresponding microscopy pictures are shown. Chromosomes are shown in blue. On the example of a multipolar spindle, tubulin is shown in red. Telomere fusion upon PARP3 depletion is visualized by FISH using PNA probe for telomeres (red); the microscopy pictures were kindly provided by Françoise Dantzer [19]. Ultrafine anaphase DNA bridges in PARP1-

depleted or inhibited cells are visualized with anti-PICH staining in green; the microscopy pictures were kindly provided by Simon Gemble and Mounira AmorGuéret [72].