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Review

How unfinished business from S-phase affects mitosis and beyond

Hocine W Mankouri[1,3], Diana Huttner[1,2,3] and Ian D Hickson[1,*]

[1]Department of Cellular and Molecular Medicine, Nordea Center for Healthy Aging, Copenhagen N, Denmark and [2]Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Copenhagen N, Denmark

The eukaryotic cell cycle is conventionally viewed as comprising several discrete steps, each of which must be completed before the next one is initiated. However, emerging evidence suggests that incompletely replicated, or unresolved, chromosomes from S-phase can persist into mitosis, where they present a potential threat to the faithful segregation of sister chromatids. In this review, we provide an overview of the different classes of loci where this 'unfinished S-phase business' can lead to a variety of cytogenetically distinct DNA structures throughout the various steps of mitosis. Furthermore, we discuss the potential ways in which cells might not only tolerate this inevitable aspect of chromosome biology, but also exploit it to assist in the maintenance of genome stability.
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Introduction

Eukaryotic cell-cycle progression must occur in an efficient and timely manner in order for cell proliferation to be sustained. Cells must precisely duplicate their chromosomes during S-phase and then accurately segregate them in M-phase (mitosis). Mitosis is a particularly hazardous time for cells because the genome undergoes very rapid and dramatic structural and organizational changes. Mitosis can be broken down into a series of well-defined steps that we will briefly review here to set the scene for the subsequent discussion (see also Figure 3A). Following the completion of bulk DNA replication, sister chromatids are connected by rings of a protein complex called cohesin, which were established during S-phase. During prophase, the first step of mitosis, DNA condensation results in a dramatic compaction of all chromosomes. This process is required to provide the necessary rigidity to counteract the pulling forces of the mitotic spindle. In the first of two waves of cohesion removal, release of the cohesin located on the chromosome arms is promoted by a Polo-like kinase (Plk1)-dependent (and proteolysis-independent) signalling event. Cohesin located at centromeres is protected from this activity and remains intact at this stage. In prometaphase, the nuclear membrane breaks down and the mitotic spindle fibres (microtubules) attach to their anchorage points (kinetochores) on chromosomes. Then, during metaphase, chromosomes become aligned on the mitotic spindle in preparation for sister chromatid disjunction. This process is tightly regulated by the spindle assembly checkpoint (SAC), which remains active until all chromosomes are correctly attached to the microtubules. Once chromosomes are correctly aligned, anaphase can then proceed, and this is trigged by proteolytic cleavage of the remaining cohesin at centromeres. Sister chromatids then migrate apart towards their respective spindle poles formed by microtubule-organizing centres (centrosomes). A new nuclear membrane then forms around the separated sister chromatids in telophase, in anticipation of cytokinesis that completes cell division. We refer readers to the following reviews for a more detailed description of mitosis (Eggert *et al*, 2006; Cheeseman and Desai, 2008).

As alluded to above, mitosis is a dangerous stage in the life of a cell, as many of the events that can cause chromosomal instability are known to occur at this time. Chromosome aberrations that can be visualized and scored during mitosis, such as chromosome missegregation events that lead to daughter cells containing an abnormal chromosome content (aneuploidy), can occur principally by two means. First, they can arise due to defects in the process of mitosis itself. For example, attempted segregation of acentric or dicentric chromosomes, abnormalities in the mitotic SAC, and defects in sister chromatid cohesion or chromosome condensation can all cause profound chromosome segregation defects. These scenarios are rare and invariably pathological. A second potential source of chromosome aberrations is from unresolved DNA structures arising during S-phase, which persist beyond interphase and then interfere with timely chromosome segregation during mitosis. Examples of these include incompletely replicated loci, topologically intertwined sister chromatids, and incompletely resolved DNA repair/recombination intermediates. Indeed, it is becoming increasingly apparent that the transition from S-phase to M-phase is perhaps not quite as orderly as once thought, and cells frequently enter mitosis with incompletely replicated, or unresolved, chromosomes. These problems arise because certain regions of the genome are inherently troublesome to fully replicate and segregate. Furthermore, a

*Corresponding author. Department of Cellular and Molecular Medicine, Nordea Center for Healthy Aging, Panum Institute, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen N, Denmark. Tel.: +45 35326738; Fax: +45 35327845; E-mail: iandh@sund.ku.dk
[3]Joint first authorship.

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plethora of obstacles can potentially impair timely DNA replication, and these probably contribute to, or exacerbate, difficulties that appear to occur naturally at intrinsically problematic loci. For example, replication forks can stall upon encountering any of a number of obstacles in the template DNA, as outlined in Figure 1. This can lead to persistently stalled replication forks or other types of unresolved DNA structures (e.g., regressed/collapsed replication forks or recombination intermediates; Figure 2) arising as a consequence of the processing of these obstacles (Lambert and Carr, 2013). Another hurdle that cells must overcome is the topological problem associated with the convergence of two opposing replication forks. This occurs either during normal DNA replication termination, or when an ongoing replication fork encounters another persistently stalled replication fork (Figure 1). This problem is an inevitable feature of eukaryotic DNA replication in which bidirectional DNA replication is initiated from multiple DNA replication origins along every chromosome. However, the molecular events occurring when two opposing replication forks converge are very poorly understood. Clearly, replication termination represents a steric and topological problem that probably requires the concerted action of a number of proteins, including type II topoisomerases and the 'Bloom's complex' (BLM (Bloom's Syndrome Helicase)-TOPOIIIα (DNA Topoisomerase IIIα)-RMI1-RMI2), which we discuss further below. Indeed, as we propose in this review, these proteins are central players in the resolution of 'unfinished business' from S-phase that impacts upon the maintenance of genome stability in mitosis.

Part 1: Underlying reasons for 'unfinished S-phase business' in mitosis

To deal with potential problems arising in S-phase (Figure 1), cells have evolved efficient checkpoint signalling pathways that become activated following DNA replication stress (Errico and Costanzo, 2012; Jones and Petermann, 2012). Through these intracellular signalling mechanisms, cells are able to respond in specific ways according to the particular problem that they encounter (e.g., by activating appropriate repair/tolerance pathways and the firing of additional replication origins). However, as discussed above, these mechanisms may perhaps not be as failsafe as once thought, particularly during the terminal steps of DNA replication. So why do cells ever enter mitosis without fully completing S-phase? We propose four, non-mutually exclusive, possibilities:

(1) There may be a fundamental problem associated with DNA replication termination, because the S-phase checkpoint machinery is unable to efficiently detect small regions of unreplicated DNA.

(2) Certain regions of the genome, particularly those with important structural functions (e.g., centromere and telomeres), are inherently difficult to replicate and to segregate in a coordinated fashion along with the rest of the bulk genomic DNA. Therefore, cells have evolved efficient ways to cope with this inevitable situation, and may in some cases even exploit it to their advantage.

(3) The carry-over of incompletely replicated/unresolved chromosomes into mitosis, despite appearing counter-intuitive, may actually be a fundamental aspect of normal

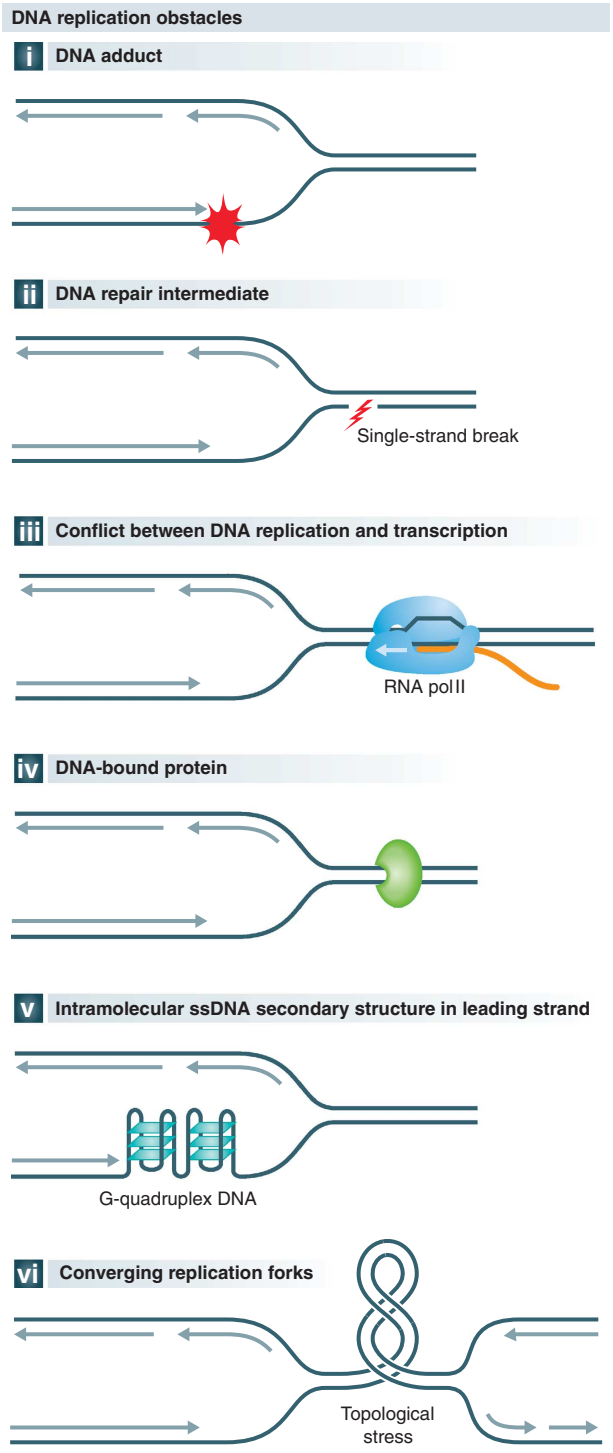

Figure 1 DNA replication problems. Schematic examples of obstacles that can hinder DNA replication forks. In some cases, these obstacles can interfere with the timely completion of DNA replication in S-phase, leading to subsequent problems in mitosis. The obstacles depicted from top to bottom are: (i) A DNA adduct (red star). (ii) A DNA repair intermediate, in this case a single-strand DNA break. (iii) A conflict between DNA replication and transcription (RNA polymerase II is depicted in blue and the transcript is shown in orange). (iv) A DNA-bound protein (shown as a green oval). (v) An intra-molecular ssDNA secondary structure in the leading strand, such as G-quadruplex DNA. (vi) Two converging replication forks with ensuing DNA topological stress that would form between them.

cell-cycle progression. This process might fulfil positive and important biological functions, at least in unperturbed cells.

(4) Cells may consider mitosis to be a far more hazardous cell-cycle stage than S-phase. Once cells have committed to mitosis, they subsequently proceed as quickly as possible to minimize the possibility of gross chromosomal aberrations (Ganem and Pellman, 2012). By comparison, the inability to fully complete DNA replication may be regarded as a minor concern in the grand scheme of things. Furthermore, cells have evolved strategies to rectify unresolved problems in the subsequent G1-phase, in the 'calm after the storm'.

The discovery that 'unfinished S-phase business' in mitosis is remarkably prevalent is a relatively new aspect in our understanding of chromosome segregation. However, we are only just beginning to understand how cells maintain genome stability in the face of such persistent, and probably ubiquitous, problems.

Part 2: Genomic regions that frequently give rise to 'unfinished S-phase business' in mitosis

The failure to fully complete DNA replication or fully resolve sister chromatids before the onset of mitosis may be due either to inherent difficulties in replicating and segregating specific types of chromosomal structures, or to late/delayed DNA replication at certain genomic loci. In this section, we will briefly review the genomic loci that most frequently give rise to 'unfinished S-phase business' in mitosis. Furthermore, the types of possible DNA structures that might persist at each of these regions will be discussed (Figure 2). Finally, we will propose potential ways in which cells may exploit these unresolved DNA structures to facilitate mitotic function.

(i) Centromeres

Centromeres are highly specialized chromosomal structures, which during metaphase of mitosis hold sister chromatids together and ensure their correct alignment on mitotic spindles (Westhorpe and Straight, 2013). The latter function is achieved by centromeric chromatin acting as an attachment point for the microtubules via kinetochore proteins. Centromeres are, therefore, important for accurate and timely chromosome segregation events. Centromeric heterochromatic is associated with specific proteins, and consists of repetitive DNA elements (α-satellite DNA in humans). It has been proposed that centromeric DNA might generate higher order looped structures via recombination between the repetitive elements (McFarlane and Humphrey, 2010). Moreover, the molecular architecture of centromeric chromatin undergoes complex cell cycle-dependent structural

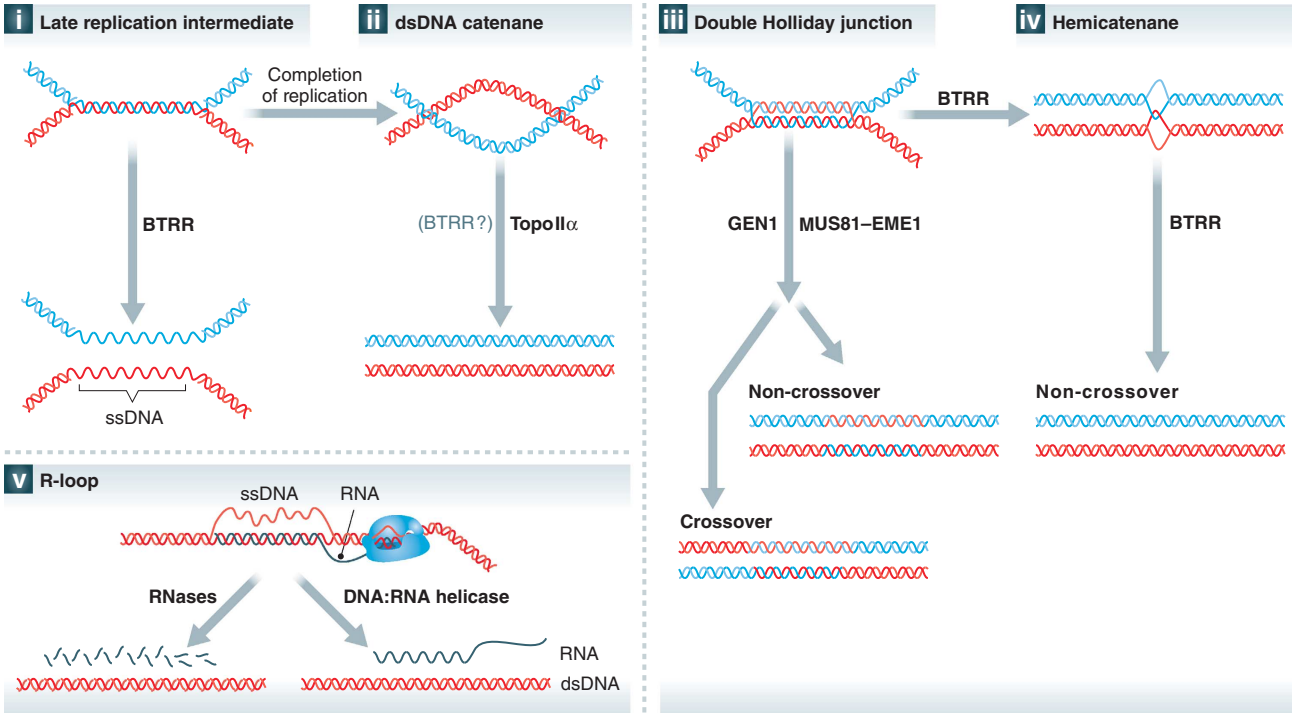

Figure 2 Examples of DNA structures arising in S-phase that may cause problems for chromosome segregation in mitosis unless resolved. (Upper left) A late replication intermediate (i), which escapes cleavage by MUS81-EME1 and contains a short stretch of unreplicated ssDNA, can be unwound by the Bloom's complex (depicted as BTRR). As a result of this, the chromosomes may contain regions of ssDNA that persist into mitosis. Fully replicated chromosomes contain dsDNA catenanes (ii), forming at replication termination sites. TopoIIα is the main enzyme that can decatenated these DNA entanglements, to produce two fully replicated dsDNA duplexes. In principle, the concerted action of the BTRR complex may also contribute to their decatenation. (Right) A double Holliday junction (iii) arises as an intermediate of homologous recombination repair of DNA gaps and breaks in S-phase. This structure can be resolved by the Bloom's complex (depicted as BTRR) in a process known as 'double Holliday junction dissolution', resulting in non-crossover (NCO) dsDNA products. Alternatively, Holliday junctions can be resolved by structure-specific endonucleases, such as MUS81-EME1 or GEN1. Crossover (CO) or NCO dsDNA products are formed depending on the relative orientation of the cleavage and ligation products. A hemicatenane (iv) can form at sites of converging DNA replication forks or as an intermediate product in the double Holliday junction dissolution reaction. Hemicatenanes are efficiently resolved by the TopoIIIα component of the Bloom's complex, resulting in NCO dsDNA molecules. (Lower left) An R-loop (v), formed during transcription, comprises an DNA:RNA hybrid with a region of displaced ssDNA. Specific DNA:RNA helicases can disrupt R-loops; the RNA transcripts dissociate and the DNA complementary strands are annealed. Alternatively, RNases may digest the RNA transcript.

alterations (Bui *et al*, 2012). All of these factors are likely to pose problems for the DNA replication machinery. Indeed, centromeres are known to comprise endogeneous sites of replication fork pausing in yeast (Greenfeder and Newlon, 1992), and they comprise hotspots for chromosome breakage and rearrangements in mammalian cells (Simi *et al*, 1998).

One particular type of unprocessed DNA structure that is proposed to persist at centromeres is the double-stranded DNA (dsDNA) catenane (Figure 2). These fully replicated, yet still intertwined, DNA molecules arise as a consequence of the need to overcome topological stress during normal DNA replication, and they must be resolved before attempted sister chromatid disjunction in anaphase (Sundin and Varshavsky, 1980, 1981). DNA Topoisomerase IIα (TOPOIIα) is the major player performing this decatenation activity in human cells (Porter and Farr, 2004). By a coupled reaction of dsDNA cleavage and passage, it can disentangle intertwined duplex DNA molecules. It has been proposed that DNA catenanes persist in centromeric regions until late in mitosis because they are shielded from TOPOIIα-mediated decatenation by cohesin. As mentioned previously, at the metaphase-to-anaphase transition, once the SAC is satisfied, the remaining cohesin protein at centromeres is proteolytically cleaved. It is only then that TOPOIIα can access centromeric regions and resolve DNA catenanes that have persisted there (Spence *et al*, 2007; Wang *et al*, 2010).

Interestingly, the Bloom's complex may also directly regulate DNA topology and prevent excessive levels of aberrant mitotic DNA structures arising at centromeres (Rouzeau *et al*, 2012; Norman-Axelsson *et al*, 2013). The Bloom's complex comprises a DNA helicase (BLM), a type IA topoisomerase (TOPOIIIα) and two OB-fold containing proteins (RMI1 and RMI2) (Mankouri and Hickson, 2007; Chu and Hickson, 2009). This complex is referred to as the 'Bloom's complex' because mutations in *BLM* cause the rare genetic disorder, Bloom's syndrome (BS), which is characterized by short stature, fertility defects, sunlight sensitivity, and predisposition to the development of all types of cancer (German, 1993). The precise functions of, and DNA substrates acted upon by, the Bloom's complex at centromeres remain unknown. In principle, the Bloom's complex could perform the same role as TOPOIIα in unlinking dsDNA catenanes persisting at centromeres. Although TOPOIIIα is single-stranded DNA (ssDNA) specific, and should not therefore efficiently unlink dsDNA catenanes, proficient dsDNA decatenation activity has nevertheless been demonstrated *in vitro* for the *S. cerevisiae* orthologues (Sgs1-Top3-Rmi1) of the Bloom's complex. This reaction proceeds through the concerted action of the Sgs1 helicase and two sequential ssDNA decatenation reactions catalysed by Top3 (Cejka *et al*, 2012). Despite this, however, it appears that TOPOIIα performs the majority of mitotic decatenation at centromeres (Wang *et al*, 2010). It therefore remains to be determined precisely what role(s) the Bloom's complex plays at centromeres, and how any putative division of labour between the Bloom's complex and TOPOIIα is normally shared and regulated.

(ii) Telomeres

Telomeres are specialized structures at the ends of chromosomes that consist of tandem sequence repeats of nucleotide bases (TTAGGG), together with their associated telomere-binding and -processing proteins (Palm and de Lange, 2008). Telomeres act as chromosomal 'caps' to prevent chromosome end-to-end fusions, and counteract the inevitable erosion of linear chromosomes caused by the 'end-replication' problem of DNA replication (i.e., the inability of the DNA replication machinery to copy the very ends of a DNA template) (Verdun and Karlseder, 2007). DNA replication problems arising at telomeres include frequent replication fork stalling due to multiple roadblocks, such as telomere-binding proteins and G-quadruplex DNA secondary structures that can form in the G-rich telomeric strand (Figure 1) (Ishikawa, 2013). Indeed, replication forks are more prone to stall in telomeric DNA repeats than throughout most other regions of the genome in yeast (Ivessa *et al*, 2002; Makovets *et al*, 2004). This increased propensity for replication forks to stall within telomeres can lead to replication fork regression, cleavage, or collapse. Importantly, telomeric replication is unidirectional and hence replication forks in telomeres are particularly sensitive to replication perturbation. For this reason, telomeres may resemble chromosome fragile sites (Sfeir *et al*, 2009, and see below). Interestingly, in addition to their structural and protective roles, telomeres may also serve important roles as 'sensors' for genomic stress. This is because telomeres can directly trigger cellular senescence once they either reach a critically short length or become dysfunctional. Indeed, DNA replication stress within telomeres causes persistent and irreparable DNA damage that can directly lead to 'telomere dysfunction-induced senescence' independent of telomere length (Fumagalli *et al*, 2012; Suram *et al*, 2012).

(iii) Fragile sites

Another source of unfinished S-phase business that can persist into mitosis is the perturbation of normal DNA replication termination events occurring at late-replicating regions of the genome. The latter phenomenon is most evident at so-called 'fragile sites', which are regions of the genome that form gaps or breaks (usually referred to as fragile site 'expression') on metaphase chromosomes in response to replication perturbation (Durkin and Glover, 2007). This is typically achieved using low doses of aphidicolin, an inhibitor of replicative DNA polymerases α, δ, and ε, to perturb DNA replication without noticeably affecting cell-cycle progression. Exposure to aphidicolin is thought to exacerbate intrinsic problems that already exist at these regions due to late/delayed DNA replication. Up to 230 aphidicolin-induced fragile sites have been described so far in human cells (Mrasek *et al*, 2010), though only a subset of these appear to be expressed in any given cell type (Letessier *et al*, 2011). Two types of these fragile sites have been characterized, termed as 'rare' and 'common'. Rare fragile sites are usually the result of nucleotide repeat expansion mutations, and are observed only in a small percentage of individuals (McMurray, 2010). Common fragile sites (hereafter denoted 'CFSs'), however, are detectable in all individuals (and even in other organisms). CFSs are considered to be a normal aspect of the genomic architecture, despite being implicated as hotspots for genome instability in cancers and neurological disorders (Smith *et al*, 2006; Durkin and Glover, 2007). The two best-studied CFS examples are FRA3B and FRA16D, which overlap with putative tumour-suppressor genes *FHIT* and *WWOX*, respectively. Indeed, these two CFSs are considered as the most sensitive sites to breakage, and comprise the most

'fragile' regions of the human genome. The reasons for fragility may be due to the presence of one or more naturally occurring, replication-perturbing DNA structures at CFSs (Figure 1), and/or due to specific properties of a particular CFS (e.g., its local chromatin structure, DNA topology, or intranuclear location) (Debatisse *et al*, 2012; Ozeri-Galai *et al*, 2012). These properties are probably aggravated by decreased usage of replication origins in/around CFSs in the cell type in which they are expressed (Palumbo *et al*, 2010; Letessier *et al*, 2011). This means that forks must travel longer distances within CFSs, and therefore are particularly sensitive to replication fork perturbation.

Given their instability and contribution to the development of human diseases, it seems remarkable that CFSs have not been eliminated by natural selection; on the contrary, CFSs are often evolutionarily conserved. For example, the mouse homologues of *FHIT* and *WWOX* also overlap with fragile sites (Fra14a2 and Fra8e1, respectively), and a similar syntenic relationship also occurs in mice for the homologues of a number of other human genes that overlap with CFSs (Durkin and Glover, 2007). This raises the possibility that CFSs fulfil positive functional roles to assist in the spatiotemporal regulation of normal cell-cycle progression. Therefore, different CSF pools may be epigenetically 'programmed' as DNA replication termination sites, depending on the relative origin usage pattern and transcriptome profile in each given cell type. Consistent with this possibility, a subset of commonly expressed CFSs has been demonstrated to exhibit reduced histone acetylation patterns (Jiang *et al*, 2009). We therefore speculate that, by ensuring that inevitable problems associated with late S-phase DNA replication termination always arise within pre-programmed loci, the cell uses the CFS as a 'chromosomal checkpoint' to monitor S-phase progression. In this way, the CFS locus itself, the encoded gene product, any (nc)RNA products, or even the mitotic DNA structure that ultimately arises there (discussed further below) could fulfil important functions in regulating the transition from S-phase to mitosis. For example, CFSs could, in principle, assist in sister chromatid cohesion following cohesin ring removal from chromosome arms in prophase by providing a set of DNA intertwinings that hold the sister chromatids in close proximity. Moreover, the RNA products caused by ongoing transcription at these CFSs might influence timely cell-cycle progression. It is notable that several CFSs contain very large (>1 Mb) genes that generally are to $>99\%$ intronic (Smith *et al*, 2006). Given the intrinsic problems associated with concurrent DNA replication and transcription (Helmrich *et al*, 2011, 2013), the seemingly unnecessary synthesis of such large transcripts that are likely to cause inevitable replication–transcription collisions appears surprising. Therefore, this may suggest that such collisions may be a normal, and physiological, aspect of CFS replication. Because the DNA replication machinery can dislodge RNA polymerases from DNA, one interesting possibility is that incoming replication forks arriving in late S-phase are utilized to spatiotemporally regulate the formation of various RNA products produced at CFS loci. When this occurs specifically in late S-phase, the release of CFS-borne pre-mRNAs and non-coding RNAs might then initiate events that ultimately promote mitotic progression. Consistent with this notion, a number of microRNAs have been reported to reside within/near many CFSs (Calin *et al*, 2004). Future studies should, therefore, be aimed at testing precisely how these microRNAs affect normal cell-cycle progression.

So what are the mechanisms that lead to the formation of gaps and breaks at CFSs? A number of precarious DNA structures have been proposed to arise/persist at CFSs, including stalled replication forks, stabilized R-loops, and/or incompletely resolved DNA repair intermediates (Figure 2). CFS expression was conventionally thought to be solely due to inadvertent rupture of chromosome structure at unreplicated regions of the genome caused by the physical forces of chromosome condensation (El Achkar *et al*, 2005; Lukas *et al*, 2011). However, recent data from both our and the Rosselli laboratory demonstrate that CFS expression can also be an active process that is promoted by structure-specific endonucleases, such as MUS81-EME1 or ERCC1, in early mitosis. Surprisingly, depletion of these structure-specific endonucleases reduces CFS expression, and this correlates with a concomitant increase in the frequency of several mitotic aberrations such as micronuclei, bulky anaphase bridges and CFS-associated ultra-fine bridges (Naim *et al*, 2013; Ying *et al*, 2013; also discussed further below). An active role for MUS81-EME1 in resolving structures at CFS loci is reinforced by our observation that MUS81-EME1 localizes to CFS loci in prophase (Ying *et al*, 2013). These data suggest that a radical re-evaluation of our models for CFS expression is required. Rather than CFS breakage *per se* being a threat to genome instability as generally thought, we propose that it is a failure to adequately cleave regions of unreplicated DNA at a CFS that poses the greater hazard to cell viability because it leads to chromosome missegregation in mitosis. Furthermore, the active formation of gaps/breaks at unreplicated regions may comprise a means to generate 'marked' DNA loci that can then be adequately tracked and protected throughout mitosis by putative intracellular signalling/sensing pathways (further discussed in Part 4). A failure to do this may mean that regions of unreplicated DNA would be overlooked by this putative tracking system, ultimately causing disastrous consequences during mitosis and/or in subsequent cell cycles.

Part 3: Mitotic structures arising due to 'unfinished S-phase business'

A number of cytogenetically distinct unresolved mitotic DNA structures have been characterized (Figure 3B). Bulky anaphase DNA bridges and lagging chromatin ('laggards') are likely to be pathological structures that arise due to aberrant chromosome morphology (and subsequent misalignment on the mitotic spindle) or defects in the mitotic machinery. These lead to aneuploidy and are rare events in unperturbed cells. More recently, a new type of much more prevalent mitotic bridge structure has been identified. Ultrafine DNA bridges (UFBs), which maintain physical links between separating sister chromatids during anaphase, have been found at this mitotic stage in all human cell types analysed so far (Baumann *et al*, 2007; Chan *et al*, 2007). They differ from the extensively studied bulky anaphase bridges and laggards in that they do not contain histones and cannot be stained with conventional intercalating DNA dyes such as DAPI; rather, they are detected using immunofluorescence microscopy visualization of proteins that bind to them. On the basis of our current understanding, the first protein that can be

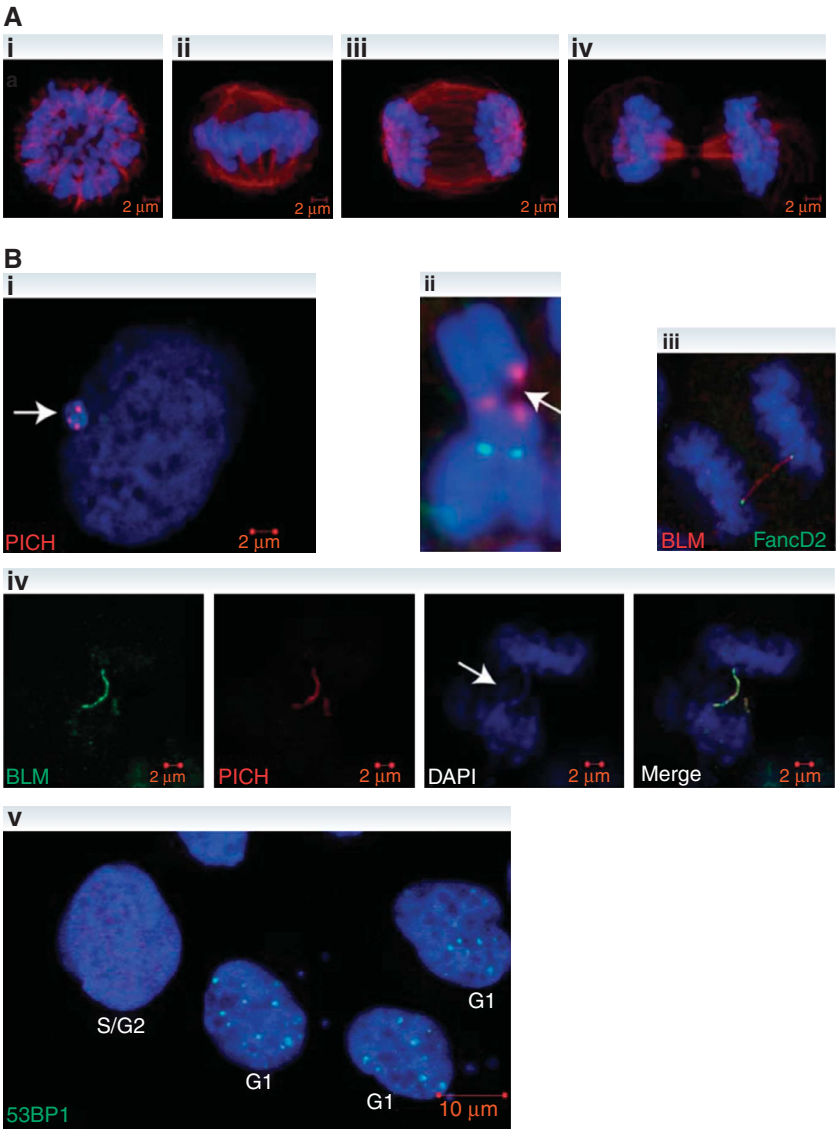

Figure 3 Normal versus aberrant mitotic structures in human cells. (**A**) Representative images of a normal mitosis in human U2OS cells. DNA is stained with DAPI (blue) and microtubules are detected using an anti-alpha-tubulin antibody (red). In prophase (not shown), chromosomes begin to condense and the centrosomes separate and move to opposite poles. In prometaphase (i) the nuclear membrane breaks down; the kinetochores assemble at centromeres and are captured by the microtubules. In metaphase (ii) chromosomes appear to be hyper-condensed; sister chromatids are attached in a bipolar fashion to spindle microtubules and aligned on the metaphase plate. In anaphase (iii) cohesin is removed from centromeres by separase-mediated cleavage, and the sister chromatids are pulled to opposite poles of the spindle. In telophase (iv) the midbody forms and cytokinesis occurs via ingression of a cleavage furrow from the plasma membrane. Following abscission, the chromosomes and nuclear components are repackaged into daughter cell nuclei. Finally, chromosomes de-condense and the nuclear envelope re-forms. (**B**) Examples of aberrant mitotic structures (i) a micronucleus, revealed by DAPI staining (blue) and shown to contain PICH foci (red). (ii) A broken common fragile site, marked by the presence of FANCD2 (red). The green 'sister foci' represent centromeres defined by Hec1 kinetochore protein. (iii) An ultrafine DNA bridge, stained positive for BLM (red) with FANCD2 foci (green) on both ends. (iv) A bulky DNA bridge, which is positive for BLM (green), PICH (red), and DAPI staining (blue). (v) 53BP1 bodies (green) in G1 cells, which are defined by being negative for cyclin A staining, as indicated in the figure.

detected on UFBs (in metaphase) is PICH, a member of the SNF2/SWI family of DNA-dependent ATPases (Baumann *et al*, 2007; Kaulich *et al*, 2012). PICH is likely to be important for accurate/timely chromosome segregation, as its depletion leads to increased micronucleus formation and an enhanced frequency of bulky anaphase bridges (Ke *et al*, 2011; Kaulich *et al*, 2012). Importantly, PICH is not normally associated with DNA in human cells until nuclear envelope breakdown in prometaphase (Baumann *et al*, 2007; Kurasawa and Yu-Lee, 2010), suggesting that the defects arising in PICH-depleted cells are a direct consequence of a defective mitosis. A number of important roles have been proposed for PICH in mitosis, including nucleosome removal, maintenance of chromosomal and chromatin architecture, and functioning as a stable platform for the recruitment of an ensemble of proteins. Evidence for the recruitment role of PICH comes from the observations that PICH is required for the binding of all other hitherto known UFB-associated proteins (Ke *et al*, 2011; Kaulich *et al*, 2012; Rouzeau *et al*, 2012). Other key UFB binding proteins identified thus far include members of the Bloom's complex, the ssDNA binding factor RPA (Chan and Hickson, 2009; Burrell *et al*, 2013), and the FANCM helicase (Vinciguerra *et al*, 2010). The roles of these proteins on UFBs are not yet fully understood, but they are implicated in the

resolution of UFBs, and/or the recruitment of other important processing/signalling factors.

Until now, three species of chromosome locus-specific UFB have been described. The most prominent form of UFBs originates from centromeres, and these are induced by treatment of cells with inhibitors of TOPOIIα, such as ICRF-193 or ICRF-159 (Baumann *et al*, 2007; Chan *et al*, 2007; Spence *et al*, 2007; Wang *et al*, 2008). A second group of UFBs co-localize with CFS loci and are revealed by foci of the Fanconi Anaemia pathway proteins FANCD2 and FANCI at their termini (Chan *et al*, 2009; Naim and Rosselli, 2009). The third group, called 'T-UFBs', arises from telomeres, and were very recently identified in skin and lung fibroblast cells (Barefield and Karlseder, 2012). As discussed in Part 2, these genomic regions all possess inherent difficulties in DNA replication that can result in the carry-over of unreplicated DNA or other unresolved DNA structures into mitosis. Given that the frequency of the various types of UFBs can be enhanced in different ways, it is likely that they are fundamentally different in their nature. Centromeric UFBs are proposed to consist of unprocessed dsDNA catenanes, which are normally shielded from TOPOIIα decatenation activity until cohesin is degraded at the metaphase–anaphase transition (Wang *et al*, 2010). CFS-associated UFBs specifically arise due to a failure of endonucleases such as MUS81-EME1 to adequately cleave putative unreplicated regions of DNA at CFSs (Naim *et al*, 2013; Ying *et al*, 2013). Assuming that CFS loci remain partially unreplicated at the onset of mitosis, we propose that CFS-associated UFBs represent late replication intermediates (LRIs) (Figure 2). T-UFBs remain poorly characterized, and it remains to be determined whether they comprise solely pathological DNA structures associated with telomere malfunction, or whether they assist with normal telomere replication and segregation events in mitosis.

Although the physiological functions of UFBs remain unclear, their presence in unperturbed cells implies that they fulfil important structural roles that assist in the spatiotemporal organization of chromosomes during anaphase. By opposing the pulling forces of the mitotic spindle, UFBs may prevent the premature rupture of chromosomes during anaphase, and/or assist the SAC machinery by maintaining the appropriate amount of tension between sister chromatids. A number of unanswered questions remain regarding UFBs. First, what is the precise nature of the various UFBs described so far? Because UFBs cannot be stained with intercalating DNA dyes there is a significant challenge in detecting them routinely. Identification of other UFB-associated proteins, and subsequent characterization of their biochemical functions, should assist in revealing the true nature of UFBs. Second, are there more subtypes of UFBs, and, if so, how are these different from the three types described in this review? Given that a number of different types of unprocessed DNA structures (Figure 2) can potentially lead to UFB formation, it may be possible to further subclassify UFBs depending on their location, the nature of the initiating lesion, and their method of resolution. Interestingly, RPA is another protein often observed on UFBs (Chan and Hickson, 2009; Burrell *et al*, 2013). However, the RPA staining pattern is not always uniform, and is usually mutually exclusive with PICH staining on UFBs. It remains to be determined whether these comprise key UFB resolution intermediates, or whether this RPA staining pattern reveals one or more novel subclasses of UFBs that arise at a specific initiating lesion or chromosomal locus. Also, depletion of XRCC3, a paralogue of the RAD51 recombinase that acts as an accessory factor in homologous recombination, leads to elevated levels of UFBs in mitosis that can be suppressed by expression of the bacterial Holliday junction resolvase, RusA (Rodrigue *et al*, 2013). These observations imply that unresolved recombination intermediates could also form precursors for at least some types of UFBs. It will be interesting to further determine the relationship between homologous recombination repair and UFB subtypes in future studies. Finally, what are the precise mechanisms by which cells resolve UFBs? Although the mechanisms remain unknown, most UFBs disappear by late telophase. Indeed, the number of UFBs decreases with progression through anaphase–telophase, while at the same time the length of the remaining UFBs increases, sometimes reaching several micrometers (Baumann *et al*, 2007; Chan *et al*, 2007). Consistent with their active resolution by the Bloom's complex, which normally resides on UFBs and which is able to process a number of DNA structures, BS cells have elevated numbers of PICH-positive UFBs and often very long UFBs, which can persist until late telophase (Chan *et al*, 2009). We therefore propose that PICH and the Bloom's complex act cooperatively to resolve UFBs and/or their precursors in mitosis.

In addition to the proposed roles of PICH and the Bloom's complex, it is possible that other enzymes can also resolve UFBs in mitosis. A prime candidate for this is GEN1, a structure-specific endonuclease that becomes maximally activated in M-phase and only gains access to DNA following nuclear envelope breakdown (Matos *et al*, 2011). Indeed, depletion of GEN1 results in elevated levels of UFBs, suggesting an active role for GEN1 in UFB resolution (Rodrigue *et al*, 2013). Furthermore, co-depletion of GEN1 and MUS81-EME1 causes severe chromosome segregation defects in BS cells (Wechsler *et al*, 2011). GEN1 activity may therefore comprise a last-ditch attempt to resolve UFBs. If so, it however remains to be determined which type(s) of DNA structures GEN1 specifically cuts during mitosis.

Part 4: Beyond mitosis

As discussed in Part 3, 'unfinished S-phase business' intermediates that persist into mitosis can potentially be cleaved by various nucleases such as MUS81-EME1 or GEN1, and/or be unwound or decatenated by the Bloom's complex. However, it is still unclear whether cells can complete the repair of these structures during this relatively short period, or whether the unrepaired DNA is carried over into the next cell cycle. Interestingly, in the beginning of the subsequent G1-phase, a protein called 53BP1 forms large foci referred to as 53BP1 nuclear bodies (53BP1-NBs; Harrigan *et al*, 2011; Lukas *et al*, 2011). These structures are defined by their equal numbers and symmetrical pattern in newly formed daughter cells (Lukas *et al*, 2011; Ying *et al*, 2013). Although they are detectable even in unperturbed cells, they are increased upon replicative stress (e.g., aphidicolin) and colocalize with CFS loci (Lukas *et al*, 2011). While 53BP1-NBs were initially proposed to form around broken DNA generated at the G2/M transition (Lukas *et al*, 2011), our recent data suggest that they may mark unresolved or broken UFBs that must be repaired in the subsequent G1- or S-phase of the cell cycle (Ying *et al*, 2013). This notion is supported by the observation

that G1 cells contain increased numbers of 53BP1-NBs upon depletion of MUS81-EME1, the enzyme proposed to cleave CFSs and to suppress CFS-associated UFB formation during early mitosis (*cf.* Parts 2 and 3) (Ying *et al*, 2013); it is furthermore consistent with the demonstration that 53BP1-NBs are elevated in BLM-deficient cells (Lukas *et al*, 2011).

Although its exact molecular role(s) remains unknown, 53BP1 is an important component of the DNA damage response that is rapidly recruited to DNA strand breaks (Schultz *et al*, 2000). Importantly, 53BP1 promotes non-homologous end-joining (NHEJ) and suppresses homologous recombination during G1-phase (Chapman *et al*, 2013; Di Virgilio *et al*, 2013; Escribano-Diaz *et al*, 2013; Zimmermann *et al*, 2013). One possibility, therefore, is that 53BP1-NBs may specify sites of ongoing NHEJ repair of broken UFBs in G1 cells. An alternative possibility is that resolved UFBs may also require the protective activity of 53BP1. For example, following the resolution of LRIs by the Bloom's complex, an ssDNA gap is predicted to occur at the unreplicated region of DNA. 53BP1 may potentially shield these ssDNA gaps through G1, until cells can efficiently repair them by evoking high-fidelity DNA polymerases in S-phase (Harrigan *et al*, 2011). In addition to shielding precarious DNA structures and promoting their timely repair, it is possible that 53BP1-NBs facilitate the restoration of chromatin structure following resolution of UFBs, which seem to be devoid of any epigenetic or chromatin marks. In future studies, it will therefore be interesting to determine which additional factors associate with 53BP1-NBs. Some proteins known to co-localize with 53BP1-NBs include the DNA damage response factors MDC1 and γH2AX, the transcription factors Oct1 and PTF, and the RNA helicase DDX1 (Pombo *et al*, 1998; Harrigan *et al*, 2011), although the functional significance of these associations remains to be established.

Part 5: The Bloom's complex is a central player in the maintenance of genome stability

A recurring theme throughout this review is the involvement of the Bloom's complex in processing various types of DNA structures. Indeed, the Bloom's complex may act as a DNA structure 'dissolvasome' that can process a variety of DNA replication and recombination intermediates arising during S-phase and mitosis (Mankouri and Hickson, 2007). It may, therefore, fulfil two temporally distinct cell-cycle functions: in S-phase, it plays a largely preventive role to suppress the formation of 'unfinished S-phase business', whereas in anaphase it also acts as a salvage mechanism to directly resolve those intertwined DNA structures that have still made it through to mitosis. Consistent with this idea, (1) the Bloom's complex can process most of the precarious DNA structures that are associated with 'unfinished S-phase business' in mitosis (Figure 2) (Wu and Hickson, 2003; Cejka *et al*, 2012); (2) the Bloom's complex localizes to all known subtypes of UFBs in mitosis and is directly implicated in their resolution (Chan *et al*, 2007, 2009; Barefield and Karlseder, 2012), and (3) BS cells display elevated levels of all types of unresolved UFBs, as well as other manifestations of aberrant mitosis such as bulky anaphase bridges, micronuclei, and 53BP1-NBs (Chan *et al*, 2007; Lukas *et al*, 2011; Figure 3B).

One notable and potentially interesting exception to the above is that BS cells do not exhibit obviously elevated CFS expression (Arlt *et al*, 2003). This observation is somewhat unexpected given that the Bloom's complex can act upon a broad range of DNA substrates that are predicted to arise at perturbed replication forks (Mankouri and Hickson, 2007). However, two important factors may complicate interpretation of the role(s) of the Bloom's complex at CFSs. First, BLM has been demonstrated to stimulate MUS81-EME1 DNA cleavage *in vitro* (Zhang *et al*, 2005) and might, therefore, promote CFS cleavage by MUS81-EME1 in a manner similar to its ability to stimulate replication fork breakage in S-phase (Shimura *et al*, 2008). Second, CFS expression in a given cell type correlates with decreased usage of replication origins at/around the respective CFSs (Letessier *et al*, 2011). Given that dormant replication origins fire more frequently in BS cells (Davies *et al*, 2007), this could in principle compensate for a lack of DNA repair/LRI processing in Bloom's cells and explain why CFS expression is not notably elevated in these cells. If true, then this implies that BS cells should be highly sensitive to loss of factors that permit such increased usage of compensatory origins around CFSs. Future studies should be aimed at testing this hypothesis, and further examining the role(s) of the Bloom's complex at CFSs. However, it should be noted that there also remains the untested possibility that there is something unique about the type, or density, of DNA replication problems arising at CFSs, which means that they are normally not adequately processed by the Bloom's complex before mitotic onset.

Part 6: Links to human health and ageing

Each of the problematic loci discussed in Part 2 of this review has implied causal links with human diseases and ageing. Indeed, age-associated shortening or dysfunction of centromeres and telomeres has been proposed to contribute to ageing and age-related diseases (Nakagome *et al*, 1984; Harley *et al*, 1990, 1992; Vaziri *et al*, 1994; McFarlane and Humphrey, 2010). CFSs have been causally implicated in tumorigenesis, tumour progression, and neurological disorders. This is largely because a number of putative tumour-suppressor genes (e.g., *FHIT* and *WWOX*), as well as a number of very large genes required for brain development (e.g., *PARKIN*, *GRID2*, and *RORA*), lie within characterized CFSs (Smith *et al*, 2006). Although CFS expression varies between individuals (Denison *et al*, 2003), as well as between different cell types (Letessier *et al*, 2011), it remains to our knowledge untested whether CFS stability varies as a function of age, and whether this contributes to the aetiology of various human diseases. In addition to the above, it is likely that mitotic aberrations in general may also be associated with premature ageing. Indeed, the mutation, or reduced expression, of an important mitotic checkpoint protein called BubR1 is associated with increased aneuploidy and the acceleration of various age-related phenotypes (including cancer), in both humans and mice (Baker *et al*, 2004; Suijkerbuijk *et al*, 2010; Wijshake *et al*, 2012). Remarkably, experimentally overriding the age-associated decline in BubR1 levels normally observed in various tissues results in attenuated tumorigenesis and life-span extension in mice (Baker *et al*, 2013). These intriguing data raise the tantalizing possibility that suppressing mitotic defects by enhancing mitotic fidelity could be an important mechanism to promote healthy ageing in humans.

Glossary

53BP1-NB	53BP1 nuclear body
BLM	Bloom's Syndrome Helicase
BS	Bloom's Syndrome
CFS	Common Fragile Site
dsDNA	double-stranded DNA
ERCC1	structure-specific endonuclease
GEN1	structure-specific endonuclease
LRI	Late Replication Intermediate
MUS81-EME1	structure-specific endonuclease
NHEJ	Non-homologous End Joining
SAC	Spindle Assembly Checkpoint
ssDNA	single-stranded DNA
TOPOIIα	DNA Topoisomerase IIα
TOPOIIIα	DNA Topoisomerase IIIα
UFB	Ultrafine DNA Bridge

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Conflict of interest

The authors declare that they have no conflict of interest.

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