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Establishment of Dependence Relationships Between Genome Replication and Mitosis

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Abstract Although budding yeast cell biology and genetics provided a powerful system to isolate S-phase checkpoint mutants, initial studies relied on a defect not likely to be relevant in higher eukaryotes. The first mutants were isolated for their inability to restrain mitotic spindle elongation in S-phase. Since most eukaryotes do not assemble spindles until prometaphase the validity of this approach might have been questioned. However, these early studies were designed with a highly valid assumption in mind; that checkpoints have a variety of targets, but comprise conserved kinase cascades that make up these signaling pathways. The task that lies ahead is to determine targets of the S-phase checkpoint relevant to mammals. One step forward might be the realization that the budding yeast S-phase checkpoint prevents loss of sister chromatid cohesion while DNA replication is ongoing. If this mechanism is conserved in mammals, it could prove vital for chromosome segregation fidelity. J. Cell. Biochem. 88: 95–103, 2003. © 2002 Wiley-Liss, Inc.

Key words: cell cycle control; S-phase checkpoint; DNA replication; sister chromatid cohesion

Checkpoint controls establish dependence relationships between cellular processes that are not biochemically integrated, but that need to be coordinated within the cell cycle. The temporal order of many such events must be strictly maintained to ensure the fidelity of cell division. Here, signaling pathways ensuring that genome duplication is completed before cell division, relationships controlled by the S-phase checkpoint, are discussed. From a historical perspective, checkpoint pathways were first conceived theoretically, then defined by pivotal genetic analyses of loss-of-function yeast mutants [Weinert and Hartwell, 1988, 1989]. A similar concept had been entertained much earlier based on mammalian cell fusion studies, revealing that mitotic or S-phase 'factors' are able to accelerate cycle progression [Rao and Johnson, 1970]. An extension of this idea was that negatively acting factors are needed to restrain cycle progression at inappropriate times. It was about 20 years later that these suspicions were confirmed by the yeast genetic experiments.

It is now known that checkpoint pathways in budding yeast and other eukaryotes have conserved signal transduction cascades but differ in terms of their targets [Weinert et al., 1994; Elledge, 1996; Smith et al., 2002]. A fundamental difference is that budding yeast checkpoints promote the activity of anaphase inhibitors rather than inhibiting the activity of cyclin/cdks that promote mitosis. A related issue is that spindle assembly occurs during S-phase in budding yeast, unlike most eukaryotes that assemble the spindle apparatus in mitosis. In budding yeast, checkpoints must inhibit spindle elongation while DNA is being replicated. In addition, sister chromatid cohesion must be maintained until the onset of anaphase. The budding yeast inhibitor of anaphase, Pds1, can perform both of these tasks [Cohen-Fix et al., 1996; Yamamoto et al., 1996a,b; Cohen-Fix and Koshland, 1997; Ciosk et al., 1998]. By inhibiting the protease Esp1, Pds1 prevents Scc1 cleavage, thereby keeping cohesion complexes intact, and restrains spindle elongation [Michaelis et al., 1997; Ciosk et al., 1998; Uhlmann and Nasmyth, 1998; Uhlmann et al., 1999; Jensen et al., 2001]. Pds1 is poly-ubiquitinated at the metaphase to

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anaphase transition by a multi-subunit enzyme complex known as the anaphase promoting complex (APC). This targets Pds1 to be degraded by 26S proteasomes [Cohen-Fix et al., 1996]. Although Pds1 is a major target of checkpoints controlling anaphase onset, a role for vertebrate homologs named securins, have not yet been demonstrated [Zou et al., 1999]. In mammals, the S-phase checkpoint no doubt prevents mitotic onset by inhibiting cyclin/cdk activity. How this is achieved is not known. Moreover, it is likely that other mammalian S-phase checkpoint pathways act in a manner similar to their budding yeast counterparts, preventing loss of sister cohesion from S-phase onwards.

S-PHASE CHECKPOINT CONTROL IN YEAST: CHECKPOINT SENSORS

The S-phase checkpoint ensures that the onset of mitosis is dependent on the completion of genome replication [Weinert and Lydall, 1993; Weinert et al., 1994; Elledge, 1996]. The genetic definition, provided by the phenotype of yeast mutants, is a checkpoint that prevents mitotic spindle elongation when DNA replication is inhibited with hydroxyurea (HU); wild type cells arrest with fully assembled short G2 spindles whereas *rad53* and *mec1* mutants initiate spindle elongation with a partly replicated genome. The S-phase checkpoint does not only control the mitotic spindle; all eukaryotes establish sister cohesion during DNA replication and it must be maintained until anaphase of mitosis. At least in yeast, cohesion is established at some loci early in S-phase and must be maintained for the remainder of the S-phase period as well as during G2 and early mitosis. Homologs of yeast S-phase checkpoint components are therefore likely to be important regulators of mammalian sister cohesion.

More yeast genetics suggested that monitoring ongoing DNA replication requires replication sensors that are integral with replication forks. Sensor components include Pol2, Rfc5, Dpb11, Drc1, and Sgs1, that are needed for DNA replication and checkpoint signaling [Araki et al., 1995; Navas et al., 1995; Sugimoto et al., 1997; Wang and Elledge, 1999; Frei and Gasser, 2000b]. For this reason, cells need to initiate DNA replication in order for a checkpoint signal to be established [Michael et al., 2000]. Experiments in the *Xenopus* egg extract system provided evidence that checkpoint activation depends on RNA primer synthesis [Michael et al., 2000]. The polymerase α-primase complex synthesizes the RNA primer once DNA has been unwound and Polα-primase has been loaded onto DNA. Based on these data, a current model (Fig. 1) can explain how the checkpoint

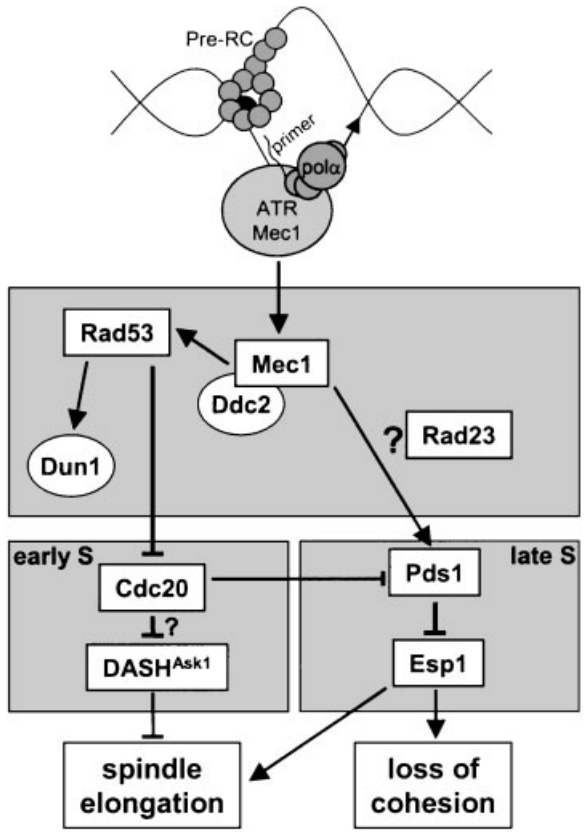

Fig. 1. Model for S-phase checkpoint signaling. Generation of the S-phase checkpoint signal requires initiation of DNA replication. Following pre-replication complex loading in G1, DNA unwinding and Cdc45 binding stimulate polα-primase complex recruitment and RNA–DNA primer synthesis by primase. Recognition of primers by Mec1/ATR activates the checkpoint signal. Budding yeast elements downstream of Mec1/ATR are shown below a schematic representation of a replication fork. Linear pathways are drawn for simplicity though more complex interactions between the checkpoint components are possible. The checkpoint signal is transmitted by kinases Mec1 and Rad53. Rad53 activation is dependent on the Mec1–Ddc2 complex. Downstream of Rad53 and Mec1, sequential pathways operate that are temporally regulated: the Mec1–Rad53 pathway operates in early S phase and may target Ask1, a component of the DASH complex that resides at kinetochores. A possible interaction is shown between Cdc20 and Ask1 based on the fact that Mec1–Rad53 are needed to prevent Cdc20 accumulation in S-phase and because Cdc20 is a kinetochore component [Topper et al., 2002]. The Mec1–Pds1 pathway is required part-way through S phase and operates by inhibition of Esp1 separase. The Mec1–Rad53 pathway also induces a Dun1-dependent transcriptional response which protects cells from replicative stress and allows replication re-start when conditions improve.

signal persists until replication is complete since primers, needed for synthesis of each lagging strand Okazaki fragment, are synthesized throughout S-phase. Other events such as origin firing perhaps do not coincide well enough with the completion of S-phase to be the molecular event that produces the checkpoint signal.

SIGNAL TRANSDUCTION

Like other checkpoint controls, transduction of the S-phase checkpoint signal from replication sensors to targets relies on a kinase cascade. In budding yeast, Mec1 (human ATR), and Rad53 (human Chk2/Cds1) kinases are the signal transduction factors [Allen et al., 1994; Weinert et al., 1994; Sanchez et al., 1996]. When replication is perturbed, these kinases are activated in a manner dependent on the sensor components. Exactly how this occurs is not known, but a putative activating factor has been identified that is known as Ddc2, Lcd1, or Pie1. Ddc2 physically associates with the N-terminus of Mec1 and is phosphorylated by Mec1 [Paciotti et al., 2000; Rouse and Jackson, 2000]. Phosphorylated Ddc2 is present in unperturbed and HU-treated cells, and is required for cell cycle arrest in HU. Phosphorylation and activation of Rad53 in response to replication arrest is Ddc2-dependent. Therefore, Ddc2 might mediate between Mec1 and Rad53 in response to ongoing DNA replication and when fork progression is blocked. How the sensor components promote the association of Ddc2 with Mec1, or modify the phosphorylation of Ddc2 by Mec1 need to be determined. Most likely, activated Ddc2 recruits Mec1 to essential sites of action (since it does not affect Mec1 kinase activity). A region of homology between Ddc2 and fission yeast Rad26, a component of the S-phase checkpoint that binds to Mec1 homolog Rad3 [Edwards et al., 1999], may be important for the interaction of Mec1–Ddc2 with targets. An attractive possibility is that Ddc2 influences loading of Mec1 onto replicating chromatin.

Once Rad53 is activated by a phosphorylation event dependent on Mec1–Ddc2, Rad53 apparently co-localize with Sgs1 in discrete nuclear foci during S-phase. Sgs1 is a homolog of *Escherichia coli* recQ helicases [Watt et al., 1995]. A physical interaction between Sgs1 and Rad53 may mirror G2 DNA damage checkpoint activation by binding of Rad9 to Rad53 [Frei and Gasser, 2000a]. In the case of both Sgs1 and Rad9, the interaction is with the Rad53 FHA domain required for checkpoint signaling [Sun et al., 1998]. Rad53-Sgs1 association may be an Sgs1-dependent loading of Rad53 onto specific chromatin regions that are actively replicating. Perhaps the putative helicase activity of Sgs1 creates a DNA topology conducive to such a loading process. This cannot be the whole story however, since the S-phase checkpoint defect of *sgs1* mutants is quite weak, not nearly as substantial as *rad53* or *mec1*. One possibility is that Sgs1 has a redundant checkpoint function, perhaps with another helicase such as Srs2. How such complexes interact with Mec1-Ddc2 at replication forks is of great interest.

S-PHASE CHECKPOINT TARGETS

Much progress has been made in elucidating mechanisms and factors involved in S-phase checkpoint activation. Even in yeast, however, checkpoint targets are less well characterized. One question is how the kinase cascade inhibits mitosis? For simplicity, onset of mitosis, or anaphase in yeast, will be equated with spindle elongation and loss of sister chromatid cohesion. One target of Mec1 and Rad53 is kinase Dun1, an effector of a complex transcriptional response. It is not clear, however, whether the Dun1-dependent transcriptional response contributes to inhibition of mitosis in the presence of HU since *dun1* mutants are not obviously S-phase checkpoint defective. Moreover, the S-phase checkpoint defects of *mec1* and *rad53* mutants are different, suggesting that there are different downstream targets. Some light was shed on the basis of this difference by analysis of *pds1* mutants. Pds1 is unlikely to be an essential checkpoint target in early S-phase because *pds1* mutants can inhibit spindle elongation when replication is blocked with HU in early S-phase. However, part-way through S-phase, when about 2/3 of the genome has been replicated, a point is reached after which Pds1 is essential [Clarke et al., 1999, 2001b]. Such studies have been made possible by improved FACS analysis of progression through S-phase [Haase and Reed, 2002]. Mec1 controls Pds1 stability in S-phase in a way that is at least partly independent of Rad53 [Clarke et al., 2001b]. Both *rad53* and *mec1* mutants prematurely accumulate Cdc20 in HU-treated cells; Cdc20 being the APC specificity factor that

promotes Pds1 degradation. But, whereas Pds1 is degraded in HU-treated *mec1* mutants, it is not in *rad53* mutants. Thus, Cdc20 protein accumulation is controlled by Mec1 and Rad53, but a separate Mec1-dependent event that prevents APC^{Cdc20} activation must promote Pds1 stability.

It remains to be determined exactly how Pds1 levels are controlled in late S-phase when DNA replication is perturbed. Unlike the case of the DNA damage checkpoint, where Pds1 stability is at least partly controlled by Pds1 phosphorylation, Pds1 phosphorylation is apparently not important for its S-phase stability. Other mechanisms must therefore exist and the link between Mec1 and S-phase Cdc20 protein levels may be one of them. Evidence has also linked two HU-inducible yeast genes to regulation of Pds1 levels [Clarke et al., 2001a]. Rad23 or Ddi1 overproduction rescue the HU sensitivity of *pds1-128* mutant cells and can stabilize Pds1-128 protein levels in S-phase cells. Rad23, a nucleotide excision repair protein, seems to have a role in regulating ubiquitin-dependent proteolysis [Ortolan et al., 2000; Bertolaet et al., 2001a,b; Chen et al., 2001; Clarke et al., 2001a]. Rad23 binds to mono- or di-ubiquitinated proteins, blocking extension of the ubiquitin chains and thus preventing recognition by proteasomes [Ortolan et al., 2000]. In fact, both Rad23 and Ddi1 bind ubiquitin via a homologous domain, the ubiquitin associated domain (UBA) [Hofmann and Bucher, 1996; Bertolaet et al., 2001b]. The Rad23 and Ddi1 UBA domains are needed for rescue of *pds1* mutant strain HU sensitivity [Clarke et al., 2001a].

SPINDLE DYNAMICS IN EARLY S-PHASE

Pds1 is not likely to be a relevant target of the S-phase checkpoint that inhibits spindle elongation in early S-phase; the null mutant can restrain spindle elongation when arrested in early S-phase with HU and overproduction of a non-degradable form of Pds1 cannot prevent premature spindle elongation seen in *rad53* mutants under the same conditions [Yamamoto et al., 1996a,b; Clarke et al., 2001b]. What then is the early S-phase target of the Mec1-Rad53 pathway? In an attempt to make progress on this issue, genetic screens were performed to find new S-phase checkpoint genes having a similar phenotype to the originally isolated *rad53* mutant [Alcasabas et al., 2001]. These studies have suggested an attractive mechanism by which mitosis might be retrained in early S-phase. Among the new class of factors needed for inhibition of spindle elongation was Ask1, a component of the DASH complex that resides at kinetochores and appears to be critical for the interaction between the kinetochore and spindle microtubules [Li et al., 2002]. Ask1 might be a regulatory component of this complex, ensuring that spindle elongation does not initiate prematurely.

MAINTENANCE OF COHESION IN S-PHASE

Control of Pds1 levels by the S-phase checkpoint is only critical when the last 1/3 of the genome is being replicated; *pds1* mutants are not only proficient in inhibiting spindle elongation in early S-phase, but they can also maintain sister cohesion in early S-phase, suggesting that another mitotic inhibitor exists. This could explain why *PDS1* is not an essential gene in yeast. While some investigators are no doubt using genetic approaches to identify mutations that confer synthetic lethality with a *pds1* null, other more fundamental experiments are important. Firstly, is there concrete proof that another factor exists other than Pds1 that prevents loss of cohesion in early S-phase? In a *pds1* null strain, loss of cohesion does not occur until late S-phase in the presence of HU, but *pds1* null mutants are also defective in transporting Esp1 separase into the nucleus. Thus, perhaps loss of cohesion is delayed until a sufficient amount of Esp1 reaches its nuclear target, the cohesin component Scc1. An experiment to resolve this issue might have been to express ESP1–NLS (an ESP1 gene fused to a nuclear localization signal) in *pds1* null cells. The expectation would be enhanced lethality in the presence of HU and maybe lethality within an unperturbed cell cycle. However, in a *pds1* null strain, an ESP1–NLS fusion did not enter the nucleus until G2 [Jensen et al., 2001]. And, in fact, the ESP1–NLS partly rescued the *pds1* null mutation; the opposite effect would be expected if Esp1 could enforce loss of cohesion before the end of S-phase. Therefore, other factors might restrict Esp1 entry into the nucleus in early S-phase. Perhaps *pds1* synthetic lethal screens will identify such a factor.

Whether or not Pds1 collaborates with an unknown inhibitor of loss of cohesion, another question is whether the Mec1/Rad53 S-phase

checkpoint pathway controls loss of cohesion in S-phase? When *rad53* and *mec1* mutants are treated with HU, no loss of cohesion is seen (Clarke and Reed, unpublished). Does this mean that loss of cohesion is controlled independently of the known S-phase checkpoint pathways? One complication with this interpretation is that DNA replication is known to proceed with different temporal order in *rad53* and *mec1* mutants. When HU-treated cells begin S-phase, replication proceeds from each replication origin until nucleotide pools are exhausted. Estimates suggest that about 10 kb of DNA can be replicated from each origin before replication forks stall. In *rad53* and *mec1* mutants, the timing of early and late firing origins is disrupted. Presumably, more origins fire than in wild type cells, thus reducing the amount of DNA replicated from each individual origin before fork stalling. Thus, there are potential differences between these S-phase checkpoint mutants and wild type cells in terms of which loci are replicated before replication arrest in the presence of HU. So far, only loss of cohesion at the *TRP1* locus in the presence of HU has been examined in *rad53* mutants, but whether this locus is replicated in *rad53* cells is not known. It would be prudent to investigate cohesion at other loci, and to determine definitively whether *TRP1* is replicated in HU-treated *rad53* cells. Another possibility is that cohesion is lost in HU-treated *rad53* cells, but that locus separation is not seen using the LacO/LacR-GFP technique that has been exclusively employed. Such an effect might be due to an aberrant chromatin structure in replicated *rad53* DNA. To determine whether this is the case, *rad53 scc1* cells should be examined in the same experiment, treating cells with HU and looking for *TRP1* locus separation.

OTHER FUNCTIONS OF THE S-PHASE CHECKPOINT

As well as the functions described above that control cell cycle progression, Mec1 and Rad53 are needed to induce transcription of genes involved in DNA repair and which deal with perturbed replication. The checkpoint response protects stalled replication forks, allowing replication re-start when conditions have improved [Desany et al., 1998]. Mec1 and Rad53 are needed to maintain fork integrity [Lopes et al., 2001; Tercero and Diffley, 2001], accounting for the large scale genome alternations caused by S-phase checkpoint failure [Myung et al., 2001a,b]. The Mec1-induced transcription pathway depends on Rad53-dependent phosphorylation of the kinase Dun1 [Allen et al., 1994; Huang et al., 1998]. Activation of Dun1 has been shown to induce transcription of genes that promote efficient DNA repair. The transcription program presumably also promotes replication fork stability, but the details of this response have not yet been determined. Mec1 and Rad53 also inhibit the firing of late replication origins during early S-phase and are involved in the regulation of telomere length and silencing [Mills et al., 1999; Craven and Petes, 2000; Longhese et al., 2000].

MAMMALIAN S-PHASE CHECKPOINT CONTROL

Little is known about mammalian proteins that might be S-phase checkpoint sensors. However, interestingly mammalian Sgs1 homologs are important for S-phase regulation [Frei and Gasser, 2000a]. Mammalian recQ helicases include WRN (mutated in Werner's syndrome patients) [Yu et al., 1996] and BLM (mutated in Bloom's syndrome patients) [Ellis et al., 1995]. Bloom's syndrome is characterized by genomic instability and a cancer predisposition; Werner's syndrome causes premature aging. Cultured cells from Bloom's syndrome patients have S-phase defects, but there is no evidence that these abnormalities include checkpoint defects. However, BLM was recently identified as a component of BRCA1-associated genome surveillance complex (BASC) that localizes to nuclear foci in HU-treated cells [Wang et al., 2000].

Mec1 homologs in mammals are the serine/threonine kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) [Savitsky et al., 1995; Bentley et al., 1996; Cimprich et al., 1996]. Ataxia telangiectasia patients have an increased incidence of cancer and suffer from neurological problems associated with defects in apoptotic pathways. Both ATM and ATR are involved in DNA damage checkpoint signaling [Kastan et al., 1992; Wright et al., 1998], but it seems ATR alone is required for preventing mitosis during S-phase. The evidence for this is not definitive, but is highly suggestive. Cultured cells expressing a kinase-dead ATR mutant (ATR-kd), that

appears to act as a dominant negative allele, are sensitive to HU [Cliby et al., 1998] and depletion of ATR from a *Xenopus* egg extract replication system caused premature activation of Cdc2-cyclin B in the presence or absence of the DNA replication inhibitor aphidicolin. In the latter case, ATR associated with chromatin in a replication-dependent manner, dissociating from chromatin when replication was complete. Perhaps the most interesting result was that loading of ATR was dependent on RNA primer synthesis. Although most evidence comes from a variety of non-mammalian systems, it seems likely that Mec1/ATR is the major S-phase checkpoint activating kinase.

Rad53 homologs in higher eukaryotes, named Chk2 [Matsuoka et al., 1998], do not seem to be critical S-phase targets of ATR. In mammals, Chk2 kinase becomes phosphorylated and activated upon HU treatment [Matsuoka et al., 1998, 2000; Chaturvedi et al., 1999], but there is no evidence that the HU-induced phosphorylation is relevant for S-phase checkpoint control. Instead, another checkpoint kinase, Chk1, appears to be the ATR target in the S-phase context. Depletion of ATR from *Xenopus* egg extracts abolished phosphorylation of Chk1 that occurs in the presence of aphidicolin and in *Xenopus* development, Chk1 is activated in post-mid-blastula transition embryonic cells treated with HU [Kappas et al., 2000]. Similarly, there is good evidence for *Drosophila* Chk1 (Grapes) coordinating embryonic DNA replication with mitosis [Sibon et al., 1999; Yu et al., 2000]. Again in *Xenopus* egg extracts, Chk1 and ATR were needed for delayed cell cycle progression in response to replication blocks. Based on ATR-kd studies, human Chk1 phosphorylation and activation in response to HU or aphidicolin treatment appear to be ATR-dependent [Liu et al., 2000]. Although it may be required for S-phase checkpoint functions in other eukaryotes, the checkpoint kinase Chk1 is not needed for S-phase checkpoint control in budding yeast.

The link between human Chk1 and inhibition of mitosis appears to be phosphorylation of Cdc25C on Ser-216 which prevents Cdc25C from activating cyclinB1/Cdc2 [Matsuoka et al., 1998; Sanchez et al., 1999]. Cdc25C is the protein phosphatase that promotes entry into mitosis by dephosphorylating Cdc2. Phosphorylated Ser-216 is a binding site for a 14-3-3 protein that inhibits Cdc25C [Peng et al., 1997]. Expression of a mutant Cdc25C that cannot be phosphorylated on Ser-216 induces mitosis

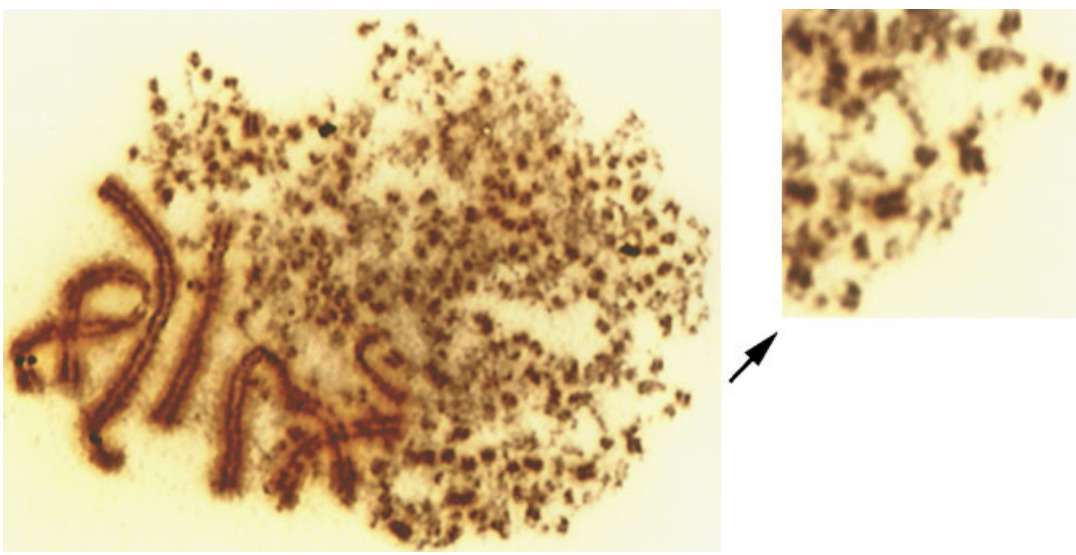

Fig. 2. Formation of a chromatid core template in S-phase. Photomicrograph of an S-phase *Muntiacus muntjak* cell fused with a mitotic cell by Sendai virus treatment. Prematurely condensed S-phase chromosomes have formed (mitotic chromosomes are seen on the left). Assembled chromatid cores stain dark brown after silver impregnation. Double (replicated) segments of core are present, interspersed with regions in which the cores are not detectable. Top right insert shows a magnification of some double core segments. The ability of mitotic factors to induce core formation only within replicated regions (double segments of core staining are always seen in S-phase, not single segments) of the chromosome suggests that the competence to assemble cores is acquired shortly after the passage of replication forks. The implication is that replication lays down a molecular template on which the chromosome scaffold can be built during mitosis. Photomicrograph courtesy of J.F. Giménez-Abián.

in the presence of unreplicated DNA [Peng et al., 1997].

CHROMATIN REPLICATION AND COHESION OF SISTER CHROMATIDS IN S-PHASE

Although the above studies in higher eukaryotes successfully trace the S-phase checkpoint pathway from the signaling kinase ATR to cyclinB1/Cdc2, there are clearly many details to be described. A determination of whether this pathway regulates sister cohesion is one important goal. A step forward has been the realization that the budding yeast S-phase checkpoint prevents loss of sister chromatid cohesion while DNA replication is ongoing. A current theory is that newly replicated sister DNA molecules become associated with protein cohesion factors in S-phase. That is, the process of chromatid cohesion is intimately associated with the progression of DNA replication forks. Little data supports this assumption however. Yeast experiments indicated that Scc1, a component of the cohesin complex, only forms functional cohesin complexes when present in S-phase [Uhlmann and Nasmyth, 1998]. Cells depleted for Scc1 during S-phase, but subsequently provided with Scc1 during G2, were able to load cohesin onto chromatin, but the resulting association did not produce cohesive sisters. Such experiments suggest that cohesion is associated with replication, but do not prove an interdependence. A competing theory might be that DNA catenations, of replicative origin [Cook, 1991], initially hold sisters together at critical sites, allowing cohesin complexes to be loaded at DNA crossovers following passage of replication forks. It is a question whether replication and sister cohesion are mechanistically linked processes, or independent events coordinated by signaling pathways.

A related question is how chromatin assembly is linked to DNA replication. In mammals, this issue has been examined from a cytological perspective [Giménez-Abián et al., 1999], yielding the proposal that a molecular 'blue print' of the protein scaffold of newly formed sister chromatids is laid down during replication (Fig. 2). The idea is that this process forms a template upon which chromatid scaffolds are built during mitotic chromosome condensation. These experiments therefore suggest a coupling between replication and chromatin assembly. As stated above for the case of cohesin loading, an issue that should be addressed by genetic analysis is whether DNA replication and chromatin assembly are physically linked processes. The alternative is that checkpoint controls coordinate replication with chromatin assembly. These are important problems because the S-phase checkpoint is unlikely to exclusively control the dependence relationship between replication and mitosis. It is a reasonable possibility that the same checkpoint machinery coordinates multiple events within S-phase.

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