Visualisation of altered replication dynamics during the S phase checkpoint response to DNA damage in human cells.

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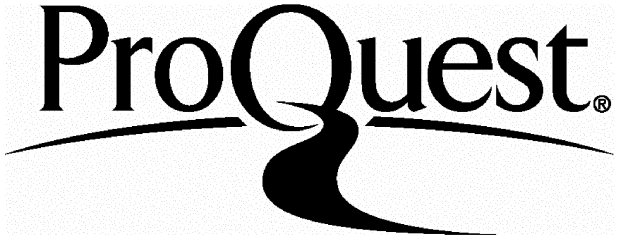

Abstract

Eukaryotic cells respond to DNA damage within S phase by activating an intra-S phase checkpoint: a response which includes reducing the rate of DNA synthesis. In yeast cells this occurs via a checkpoint-dependent inhibition of origin firing and stabilisation of ongoing forks, together with a checkpoint-independent slowing of fork movement. In higher eukaryotes, however, the mechanism by which DNA synthesis is reduced is less clear.

This work describes DNA fibre labelling strategies that offer a quantitative assessment of rates of replication fork movement, origin firing and fork stalling throughout the genome by examining large numbers of individually labelled replication forks. It shows that exposing S phase cells to ionising radiation (IR) induces a transient block to origin firing but does not affect fork rate or fork stalling. Exposure to alkylating agents or UV light causes a slowing of fork movement and a high rate of fork stalling in addition to a sustained block to origin firing. Nucleotide depletion also reduces fork rate, increases stalling and suppresses new origin firing.

The block to new origin firing depends on the central checkpoint kinases ATM and ATR in response to damage by IR and UV respectively. Both responses are transduced jointly by the CHK1 and CHK2 kinases. ATR also has a role in preventing irreversible fork stalling but this appears to be independent of CHK1. Finally, the slowing of replication forks is independent of both ATR and CHK1. Thus, this work provides a detailed picture of the mechanics of the replication response to DNA damage in human cells, and clarifies the relative checkpoint dependencies of each aspect of this response.

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Abbreviations

ACS	ARS Consensus Sequence
ARS	Autonomously Replicating Seequence
AT	Ataxia Telangectasia
ATLD	Ataxia Telangectasia-Like Disorder
ATM	Ataxia Telangectasia Mutated
ATR	ATM and Rad3 Related
BASC	BRCA1-Associated Complex
BER	Base Excision Repair
BRCT	BRCA1 C-Terminal
BrdU	Bromodeoxyuridine
CDK	Cyclin Dependent Kinase
ChIP	Chromatin Immunoprecipitation
CHO	Chinese Hamster Ovary
CldU	Chlorodeoxyuridine
dNTP	Deoxynucleotide phosphate
dsb	double strand break
dsDNA	double stranded DNA
FA	Fanconi Anaemia
FHA	Forkhead Associated
Gy	Grays
H2AX	Histone 2AX
HR	Homologous Recombination
HU	Hydroxyurea

IdU	Iododeoxyuridine
IR	Ionising Radiation
MBT	Midblastula Transition
MMR	Mismatch Repair
MMS	Methyl Methane Sulphonate
NBS	Nijmegan Breakage Syndrome
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NMD	Nonsense Mediated Decay
PIKK	Phosphatidylinositol 3 Kinase-like Kinase
RDS	Radioresistant DNA Synthesis
RFB	Replication Fork Barrier
RSZ	Replication Slow Zone
S.cerevisiae	*Saccharomyces cerevisiae*
S.pombe	*Schizosaccharomyces pombe*
SCE	Sister Chromatid Exchange
ssb	single strand break
ssDNA	single stranded DNA
TLK	Tousled-Like Kinase
UV	Ultraviolet

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Chapter 1: Introduction

The eukaryotic cell cycle consists of four phases: M phase or mitosis, S phase or the DNA synthetic phase, and two intervening 'gap' phases termed G1 and G2. During each S phase, the cell's entire genome must be duplicated to yield two identical copies destined for the two daughter cells which will be formed at mitosis. Eukaryotic genomes can range in size from around ten megabases to hundreds of thousands of megabases (12Mb, for example, in the budding yeast *Saccharomyces cerevisiae* and 670,000Mb in *Amoeba dubia* (*http://www.cbs.dtu.dk/databases/DOGS*)) and accordingly, the task of duplicating the genome can take anything from a few minutes to several hours (20 minutes in *S.cerevisiae* compared to upwards of 8 hours in cultured human cells). Even so, replicating the whole eukaryotic genome within such a time frame requires multiple points of initiation (Huberman and Riggs, 1968), in contrast to the canonical single replicon which operates in all prokaryotes (Cairns, 1966).

Thus, in the eukaryotic cell cycle, the factors which dictate the duration of S phase are relatively complex: the overall rate of DNA synthesis depends on the number of active replication origins in the genome, the temporal programme of origin firing and termination, the rates of movement of all the active replication forks and the occurrence of any non-termination 'fork stalling' events. Any or all of these parameters may vary over the course of a normal S phase, and furthermore may be affected by DNA damage, either as a direct result of DNA lesions or via the action of the cell cycle checkpoint proteins which are discussed in more detail below.

The basic mechanism of DNA replication is conserved in all eukaryotes although the factors that determine how it operates have changed significantly over evolution. So, for example, the central protein component at replication origins, the origin recognition complex (ORC), is closely conserved from yeast to man (Bell and Dutta, 2002; Stillman et al., 1992) but the factors governing where in the genome it actually binds to DNA have changed.

The six-subunit ORC complex binds to DNA in an ATP-dependent manner, specifying the location of a potential replication origin (Bell and Stillman, 1992). In the budding yeast *S.cerevisiae,* ORC remains bound to DNA throughout the cell cycle (Diffley et al., 1994), whereas in higher eukaryotes it dissociates at mitosis and the Orc1 subunit is released during S phase (Kreitz et al., 2001; Mendez et al., 2002). All the other components of a replication complex are assembled around ORC in a dynamic manner in each cell cycle. In early G1, the ATPase Cdc6 (Liang et al., 1995) and a second protein Cdt1 (Hofmann and Beach, 1994) are recruited to ORC, followed by the six-subunit MCM2-7 complex which is thought to act as the replicative helicase (Ishimi, 1997; Lee and Hurwitz, 2001; Masuda et al., 2003). This assembly is termed a pre-replicative complex (pre-RC) (Diffley et al., 1994): it is competent to act as an initiation point for replication but will not necessarily actually be activated during S phase. Further possible pre-RC components including Noc3 (Zhang et al., 2002) and Yph1 (Du and Stillman, 2002) have recently been identified in yeast but their exact roles remain unclear.

In *S.cerevisiae (*and also in *Schizosacchromyces pombe (S.pombe)),* Cdc6 and Cdt1 are then displaced from the pre-RC (Drury et al., 1997; Piatti et al.,

1995; Tanaka and Diffley, 2002), whereas in mammalian cells Cdc6 remains associated with DNA throughout S phase (Coverley et al., 2000; Fujita et al., 1999; Mendez and Stillman, 2000; Mendez et al., 2002). Additional proteins are added to the preRC, including Cdc45 (Zou et al., 1997), Mcm10 (Homesley et al., 2000), Sld2 (Masumoto et al., 2002; Wang and Elledge, 1999), and Sld3 (Kamimura et al., 2001), Dpb11 (Araki et al., 1995) and the GINS complex (Takayama et al., 2003). All of these have homologs in higher eukaryotes such as *Xenopus laevis* although not all the homologs are firmly established in mammalian cells. Furthermore, even in yeast model systems, the exact role of most of these proteins remains unclear. Some, such as Cdc45, are required for both initiation and progression of DNA replication (Tercero et al., 2000): Cdc45 is thought to activate the putative replicative helicase MCM2-7 (Masuda et al., 2003). Other components appear to have distinct roles in initiation or pre-initiation: Mcm10, for example, forms transient foci in human cells *preceding* the ordered appearance of replication foci (Izumi et al., 2004).

The assembly of all these components occurs in a temporally regulated manner correlated with asynchronous origin firing throughout S phase (Aparicio et al., 1999; Dimitrova et al., 1999). The actual firing process requires the activity of two S phase promoting kinases CDK2 and Cdc7/Dbf4 (Bell and Dutta, 2002; Donaldson and Blow, 1999). Factors recruited at the point of origin firing include the single-stranded DNA (ssDNA) binding protein RPA and polymerases α and ε (pol α/ε), possibly through interaction with Cdc45 (Zou and Stillman, 2000) or Dpb11 (Masumoto et al., 2000). RPA binds to the newly-unwound origin DNA and pol α synthesises an RNA primer for DNA synthesis by processive polymerase(s). Both pol δ and pol ε appear to play roles here, although exactly

how the task of replication is shared between them is not known. Several components of the replication complex including Cdc45 and MCM2-7 travel away from the site of the origin with the replication fork (Katou et al., 2003; Tercero et al., 2000), and processive DNA synthesis additionally requires the ring-shaped polymerase clamp PCNA and its clamp-loading complex RFC (Maga and Hubscher, 2003), as well as topoisomerases I and II to restore the topology of the DNA (Lucas et al., 2001) and the endonuclease and ligase functions of Fen1, Dna2 and Lig1 to process Okasaki fragments into a continuous DNA strand (Bae et al., 1998).

Surrounding this highly conserved replicative machinery, certain regulatory mechanisms have been elaborated between yeast and higher organisms. Most of these relate to the ways of preventing re-initiation: this may require further levels of control as genome size, and therefore the number of origins, increases. ORC, for example, is regulated differently in yeast and mammalian cells throughout the cell cycle (Kreitz et al., 2001; Mendez et al., 2002) and the same is true of Cdc6 (Jiang et al., 1999; Saha et al., 1998) which may possess additional checkpoint roles in mammalian cells (Clay-Farrace et al., 2003; Oehlmann et al., 2004). Yeast also lack geminin, a protein which inhibits Cdt1 and so prevents inappropriate MCM2-7 loading at pre-RCs (McGarry and Kirschner, 1998; Tada et al., 2001). Thus the basic form of a replicon is very similar in all eukarya but it is distributed and activated in rather different ways to meet the demands of replicating the 12Mb genome of *S.cerevisiae* and the 3400Mb genome of *Homo sapiens*.

S phase parameters in *S.cerevisiae*

The parameters of S phase have been extensively studied in the budding yeast *S.cerevisiae.* In this organism, unlike higher eukaryotes, origins of replication are defined by specific DNA sequences called Autonomous Replication Sequence elements or ARSs. Such sequences generally encompass about 200bp, including an 11bp consensus sequence (ACS) which is essential, but not sufficient, for origin function (Kearsey, 1984; Van Houten and Newlon, 1990). The 200bp flanking the ACS, although they are also found to be essential in functional plasmid maintainance assays, share no obvious consensus sequence (Newlon and Theis, 1993).

The *S.cerevisiae* genome appears to contain about 400 ARSs spaced at irregular intervals with an average spacing of 35kb (Newlon et al., 1993), but not all ARSs are active in every S phase. 'Efficient' origins are reproducibly used in almost every S phase while others are used only rarely, or are even entirely dormant (Santocanale and Diffley, 1996). Furthermore, specific origins always fire either early or late in S phase (Friedman et al., 1997; Yamashita et al., 1997). A microarray study characterising replication dynamics across the entire yeast genome (Raghuraman et al., 2001) has shown that there is a reproducible timing sequence for all active origins, with a continuum of firing events occurring throughout S phase. It appears, however, that at least half of the origins fire early in S phase since DNA fibre immuno-labelling has shown that about 190 origins, with an average spacing of only 46kb, are activated before the drug hydroxyurea can act to block S phase (Lengronne et al., 2001). The mechanism determining the temporal program of origin firing remains poorly understood, but appears to involve trans-acting factors such as the Clb5 cyclin (Donaldson et al., 1998b), the

Sir3 telomeric chromatin protein (Stevenson and Gottschling, 1999) and the Ku proteins (Cosgrove et al., 2002) as well as cis-acting DNA sequences surrounding the ARS itself (Ferguson and Fangman, 1992; Friedman et al., 1996). Whatever dictates the program, it is already established in G1 - well before any origins are actually activated – since an origin's timing can only be modified by moving it to a new location before this point (Raghuraman et al., 1997). It has been proposed that the timing mechanism may result in certain components such as Cdc45 associating with early origins before late origins (Aparicio et al., 1999; Diffley and Labib, 2002): possibly the existing chromatin context simply limits the access of such factors to a subset of origins at the start of S phase and they can only be assembled upon the 'late' origins when progressive replication has modified the chromatin structure sufficiently. Telomeric heterochromatin, for example, imposes late firing upon origin sequences which can be rendered early-firing by either moving them to new locations or modifying the chromatin characteristics of telomeres (Stevenson and Gottschling, 1999). However, chromatin structure is unlikely to be a complete explanation: the rDNA array should have relatively uniform chromatin characteristics but replication is still only initiated at clustered subsets of origins within the array (Pasero et al., 2002).

Microarray analysis has also shown that the parameter of fork rate varies widely between different regions of the yeast genome, with a mean of 2.9kb/min under the growth conditions used (Raghuraman et al., 2001). An alternative method of determining fork rate, fibre autoradiography, also yielded a wide distribution with a mean of 2.1μm/min, probably equivalent to about 6kb/min (Rivin and Fangman, 1980b). This study further showed that the fork rate could be significantly altered by nutrient conditions and that this change in a single

replication parameter was sufficient to alter the overall length of S phase several-fold.

Finally, the parameter of non-termination stalling cannot be easily examined by the microarray technique, nor by any method relying on homogenous S phase kinetics in a whole population, because such stalling is likely to be a stochastic, unscheduled event occurring only as a result of DNA lesions, collisions between the replication fork and transcriptional machinery or other blocks to normal fork progression. However, certain regions have been defined within the yeast genome where stalling seems to be especially likely even in the absence of exogenous DNA damage. These are termed Replication Slow Zones (RSZs) (Cha and Kleckner, 2002). They were originally defined as areas in which chromosome breakage occurs at a high rate after cells attempt S phase in the absence of the Mec1 checkpoint protein. RSZs do not share any obvious sequence similarities or chromatin characteristics but they do take an unusually long time to replicate even in wild-type cells, so the chromosome breakage which occurs in these regions in Mec1 mutants may be a consequence of terminally stalled or incomplete replication. This suggests that some chromosomal regions are inherently more difficult to replicate than others, predisposing them to fork stalling during normal replication, but that this is only catastrophic in the absence of a functional checkpoint system. Another example of fork stalling being induced by specific chromatin features is seen at the replication fork barrier (RFB) within rDNA arrays. Normal replication through this region requires the helicase Rrm3 but this requirement is eliminated in the absence of the RFB-binding protein Fob1, suggesting that this particular chromatin-bound protein blocks replication forks. Rrm3 also appears to be required in other areas of the

genome which do not bind Fob1, so stalling at chromatin-bound proteins may occur throughout the genome and the cell may have evolved specialised helicases to resolve such problems (Ivessa et al., 2003; Torres et al., 2004).

S phase parameters in higher eukaryotes

In mammalian cells, replication is theoretically subject to exactly the same parameters as those outlined above for *S.cerevisiae*, although most of these have been less precisely studied in higher eukaryotes. This is partly due to the far larger genome sizes involved and the problems of synchronising and genetically manipulating mammalian cells. Furthermore, the sequence-defined origins which have greatly facilitated the study of yeast replication appear to be unique to *S.cerevisiae*: in all other eukaryotes, the equivalent of an ACS has yet to be identified and there is good evidence that at least in some situations, any DNA sequence placed on a plasmid can act as an origin (Kim et al., 1992; Krysan et al., 1993; Mello et al., 1991; Smith and Calos, 1995). Thus, while the mechanism of replication and the proteins which carry it out are closely conserved from yeast to man, the way in which origins are laid down appears to have changed. Nevertheless, empirical data does exist on the sequences of certain specific origins as well as on origin spacing, temporal programs of initiation and rates of fork progression. These factors have been studied most extensively in human and rodent cells and in *Xenopus laevis* egg extracts (an easily manipulated model system for replication in higher eukaryotes which may not, however, be comparable in all respects to the S phase which occurs in discrete cells at later developmental stages, either in *Xenopus* or in mammals).

Origin specification

Regarding origin specification, although any DNA sequence may theoretically act as an origin, certain loci have been identified in mammalian genomes where replication is known to initiate reproducibly *in vivo*. These include the human β-globin locus, the lamin B2 locus and the Chinese hamster *DHFR* locus. In the case of β-globin, replication initiates within the few kb between the β and δ-globin genes and this sequence can be sufficient for origin activity if moved to an alternative location (Aladjem et al., 1998). However, a wide area of sequence context remains important since deletions up to 50kb away from the origin region can abolish its activity (Aladjem et al., 1995; Cimbora et al., 2000). In lamin B2, initiation has actually been narrowed down to the site of a single nucleotide (Abdurashidova et al., 2000) but again, the surrounding sequence context is also required.

The *DHFR* locus has been proposed to represent a second 'class' of mammalian origin in which multiple sites within a large intergenic region can all initiate DNA replication (Gilbert, 2001). Again, there are specific elements within this region where replication is preferentially initiated, e.g. the ori-β locus (Altman and Fanning, 2001). This sequence retains its function at ectopic locations but its activity *in situ* depends on distant sequences in the 3' end of the adjacent *DHFR* gene. It is not known whether this general model of preferred initiation sites being associated with wider regions of 'accessory sequence' applies to origin specification in the entire mammalian genome, but certain factors have been more generally linked to origin function, such as AT richness (Anglana et al., 2003) and being in an intergenic region (Brewer and Fangman, 1994). These factors would make sense, since AT base pairs melt more easily than GC

base pairs, and replication and transcription may interfere with each other on DNA.

Origin spacing and clustering

Clearly, while any sequence may be *able* to initiate replication, not every sequence actually does so in any single S phase. The spacing of active origins has been assessed both in *Xenopus* and in human Hela cells and in both systems spacing is found to vary with cell cycle conditions. In the *Xenopus* genome, origins spacing and specification alters dramatically during development: in the first embryonic S phases, origins are closely spaced at 5-15kb (Blow et al., 2001) and they become more widely spaced at the mid blastula transition (MBT), corresponding to a lengthening of the whole cell cycle. The initial close spacing is thought to represent 'saturation' of the genome with pre-RCs (Rowles et al., 1996; Rowles et al., 1999), although the mechanism that dictates this saturation is unknown. Most or all of the pre-RCs are then activated in a very rapid S phase, although even within this brief period origins fire asynchronously, as they do in the *S.cerevisiae* S phase (Herrick et al., 2000). After the MBT, although the same number of pre-RCs are laid down, fewer actually fire (Walter and Newport, 1997). This may be due to the start of embryonic transcription: for example, specific variants of replication factors may be synthesised or the onset of transcription may impose constraints on origin firing within active genes. This has been specifically demonstrated in the ribosomal RNA genes (Hyrien et al., 1995).

In human cells, a similar situation exists in which more pre-RCs are probably formed than are actually activated in a normal S phase (Okuno et al.,

2001) and again, there are examples of origin choice being developmentally regulated. In murine pre-B cells, for example, the IgH locus is replicated early in S phase from more than one origin while in non-B cells it is replicated by a single, much larger replicon (Hyrien et al., 1995). In Chinese hamster cells selected for coformycin resistance, nucleotide depletion can alter origin spacing, although this phenomenon may be unique to this cell line (Anglana et al., 2003). More generally, the total number of replicons active at any time during S phase and also their spacing throughout the genome has been assessed in cultured cells by a combination of density substitution and immuno-labelling of replication foci or of replication tracks on isolated DNA fibres.

In mammalian cells, S phase takes 8 hours or more and origins are fired asynchronously throughout this period. Using DNA fibre immuno-labelling, origin spacing at the start of S phase has been measured as 144+/-66kb in Hela cells (Jackson and Pombo, 1998) and 59kb, diminishing to 46kb over the first hour of S phase in mouse m5S cells (Takebayashi et al., 2001). It is unclear whether the three-fold difference between these figures is due to the cell lines used or to the labelling method (Takebayashi et al used short pulses of bio-dUTP instead of BrdU, which theoretically gives higher resolution and may help to distinguish very small replicons). Using an entirely different method of measuring average replicon sizes by density substitution followed by shearing of DNA, a relatively small replicon size of 13μm (approximately 39kb) was calculated for human diploid fibroblasts (Roti Roti and Painter, 1977). However, this method is not able to measure the range of replicon sizes which contribute to this average. By DNA fibre labelling, a wide range of replicon sizes is observed in many different cell lines (reviewed (Berezney et al., 2000). For example, the

DNA fibre immuno-labelling studies described above yielded size ranges of 21-406kb in Hela cells and 10-200kb in mouse cells, while earlier investigations using fibre autoradiography revealed even wider ranges of up to 400μm (probably in excess of 1Mb) in some human cell lines (Yurov and Liapunova, 1977). For technical reasons, it is likely that most DNA fibre techniques fail to detect both very large and very small replicons, a problem extensively discussed in (Liapunova, 1994) and in (Berezney et al., 2000).

Nevertheless, given that very large replicons are probably relatively rare, the size of a mammalian genome dictates that several thousand origins must fire in a normal S phase. Due to the sizes of mammalian genomes and the absence of defined origin sequences, no comprehensive assessment has been made of origin firing kinetics throughout S phase, but detailed studies have been made of the number of origins fired at the onset of S phase. This is achieved by counting replication foci labelled by BrdU incorporation into nascent DNA (Nakamura et al., 1986). The co-localisation of these BrdU foci with replication proteins such as PCNA and RPA supports their being authentic sites of DNA replication (Kill et al., 1991; Krude, 1995). In a variety of mammalian cell types, at least 5 distinct focal patterns appear as S phase proceeds, from a large number of small foci dispersed throughout the nucleus at the start of S phase to a small number of large foci at the nuclear periphery in the final stages (Humbert and Usson, 1992a; Okeefe et al., 1992). More recent analyses suggest that in untransformed cells, the initial 'type 1' pattern is actually preceded by a phase of replication occurring at only a small number of foci surrounding the nucleolus, but in most transformed cells this initial pattern is not observed (Kennedy et al., 2000).

At the start of S phase, each discernable focus is thought to represent an individual cluster of replicons. The intensity of such foci compared to mitochondrial DNA indicates that most foci must contain more than one replicon (Nakamura et al., 1986); furthermore, early studies by fibre autoradiography revealed tandem arrays of up to 4 replicons of similar size, (Hand, 1975; Hand, 1977). More recently, this clustered arrangement of replicons has been supported by more detailed analyses of DNA fibres from a variety of mammalian cell lines. In HeLa cells, which contain approximately 62 chromosomes, 749+/-154 foci, about 12 per chromosome, were observed after BrdU labelling for 20 minutes at the start of S phase. Analysis of the segregation of these foci over subsequent cell cycles showed that they were not fragmented by crossing-over at mitosis, so they probably contained closely clustered replicons. DNA fibre immuno-labelling further supported the existence of these clusters as tandem arrays of 2-10 synchronously-initiated replicons (Jackson and Pombo, 1998). It is not known what dictates this clustered pattern of origin firing but it appears to be unique to (or perhaps only detectable in) higher eukaryotic genomes undergoing relatively long S phases. By contrast, significant clustering in the *S.cerevisiae* genome has only been detected in the rDNA array, as discussed above, and in the *Xenopus* system, clustering is increasingly observed with increasing S phase length (Marheineke and Hyrien, 2004).

The average time for replicon activity is about one hour because the first replicons fired at the onset of S phase remain distinguishable on DNA fibres for about one hour before fusing together, at which point new, smaller replicons appear on DNA fibres, both adjacent to earlier clusters and at more distant sites (Jackson and Pombo, 1998). Within a similar timeframe, new foci appear in

whole nuclei sequentially labelled with two modified nucleotides (Ma et al., 1998; Manders et al., 1996). For the first three hours of S phase, the number of foci remains at about 750/cell, each active for about one hour (Jackson and Pombo, 1998). At later stages of S phase, replication foci are fewer but much larger and it is no longer clear whether each focus represents a single cluster of replicons.

A very similar picture of replicon dynamics emerged from the analysis of a primary mouse cell line, although the greater resolution of the bio-dUTP labelling method revealed significant asynchrony in initiation events within replicon clusters (Takebayashi et al., 2001). Some degree of asynchrony is supported by extensive pairwise comparisons of adjacent origins in the genomes of mole rat cells (Dubey and Raman, 1987b) and also by the density substitution method used by Roti Roti and Painter (1977).

The general model of clustered, synchronous or near-synchronous origin firing has been called into question by certain fibre autoradiography experiments (Yurov and Liapunova, 1977) which showed that only 15% of replicons in asynchronous cells existed in closely-spaced clusters. The disparity between this and the figure of 84% clustering in synchronised HeLa cells (Jackson and Pombo, 1998) is probably due to cell cycle differences as well as to the general under-detection of very large replicons: Jackson and Pombo analysed only the first two hours of S phase so very large, long-running replicons would not be detected (and the outermost forks of a cluster *were* able to continue for at least two hours). It is also possible that the synchronisation of cells with aphidicolin contributed to the synchrony of clustered origin firing upon release from the drug. No data is available dealing specifically with the later stages of S phase but it is possible that very long-running replicons would make up a more significant proportion of the

total at these stages. Isolated examples which have been mapped in detail, such as the IgH locus (Ermakova et al., 1999), show that small clustered replicons can be found directly adjacent to long regions of DNA which are replicated over several hours by a single fork.

The temporal program of origin firing

In *S.cerevisiae*, although individual origin sequences fire at a reproducible times during S phase, the temporal program is poorly understood and appears to be dictated not by the origin sequence itself but by surrounding sequences and general chromatin context, as discussed above.

In mammalian cells, the factors dictating the temporal program of origin firing are even less clear but reproducible timing has been demonstrated in a few specific origins. Replication at the lamin B locus, for example, is reproducibly early. Two variants of the rDNA locus replicate at distinct times – one variant about three hours later than the other - despite being in the same amplified locus (Larner et al., 1999). This suggests, as in *S.cerevisiae*, that timing is at least partly determined by specific sequences in or around the origin locus. However, the wider chromatin context is also relevant since focal replication patterns show that euchromatic regions replicate early in S phase and heterochromatic regions, late (Humbert and Usson, 1992b). Furthermore, the same regions of chromosomes replicate at the same time in successive S phases, since early-replicating foci labelled in one S phase co-localise with early foci labelled in the next S phase (Dimitrova and Gilbert, 2000b).

This remains true at single-replicon resolution: at least some of the replicons which appear in HeLa cells at the start of S phase are re-labelled at the

outset of the next S phase (Jackson and Pombo, 1998). A similar phenomenon was observed in primary mouse cells, although in this higher-resolution study, many replicons initiated with a slight shift of a few kb in the next S phase (Takebayashi et al., 2001). Thus, early-firing origins are not picked at random from a large pool of potential origins but rather have specific features – either in their actual sequence or in some feature of their chromatin which is conserved from S phase to S phase. There is circumstantial evidence that transcriptional activity may be important: gene-rich R-bands on chromosomes are known to replicate early and gene-poor G-bands late (Drouin et al., 1994). Furthermore, early replication foci labelled with BrdU closely resemble active transcription sites (Jackson and Pombo, 1998).

It is not known whether late origins are defined with the same degree of specificity as early origins and if so, what factors might dictate late-firing. Possibly origin choice becomes more relaxed later in S phase in order to fill in the gaps left by earlier replicons which may have expanded at variable rates. In yeast, the removal of efficient origins increases the probability that late or inactive origins in adjacent DNA will fire before S phase ends (Ivessa et al., 2002; Vujcic et al., 1999). On the other hand, however, checkpoint modification of the normal origin firing program can act in the opposing direction to *prevent* late origin firing if replication from earlier origins is incomplete (Santocanale and Diffley, 1998b). Similarly, in mammalian cells, if replication is blocked in early S phase with aphidicolin (Dimitrova et al., 1999; Jackson, 1995) or if early-replicating DNA is specifically damaged (Hamlin, 1978) then late replication patterns do not appear at the expected time.

Replication Fork Rate

Most of the methods described above (DNA fibre autoradiography or immuno-labelling and also density substitution) have been used to derive figures for the rate of replication fork movement in mammalian cells.

As in *S.cerevisiae,* fork rates in mammalian cells are generally found to be widely variable: variation has even been documented within the genomes of single cells at a single stage in S phase (0.2-1.2μm in human fibroblasts (Yurov, 1980) between the two forks of single replicons (Dubey and Raman, 1987a) and between the leading and lagging strands of the same fork (Takebayashi et al., 2001). Fibre immuno-labelling in HeLa cells synchronised with aphidicolin showed that forks move at an average of 1.7+/-0.3kb/min. In unsynchronised cells, an even wider range of 0.2-2μm/min was observed by fibre autoradiography. This is equivalent to 0.6-6kb/min if DNA stretches at 3kb/μm (Edenberg and Huberman, 1975). More recent reports have included spreading controls to confirm this DNA-stretching factor: immuno-labelling of stretched adenovirus genomes showed that DNA was stretched at 2.59kb/μm (Jackson and Pombo, 1998) and FISH within the mouse genome yielded a similar factor of 3.5kb/μm (Takebayashi et al., 2001)). The density substitution method, which should be not be affected by DNA stretching factors, also yielded comparable average fork rates: 0.55μm/min in HeLa cells, 0.9μm/min in human fibroblasts, 0.7μm/min in CHO cells, 0.8μm/min in rabbit CBL cells and 1.8μm/min in mouse L cells (Painter and Schaefer, 1969). Since all these cells were analysed under identical conditions, it appears that there are up to 3-fold differences between fork rates in different cell lines, even though the replication machinery is essentially the same.

In *S.cerevisiae*, the mean fork rate does not vary from early to late S phase (Rivin and Fangman, 1980a) but several reports suggest that forks speed up towards the end of S phase in mammalian cells. Fibre autoradiography showed a three-fold increase in average track length during S phase in synchronised CHO cells (Housman and Huberman, 1975) and density substitution showed a similar two-fold increase in HeLa cells synchronised by fluorodeoxyuridine (Painter and Schaefer, 1971). This may be partially due to slow recovery of normal replication rates after cells are released from the FUdR block, but an increase in fork rates was also observed in HeLa cells synchronised without any drug treatment (see results section, fig.5).

It remains unclear what causes the wide variation in fork rates, but chromatin structure may play a role: late-replicating sequences are generally heterochromatic and contain fewer active genes, which may mean that replication forks are not impeded by transcriptional machinery. dNTP levels also increase significantly during S phase, which may promote increasingly rapid fork movement (Walters et al., 1973). Additional dNTPs can force fork rates up in early S phase but not in late S phase in mammalian cells (Leeds et al., 1985; Malinsky et al., 2001). Interestingly, the opposite is true of replication in the *Xenopus* extract system, where fork rate slows down towards late S phase (Marheineke and Hyrien, 2004). Here dNTPs are presumably depleted from the extract as replication proceeds, so this would further support the idea that dNTP pools directly modulate fork rate.

Non-termination fork stalling

The stalling of replication forks during normal replication is one of the hardest S phase parameters to assess since it is generally unlikely to follow any reproducible pattern. Accumulating evidence does suggest, however, that some degree of non-termination fork stalling does occur during the course of a normal S phase, both in *S.cerevisiae* and in mammalian cells, and that cells have evolved to cope with a certain level of such events. Forks may stall as a consequence of endogenous DNA damage (spontaneous depurination of DNA bases, for example, has been estimated to generate up to 10,000 abasic sites in the genome per day (Lindahl, 1993)). Forks may also be impeded by transcriptional machinery or other chromatin proteins, or by secondary structures in particular DNA sequences.

Mammalian genomes contain a number of 'fragile sites' which are especially prone to chromosome breakage and which may be analogous to the RSZs found in the yeast genome (Glover and Stein, 1988). These regions are frequently broken in conditions of 'replicative stress' such as polymerase inhibition by aphidicolin, but they also show rare breakage events during unstressed proliferation. Like RSZs, fragile sites do not share any obvious sequence features but they are generally late-replicating (Hansen et al., 1997; Subramanian et al., 1996), although whether this is because forks actually traverse them especially slowly, as is the case in RSZs, remains unclear. Aphidicolin delays their replication even further and can cause them to remain unreplicated into G2 (Le Beau et al., 1998) perhaps leading to breakage when mitosis is attempted. Certain fragile sites *do* contain specific DNA sequences: expanded tri-nucleotide repeats such as the CGG repeat in the disease-associated FRAXA site (Warren, 1996), and these repeats are thought to form secondary structures at

replication forks (Krasilnikova and Mirkin, 2004; Usdin and Woodford, 1995). This lends further weight to the hypothesis that it is fork stalling that actually causes fragile sites to break.

While some fragile sites are associated with heritable diseases (such as the Fragile X Syndrome caused by FRAXA), the majority are not. This suggests that if cells do experience difficulties in replicating these sites, such problems are either resolved without mutagenic consequences or are tolerated, giving rise to phenotypically silent mutations, or to an undetectably low mutation rate. Fragile sites are expressed at much higher levels in the absence of the mammalian Mec1 homolog ATR (Casper et al., 2002) and if a trinucleotide repeat characteristic of certain human fragile sites is inserted into the yeast genome, it too shows fragility in the absence of the Mec1-Rad53-Rad9 pathway (Lahiri et al., 2004). Therefore, as in yeast, a functional checkpoint system is required to facilitate the replication of fragile sites.

Cell Cycle Checkpoints in S phase Regulation

In addition to the endogenous DNA damage described above, many types of exogenous DNA damage can cause mutations in the genome of a cell, both by direct mutagenesis and also by generating lesions which are processed into mutations when DNA is replicated during S phase. Whatever the origin of such DNA damage, eukaryotic cells have evolved a complex set of systems to guard against it. These include multiple DNA repair systems and also cell cycle checkpoints. Checkpoints exist to monitor a cell's progress through each phase of the cell cycle and to sense and respond to DNA damage by arresting the cycle when damage is detected. This allows sufficient time for DNA repair before the cell cycle progresses any further (Zhou and Elledge, 2000). Checkpoints are therefore vital to maintaining the genetic integrity of cells: their failure can lead to the creation and propagation of mutations (Myung et al., 2001) and is frequently linked to cancer development or cancer predisposition (Kastan, 1997).

Checkpoints can act at any stage of the cell cycle and are frequently classified according to the stage at which the cycle is arrested (the 'G1', 'intra-S' and 'G2' checkpoints); or according to the cell cycle transition that is prevented (the G1-S and G2-M checkpoints). However, it is frequently unclear whether these classifications refer to the phase in which DNA damage is sensed as well as the phase in which the response occurs, and it is becoming increasingly clear that this may depend on the form of DNA damage in question. For example, some forms of replication-blocking damage may require processing through S phase or an attempted mitosis before an efficient checkpoint response can be mounted, whereas other forms of damage can be sensed immediately regardless of cell cycle phase. Furthermore, if experiments investigating checkpoint responses are carried

out on asynchronous populations of cells, the situation may be further complicated by the fact that although the population eventually arrests at a single stage, subpopulations of cells will have experienced the damage at different stages in their own individual cycles and may have sensed and responded to it in different ways. Finally, most checkpoint proteins have roles in several different phases of the cell cycle. It may therefore be more realistic to regard the whole checkpoint system as a single network rather than as multiple phase-specific pathways, with the upstream elements of this network having central roles throughout the cell cycle while the downstream effectors vary according to what is appropriate at a particular stage of the cell cycle.

This project focuses on the effects of the checkpoint network upon DNA replication when DNA damage occurs specifically during S phase. It is well-established that the rate of DNA synthesis is reduced in response to various types of damage, presumably to minimise the risk of any lesions being fixed into potentially dangerous mutations before they can be repaired. (During S phase, for example, single stranded DNA breaks (ssbs) can be processed into double stranded breaks (dsbs) and mismatches can be fixed into permanent point mutations by the passage of a replication fork.) The 'intra-S phase' checkpoint has been conserved from yeast to man (Boddy and Russell, 2001) and many of the central proteins also remain closely conserved, making the budding yeast *S.cerevisiae* a suitable model system for the study of this checkpoint.

The S phase checkpoint in *S.cerevisiae*

The actual mechanisms that bring about the reduction in DNA synthesis after DNA damage have been extensively studied in *S.cerevisiae*. The responses to a range of DNA damaging agents, methyl methane sulphonate (MMS), hydroxyurea (HU) and ionising radiation (IR) have all been assessed, using a combination of flow cytometry, Southern blotting and 2D gel analysis of replication intermediates, density transfer to monitor fork progression from defined origins and electron microscopy to visualise replication structures. These techniques can separate effects on origin firing from effects on fork progression, at least on a population level, and they have shown that origin firing is blocked in response to MMS or HU (Santocanale and Diffley, 1998b; Shirahige et al., 1998) and that rates of fork movement are also reduced after MMS damage (Tercero and Diffley, 2001). After IR damage, DNA synthesis is again reduced (King et al., 2003), although the mechanism(s) in this case have not been elucidated.

The block to origin firing depends on the central checkpoint kinases in *S. cerevisiae*, Mec1 and Rad53, whereas the reduction in fork rate appears to be independent of these kinases. Mec1 – the central transducer in the checkpoint pathway - belongs to the PIKK family: a group of very large (>300kDa) proteins with C-terminal homology to phosphoinositide 3' kinases. Despite this homology, Mec1 is a protein kinase which phosphorylates and activates several downstream effector proteins, amongst them Rad53 (reviewed (Foiani et al., 2000; Lowndes and Murguia, 2000)). *S. cerevisiae* also possesses a second PIKK, Tel1. Tel1 appears to be non-essential for checkpoint activity but a redundant role in some aspects of the checkpoint is revealed if both Tel1 and

Mec1 are mutated (D'Amours and Jackson, 2001; Giannattasio et al., 2002; Nakada et al., 2003).

Both Mec1 and Rad53 are essential for viability in *S.cerevisiae,* but their essential role does not appear to be in the S phase checkpoint described above, nor in other cell cycle checkpoints. Rather, Mec1 and Rad53 also act to regulate the production of dNTPs, both by transcriptional regulation of RNR (Huang and Elledge, 1997b) and by S phase-specific suppression of Sml1, a repressor of RNR activity (Zhao et al., 2000). Mutations in Mec1 and/or Rad53 can thus be suppressed by deleting Sml1 and this has greatly facilitated the study of the S phase checkpoint in yeast, since, in contrast to the analogous situation in mammalian cells, mutants in Mec1 and/or Rad53 can easily be studied.

It is important to note that Mec1 and Rad53 are also central to several other aspects of the S phase checkpoint: the induction of a transcriptional programme of damage response genes (Aboussekhra et al., 1996), the prevention of irreversible fork stalling after MMS damage (Lopes et al., 2001b; Sogo et al., 2002; Tercero and Diffley, 2001) and the dramatic increase of intracellular dNTP levels after damage (Chabes et al., 2003). It is not clear, however, whether these additional checkpoint responses actually affect the rate of DNA synthesis.

The S phase checkpoint in mammalian cells

By contrast to the picture outlined above, the mechanism of the S phase checkpoint in mammalian cells remains poorly understood. The standard assay for an S phase checkpoint response in mammalian cells is the radioresistant DNA synthesis (RDS) assay, in which a population of cells is pulse-labelled with tritiated thymidine after being exposed to DNA damage. The incorporation of TCA-precipitable ^{3}H counts is then taken as a measure of the rate of DNA synthesis (normalised to the ^{14}C counts present in the DNA from a pulse of ^{14}C-labelled thymidine which was added to the cells in the previous cell cycle). This assay cannot distinguish effects on origin firing from either fork movement or fork stalling and it also includes not only intra-S-phase changes to DNA synthesis but also complete prevention of the G1-to-S transition. In addition, the RDS assay could be affected by any changes in dNTP balance within cells since it relies on the uptake of tritiated thymidine via the nucleotide salvage pathway. Therefore, in the absence of a good range of sequence-defined early and late origins in mammalian genomes (which might facilitate the use of the same techniques employed to study *S.cerevisiae*), various alternative assays have been used to further investigate specific aspects of the mammalian S phase checkpoint.

Size separation of ^{3}H-labelled DNA on an alkaline sucrose gradient after treating cells with IR led to the inference that origin firing is blocked since the proportion of small DNA fragments – assumed to represent recently fired origins – is reduced (Painter, 1985a; Painter and Young, 1980). Longer fragments of labelled DNA – assumed to represent ongoing forks - were also reduced in size but only after much higher doses of IR. This was interpreted as a reduction in fork movement. A similar reduction in the number of short DNA fragments was

observed after MMS and UV damage, with longer fragments again being affected to a lesser extent and only after longer time periods (Painter, 1977; Painter, 1985b). The data regarding IR is supported by DNA fibre autoradiography showing that the number of short labelled tracks on DNA fibres is reduced after IR treatment (Watanabe, 1974). It is important to note, however, that alternative interpretations of much of this data could be made since the actual identities of the DNA fragments are unknown and time resolution is relatively poor. Furthermore, like the RDS method, the size-separation assay could be skewed by cell cycle effects outside S phase and also by any changes to dNTP levels (although an attempt to quantify tritiated thymidine incorporation before and after IR suggested that at least in the mouse L5178Y cell line, no such change occurred (Watanabe, 1974)).

A subsequent investigation of ^{3}H labelling of DNA in asynchronous versus synchronised cell populations showed that at least 50% of the reduction in ^{3}H labelling which follows exposure to IR in an asynchronous population was indeed due to the complete prevention of S phase entry via a G1/S checkpoint, as opposed to any intra-S-phase change in replication dynamics (Lee et al., 1997). Nevertheless, the existence of a block to origin firing which is genuinely intra-S phase has been corroborated by a second method: 2D gel analysis of replication in late-replicating versus early-replicating rDNA variants. This showed - at least qualitatively and on a population level - that unfired origins could be blocked following IR damage *within* S phase while fork movement appeared to be minimally affected after moderate IR doses (Larner et al., 1999).

Replication dynamics have not been tested using the 2D gel method after other forms of DNA damage such as MMS. However, an alternative approach

has been used to test for an origin firing response to another agent which is thought to stall replication: the polymerase inhibitor aphidicolin. This technique, involving the fluorescent labelling of early and late replication foci in CHO cells, revealed a block to the appearance of late replication patterns when the cells are treated with aphidicolin (Dimitrova and Gilbert, 2000b; Zachos et al., 2003). This was interpreted as a checkpoint-dependent block to origin firing. However the method does not yield quantitative data on the numbers or proportions of affected origins in the labelled foci, nor can it address other parameters such as replication rate or collapse at ongoing forks.

In comparison to *S.cerevisiae*, where the roles of Mec1 and Rad53 (if not of their downstream effectors), are relatively well understood, the proteins responsible for the various phenomena of the intra S phase checkpoint in mammalian cells remain poorly defined. This is partly due to the essential nature of many of the proteins involved, the difficulties of generating targeted knockouts in mammalian cells and the problems of making direct comparisons between different disease cell lines and between different mammalian species. In addition, the checkpoint network in higher eukaryotes is more complex than the network which exists in *S.cerevisiae*. Two central PIKKs share the role of the yeast Mec1 kinase, responding to two different classes of DNA damage. The ATM kinase is actually most homologous to Tel1 but in mammalian cells ATM has a major role in responding to ionising radiation and to drugs that causes dsbs. ATR, which is most homologous to Mec1, responds to bulky adducts, crosslinks and other forms of damage which could block replication forks, as well as to drugs such as

aphidicolin and hydroxyurea which directly stall replication by nucleotide depletion or by inhibiting polymerases.

The discussion below describes the mammalian checkpoint network in terms of the proteins associated with each of these two central kinases, although in reality there is probably considerable crosstalk between the two and they certainly share many substrates in common. Each section concludes with a review of what *is* known so far about how these checkpoint proteins actually enforce the suppression of DNA replication.

The S phase checkpoint: ATM pathway

The ATM kinase was first identified as the factor lost or mutated in the inherited disease ataxia telangiectasia (AT) (Savitsky et al., 1995), reviewed (Shiloh, 2003). AT patients show a variety of developmental and neurological abnormalities and also cancer predisposition (Swift et al., 1991). At a cellular level, cells from AT patients are hypersensitive to IR and do not show characteristic IR-induced checkpoint arrests at any stage of the cell cycle. Together, these phenotypes suggest that the loss of ATM leads to improper repair of IR-induced dsbs and/or failure to arrest the cell cycle or induce apoptosis in the presence of dsbs. These failures may result in the creation and propagation of cancer-causing mutations and genomic instability.

A number of studies have confirmed that ATM does indeed respond to IR, via an increase in its kinase activity towards substrates such as p53 (Banin et al., 1998) and CHK2 (Ahn et al., 2000). ATM also responds to radiomimetic drugs like bleomycin (Banin et al., 1998), but not to DNA damage caused by UV (Canman et al., 1998). This suggested that dsbs are the specific primary signal for ATM activation (as opposed to the base damage or ssbs which are also associated with irradiation of DNA (Sutherland et al., 2000a; Sutherland et al., 2000b)). More recently, the mechanism by which ATM is activated has been elucidated at the molecular level. ATM is present in the nucleus throughout the cell cycle and is not transcriptionally induced after DNA damage (Watters et al., 1997). Instead, a method of rapid, conditional activation operates on the existing nuclear pool of ATM (Bakkenist and Kastan, 2003). In undamaged cells ATM exists as an inactive homodimer but when cells are irradiated, a specific serine residue

(Ser1981) in the conserved FAT domain which flanks the catalytic domain is phosphorylated in an intermolecular reaction, leading to the dissociation of the protein homodimer and activation of ATM towards its substrates. This mechanism may explain the observation that heterozygous carriers of missense mutations in ATM show an increased risk of certain cancers (reviewed (Meyn, 1999): mutant ATM could have a dominant negative effect and this has recently been confirmed *in vitro* (Lee and Paull, 2004). Although Ser1981 phosphorylation is clearly an important step in ATM activation, it is probably not the only event that activates ATM: mutating Ser1981 does not have any effect on ATM's *in vitro* kinase activity. A protein phosphatase PP5 has recently been reported to bind to ATM in an IR-inducible manner and promote ATM activation, perhaps by removing inhibitory phosphates (Ali et al., 2004).

The whole process of ATM activation occurs within minutes of as little as 0.5 Grays (Gy) of IR, a dose which is estimated to cause only 18 dsbs per genome (Rothkamm and Lobrich, 2003). This may mean that activation is unlikely to be triggered by direct contact between individual ATM dimers and broken DNA ends: instead, some major change in chromosome structure is thought to occur which is rapidly transmitted throughout the nucleus. Although no direct evidence has yet been produced to support this model, ATM is also activated when cells are treated with hypotonic buffer, chloroquine or the histone deacetylase inhibitor trichostatin A, all agents that disturb chromatin structure without apparently causing dsbs.

This activation mechanism does not require ATM to be localised to dsbs in order to become active; however, recent evidence suggests that ATM does subsequently become concentrated at dsbs and this may be important for its

activity towards at least some of its substrates. ATM foci co-localising with H2AX (an established indicator of dsb sites – see below) can be detected if the masking pool of free ATM is first removed from the nucleus by detergent extraction (Andegeko et al., 2001). ATM foci are also seen at other sites containing DNA ends such as uncapped telomeres (Takai et al., 2003) and V(D)J-associated breaks (Perkins et al., 2002). Furthermore, the interaction of ATM with DNA ends can be detected *in vitro* in both human (Suzuki et al., 1999) and *Xenopus* (Costanzo et al., 2000) extracts, and also observed directly by atomic force microscopy (Smith et al., 1999). That the ATM-dsb interaction is functionally important is demonstrated by the tethering of a normally nucleoplasmic ATM substrate, CHK2, to chromatin via a histone fusion (Lukas et al., 2003). Tethered CHK2 is only activated at dsb sites, suggesting that ATM only actually acts on CHK2 at these sites, although activated CHK2 then diffuses rapidly throughout the nucleus if it is allowed to do so.

The ATM pathway: substrates of ATM

Although ATM clearly responds very rapidly to the presence of dsbs, considerable controversy remains as to whether this activation is actually sufficient for all of ATM's functions. ATM is either disabled or considerably less efficient in some aspects of checkpoint arrest and repair if any one of a number of so-called 'adaptor' proteins is missing, and this coincides with inefficient phosphorylation of known substrates. Thus it appears that ATM can only act on some of its substrates if it is associated with adaptor proteins in the context of damage-induced foci (Bakkenist and Kastan, 2004). Such foci are also required to actually repair at least a subset of dsbs (Kuhne et al., 2004) explaining the radiosensitive phenotype of both AT cells and various adaptor-deficient cells.

Since there may be multiple mutual dependencies between ATM (and perhaps also some of its substrates) and these vital adaptor proteins, both for localisation and for subsequent activity, it is probably inaccurate to regard either focus formation or the 'ATM pathway' itself as a linear series of events. Certainly, attempts to order such a pathway have given rather confusing results. This may be partly due to the problems of comparing different disease cell lines or siRNA-mediated partial knockdowns, and also to the qualitative nature of readouts such as phosphorylation shifts or immunofluorescent foci. All these readouts depend on the strength and specificity of the antibodies used: a particular antibody, for example, may not recognize a particular phosphorylated form of a protein, or an activating phosphorylation may not induce a detectable electrophoretic shift. With regard to focus formation, most proteins must be present at several hundred copies to become detectable as an immunofluorescent focus and proteins with a high free concentration and/or relatively weak focal

binding may never be detected in damage-induced foci. However, despite all these issues, it is also likely that the checkpoint response genuinely does not operate as a linear cascade but rather as an iterative process of protein interactions, assembly of foci and progressive signal amplification. There is accumulating evidence that foci are not the static structures implied by fixed immunofluorescence, but rather are highly dynamic in terms of both the proteins and the DNA that they contain. For example, FRAP analysis in live cells demonstrates that the repair protein Rad51 is a relatively stable component of foci while Rad52 and Rad54 diffuse rapidly in and out (Essers et al., 2002). A single dsb is sufficient to nucleate a focus (Sedelnikova et al., 2002) but if a cell contains several foci, these can be brought together at later times into aggregates which may contain several different broken DNA ends (Aten et al., 2004). Again, this dynamic organization depends on adaptor proteins.

In view of this non-linear model for the ATM pathway, the substrates of ATM are discussed below under the general headings of 'dsb sensors or markers', 'adaptor proteins', 'signal transducers', and finally 'effectors of cell cycle arrest or DNA repair'. All the substrates are phosphorylated by ATM on one or more S/T-Q motifs (Kim et al., 1999) and with few exceptions, this activates their function and/or promotes their correct localisation. The mechanism of the actual phosphorylation, however, varies between different ATM substrates: some appear to require a stable, DNA-bound association of the protein with active ATM in the context of a damage-induced focus while others can be at least partially activated via transient associations with free ATM.

p53

The canonical ATM substrate p53 lies outside of the network described above since its principal role appears to be in G1 rather than in the intra-S phase checkpoint: it acts to prevent cells from entering S phase in the presence of DNA damage. p53 is phosphorylated by ATM on Ser15 (Banin et al., 1998; Canman et al., 1998), stimulating p53 binding to the transcriptional co-activator p300 (Dumaz and Meek, 1999) and also stimulating further phosphorylation events which cause p53 to dissociate from MDM2 and thus prevent p53's normally rapid proteosomal turnover (Freedman and Levine, 1999). The accumulated p53 then transcriptionally up-regulates p21, which in turn suppresses the cyclin-dependent kinase activity necessary for the G1 to S transition (Giaccia and Kastan, 1998).

Several lines of evidence indicate that the phosphorylation of Ser15 in p53 does not require adaptor proteins or the focal recruitment of either p53 or ATM. p53 interacts directly with ATM after DNA damage (Watters et al., 1997), its phosphorylation is not correlated with the level of dsbs in the cell but rather becomes maximal after very low IR doses similar to those which maximally activate ATM and finally, non-dsb inducing treatments which activate ATM also stabilise p53 (Bakkenist and Kastan, 2003). p53 activation *does* depend on the MRN complex, but probably in its role as an ATM activator not as a focus-forming adaptor (Lee and Paull, 2004). The only factor arguing against this general model is the finding that p53 phosphorylation *is* affected when certain adaptors such as 53BP1 (Wang et al., 2002) and BRCA1 (Fabbro et al., 2004; Foray et al., 2003) are absent. However, there are also indirect routes by which ATM stabilises p53 and some of these may be promoted by the assembly of IR-responsive foci. For example, the BRCA1/BARD1 complex – which does require

the action of ATM within foci - is somehow in turn required for efficient p53 phosphorylation (Fabbro et al., 2004). ATM also directly phosphorylates MDM2 (Maya et al., 2001) and activates CHK2 which then phosphorylates a second site in p53, Ser20 (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). The *in vivo* significance of this latter phosphorylation of p53 remains controversial, a matter discussed in more detail under CHK2.

In addition to its central role in the G1 checkpoint, p53 has been proposed to cause a delay within S phase in mouse zygotes, although data on this pathway is still very limited. When zygotes are fertilized with damaged sperm DNA, DNA synthesis is suppressed only after S phase entry, rather than via G1 arrest, and this occurs in a p53-dependent but p21-independent manner (Shimura et al., 2002). This particular cell system is not capable of an IR-induced G1 arrest (Kim et al., 2002a) so it is possible that the S phase delay is a backup mechanism which only acts in this special situation: in somatic cells the S phase checkpoint is well established as being p53-independent (Lee et al., 1997; Xie et al., 1998). Finally, p53 may also contribute to the arrest of cells in G2 by up-regulating targets such as 14-3-3 proteins and GADD45 (Taylor and Stark, 2001). However, this is not essential because p53-negative cells still possess a G2 checkpoint.

Damage sensors or markers: H2AX and MRN

H2AX

H2AX is a minor variant of the histone H2A. It is one of eight H2A variants, it differs from the major form mainly in its C-terminus, and it is conserved from yeast to man (West and Bonner, 1980). A conserved C-terminal

SQ motif in H2AX is phosphorylated by ATM within three minutes of IR damage (Burma et al., 2001; Rogakou et al., 1999; Rogakou et al., 1998), although another dsb-responsive PIKK, DNA-PK, also contributes to H2AX phosphorylation (Park et al., 2003; Stiff et al., 2004). The phosphorylated form is denoted γ-H2AX and is considered a sensitive indicator of the presence of dsbs. Studies using laser scissors or V(D)J recombination to induce dsbs have demonstrated the close co-localisation of γ-H2AX foci with all sites containing dsbs (Chen et al., 2000a; Petersen et al., 2001; Rogakou et al., 1999) and quantitative dsb induction using radioactive IdU has shown that a single γ-H2AX focus indicates a single dsb. Furthermore, the phosphorylation must occur *at* the dsb because H2AX is essentially immobile in chromatin (Siino et al., 2002). Thus, in contrast to p53, H2AX is a substrate that requires localised, active ATM. Phosphorylation of H2AX is processive for up to 30Mb distal to a dsb, with approximately one in ten H2AX molecules being phosphorylated (Rogakou et al., 1999). This supports an iterative model for focal recruitment of damage-responsive proteins, since one of γ-H2AX's principal roles is the recruitment of adaptor proteins, which can then recruit more ATM to act on further H2AX molecules (Paull et al., 2000). γ-H2AX interacts *in vitro* with adaptors including NBS1 (Kobayashi et al., 2002a), MDC1 (Stewart et al., 2003), 53BP1 (Ward et al., 2003a), and BRCA1 and the lack of H2AX *in vivo* abolishes the formation of stable, long-term damage-responsive foci containing these proteins (Celeste et al., 2002). However, γ–H2AX may only be a signal-amplifier rather than the elusive primary sensor of dsbs, since the initial, transient recruitment of these adaptors does not require γ-H2AX (Celeste et al., 2003). Consistent with this, γ–H2AX is not needed for the activation of the S phase checkpoint (Redon et al., 2003) though it is required for a subsequent G2

arrest after low-dose IR, perhaps because a G2 arrest requires more sustained amplification of a weak DNA damage signal (Fernandez-Capetillo et al., 2002).

In addition to its checkpoint-promoting role, γ-H2AX may facilitate dsb repair, perhaps by keeping broken ends together (Bassing and Alt, 2004) or by decondensing chromatin structure. Exposing cells to hypertonic salt, which condenses chromatin, reduces repair efficiency and also causes γ-H2AX to accumulate into larger, more sustained foci (Reitsema et al., 2004). In yeast, a mutant mimicking constitutive H2AX phosphorylation has relatively decondensed chromatin and the opposite, non-phosphorylatable mutant has the reverse effect and is deficient in non-homologous end joining (NHEJ), although not in homologous recombination (HR) (Downs et al., 2000). In contrast, an H2AX knockout mouse (Bassing et al., 2003; Celeste et al., 2003; Celeste et al., 2002) is deficient in HR (immunological class switching) but not in NHEJ (V(D)J recombination). Whatever the exact repair defect, however, it results in genomic instability and IR sensitivity in both yeast and mice, and the mice are accordingly cancer prone in a p53-negative background.

The MRN complex: MRE11/RAD50/NBS1

The MRN complex consists of three components which are conserved from yeast to man and are all linked to AT-like inherited disorders. The genes for NBS1 or RAD50 are mutated in Nijmegen Breakage Syndrome and MRE11 in AT-like disorder (ATLD) (reviewed (Tauchi et al., 2002)). MRE11 is also found mutated in certain sporadic cancers (Giannini et al., 2002). Knockout mice for any of the three genes are inviable (Luo et al., 1999; Xiao and Weaver, 1997; Zhu et al., 2001) but mice with hypomorphic alleles similar to those found in NBS and

ATLD can survive and these mice reproduce many of the AT-like phenotypes including radiosensitivity and cancer predisposition (Bender et al., 2002; Kang et al., 2002; Theunissen et al., 2003; Williams et al., 2002). All this suggests close functional links between MRN and ATM and indeed, MRN does play a central role in initiating IR-induced focus formation and in localising and perhaps stimulating active ATM to act on many of its substrates. It still remains unclear whether MRN also acts upstream of ATM as a dsb sensor or whether it simply stabilizes ATM which is already activated (reviewed (Bakkenist and Kastan, 2004)). Either way, such a role is only revealed at very low levels of dsbs (Cerosaletti and Concannon, 2004) but this is perhaps closer to a physiological situation than the severe IR treatments employed in most experiments.

NBS1 is a 95kDa protein containing the BRCA1 C-terminal domain (BRCT domain) which is characteristic of all adaptor proteins. BRCT domains bind to DNA ends and mediate protein-protein interactions (Huyton et al., 2000; Yamane and Tsuruo, 1999). NBS1 also contains three nuclear localisation sequences and a forkhead associated (FHA) phosphothreonine binding domain which mediates phosphoprotein interactions (Durocher et al., 2000). Together these domains allow NBS1 to locate the whole MRN complex in the nucleus and to bind to γ-H2AX at dsbs (Desai-Mehta et al., 2001; Kobayashi et al., 2002a). MRE11 has DNA binding motifs and a phosphoesterase motif. It acts as a limited DNA helicase, a 3'-5' exonuclease and also an endonuclease, these activities being stimulated by NBS1 and RAD50 (de Jager et al., 2002; Paull and Gellert, 1999). RAD50 has ATPase motifs and paired coiled-coil domains which allow it to bridge DNA ends in the form of a RAD50 dimer together with two MRE11 molecules (Hopfner et al., 2002). In combination, these features allow the MRN

complex to bind dsbs and form damage-responsive foci as well as modifying the broken DNA ends and also holding them together for efficient repair.

Thus the MRN complex has both checkpoint signalling and DNA repair functions. MRN forms foci at dsbs within minutes of IR damage (Maser et al., 1997; Mirzoeva and Petrini, 2001; Nelms et al., 1998) and acts as a crucial adaptor to recruit ATM to these foci (Uziel et al., 2003). Both NBS1 (Gatei et al., 2000)Lim, Kim et al. 2000) and MRE11 (Dong, Zhong et al. 1999) are then phosphorylated by active ATM. As is the case for the other adaptor proteins, MRN is only phosphorylated after its initial recruitment to dsbs (Mirzoeva and Petrini 2001) but the phosphorylation is subsequently required for some of the downstream activities of the complex. For example, MRN is required for an efficient intra-S phase checkpoint and for this, NBS1 must be phosphorylated (Lim et al., 2000; Wu et al., 2000). The reason for this remains unclear, but it is possible that the checkpoint-transducing kinase CHK2 is most efficiently recruited to dsb sites by phosphorylated NBS1: a similar interaction has been reported, for example, between another adaptor, MDC1 (see below) and phosphorylated FHA domains of CHK2 (Lou et al., 2003). In addition, the second Chk kinase, CHK1, also requires MRN for its activation by ATM (Gatei et al., 2003). However, the checkpoint defect is only partial if NBS1 is absent or non-phosphorylatable (Falck et al., 2002), so other transducers of the S phase checkpoint such as SMC1 may be recruited to ATM-containing foci by unphosphorylated NBS1 and/or by other adaptors. Alternatively, the requirement for NBS1 to recruit CHK2 may be dose-dependent: a sufficient dose of dsbs may simply render MRN-mediated focal recruitment of CHK2 unnecessary. Consistent with this latter explanation, CHK2 activation has been reported to be

NBS1-dependent after low-dose IR (Buscemi et al., 2001; Girard et al., 2002; Uziel et al., 2003) but NBS1-independent after high-dose IR (Falck et al., 2002).

A similar debate surrounds MRN's role in the G2 checkpoint. Some reports suggest a partial defect in G2 arrest in NBS1-deficient cells (Buscemi et al., 2001; Carson et al., 2003; Williams et al., 2002), while others report that the G2 checkpoint is MRN-independent (Xu et al., 2001; Xu et al., 2002a). Again, this could be due to redundant mechanisms for activating CHK1 and CHK2, or to dose-dependent activation of the Chk kinases in non-null MRN mutants. Neither the intra-S nor the G2 checkpoint requires the RAD50 component (Bender et al., 2002) so it appears that the checkpoint signalling role of MRN is restricted to the ATM phosphorylation targets within the MRN complex – NBS1 and MRE11.

The role of MRN in dsb repair *does* require the RAD50 component and this role is separable from the checkpoint signalling role. The MRN complex specifically promotes homologous recombination (HR): NBS1-deficient cells lack sister chromatid exchange and HR-mediated gene targeting but not plasmid end-joining via non-homologous end joining (NHEJ). MRN also suppresses inter-chromosomal recombination in favour of sister chromatid exchange, probably because RAD50 can keep nearby DNA ends together (Tauchi et al., 2002). However, during G1 when sister-chromatid HR is not possible, MRE11 may actually promote the clustering of dsbs from different chromosomes (Aten et al., 2004). MRN also associates with the RecQ helicase WRN in an IR-inducible fashion (Cheng et al., 2004) and, like the BLM helicase discussed below, WRN promotes HR over NHEJ (Prince et al., 2001). Evidence from yeast and *Xenopus* suggests that this entire repair role may be an extension of a normal replication function for MRN, resolving DNA hairpins that could stall replication forks

(Farah et al., 2002) and/or repairing endogenous dsbs that may arise at stalled forks (Costanzo et al., 2001). Consistent with this, MRN in mammalian cells co-localises with some replication foci during undamaged S phases.

A final, more controversial role for MRN is in the actual activation of ATM itself. MRN is needed for most of the *readouts* of ATM activity in terms of substrate phosphorylation, but this could simply be explained by an adaptor role for MRN in facilitating ATM's access to its substrates or promoting ATM's catalytic activity. However, the extent of ATM's self-phosphorylation on Ser1981 also depends on MRN after low doses of IR (Carson et al., 2003; Horejsi et al., 2004; Kitagawa et al., 2004) or radiomimetics (Uziel et al., 2003). For this, MRE11 and RAD50 but not NBS1 are required, suggesting that it is the DNA binding or processing roles of MRN which mediate ATM activation (Cerosaletti and Concannon, 2004). It is not known whether MRN somehow senses dsbs and then promotes ATM's activation or whether it only stabilizes previously-activated ATM – for example, by complexing with ATM and preventing its dephosphorylation and re-association into inactive homodimers. The IR-stimulated formation of MRN/ATM complexes has been reported *in vitro* (Lee and Paull, 2004). In either model, MRN must act via nucleoplasmic as opposed to focus-bound interactions with ATM, or via only transient interactions with dsbs, because MRN which cannot bind to foci retains its ATM activating function.

Adaptor proteins: MDC1, 53BP1, BRCA1, TopBP1 and MSH2

MDC1

MDC1 was recently identified as a large (226kDa) protein which binds to the MRN complex (Goldberg et al., 2003; Stewart et al., 2003). MDC1 is phosphorylated by ATM in response to IR (showing some degree of redundancy with other PIKKs as is the case for most ATM substrates). The C-terminus of MDC1 contains repeated BRCT domains which may facilitate its constitutive association with other checkpoint proteins including 53BP1, SMC1 and FANCD2 (Stewart et al., 2003). MDC1 also contains an FHA domain which mediates phosphoprotein interactions with the CHK2 kinase (Lou et al., 2003) and with the MRN complex (Xu and Stern, 2003).

MDC1 forms γ–H2AX-dependent foci, co-localising with other adaptors like 53BP1 and MRN. It also promotes further H2AX phosphorylation (Goldberg et al., 2003) and has a feedback role in promoting ATM's own activation (Mochan et al., 2003), suggesting MDC1 may have a direct role as part of the MRN complex.

Cells treated with siRNA to knock down MDC1 fail to recruit MRN, 53BP1 and BRCA1 to stable foci but ATM is still able to act on at least some of its substrates including NBS1 and CHK2 (Goldberg et al., 2003). This is consistent with MDC1 acting partially redundantly with MRN and other adaptors as a scaffold around which foci are assembled – a theory further supported by examining the kinetics of the proteins which are still phosphorylated in the absence of MDC1. NBS1 is recruited transiently to dsbs, and thus phosphorylated by ATM, but it is not retained at the dsb site (Lukas et al., 2003). Similarly,

although CHK2 can still be activated, focal recruitment of the activated protein is impaired in the absence of MDC1 (Shang et al., 2003).

With respect to cell cycle arrest, MDC1 knockdown cells do show radioresistant DNA synthesis (Goldberg et al., 2003) and also a G2 checkpoint failure but the mechanism of this remains unclear. CDC25A degradation is unaltered, which argues against a crucial role for MDC1 in Chk kinase activation (Goldberg et al., 2003; Stewart et al., 2003). Furthermore, although MDC1-knockdown cells are IR-sensitive no clear role for MDC1 in DNA repair has been reported and the lack of MDC1 actually *decreases* CHK2-dependent apoptosis (Lou et al., 2003) so the sensitivity cannot be due to increased cell death by the apoptotic route. Perhaps the cell cycle and repair phenotypes of MDC1-knockdown cells are indirect effects of general inefficiency in the signalling network which recruits checkpoint proteins into damage-responsive foci.

53BP1

53BP1 was originally identified as a protein interacting with p53 in human and *Xenopus* embryonic cells but this interaction does not occur in adult somatic cells (Xia et al., 2001). Instead, 53BP1 acts as an adaptor in the ATM pathway and is thought to be a functional homolog of the adaptor proteins Crb2 and Rad9 in *S.pombe* and *S.cerevisiae* respectively.

53BP1, like NBS1 and MDC1, contains repeated BRCT domains, is phosphorylated by ATM (Rappold et al., 2001) and rapidly co-localises with γ-H2AX via its C terminus (Schultz et al., 2000). This interaction does not require prior phosphorylation of 53BP1 by ATM, placing 53BP1 upstream of ATM recruitment in the process of assembling a focus (Ward et al., 2003a).

53BP1 then plays an important role in the focal recruitment of activated ATM: the two proteins interact specifically in IR-damaged cells (DiTullio et al., 2002) and 53BP1 allows ATM to act on some of its substrates, including SMC1 and BRCA1 (DiTullio et al., 2002; Wang et al., 2002). In fact, siRNA-mediated knockdown of 53BP1 completely abolishes the formation of visible foci containing S/T-Q phosphorylated proteins (DiTullio et al., 2002). Despite this, 53BP1 is not actually crucial for the activation of all ATM's substrates. CHK2, for example, can still be phosphorylated to some extent when 53BP1 is knocked down with siRNA (Fernandez-Capetillo et al., 2002; Wang et al., 2002) or even when 53BP1 is completely absent in a knockout mouse (Peng and Chen, 2003; Ward et al., 2003b). Indeed after doses >5Gy of IR, CHK2 is activated at normal levels regardless of 53BP1. This is further evidence that CHK2 can be phosphorylated relatively well via only transient associations with ATM at dsbs. In addition, 53BP1 and MDC1 probably form two partially-redundant parallel pathways for recruiting ATM to dsbs. MRN/MDC1 forms foci independently of 53BP1 and MRN/MDC1 recruitment is actually up-regulated if 53BP1 is absent. The loss of both pathways, however, causes a decrease in ATM activation and ATM activity towards its substrates (Mochan et al., 2003).

53BP1 has been implicated in both cell cycle arrest and DNA repair, although as for MDC1, this may be an indirect consequence of its structural role in promoting the phosphorylation of other ATM substrates. Cells lacking 53BP1 show both RDS and a partial G2 checkpoint defect (Wang et al., 2002). Knockout mice have a phenotype similar to $ATM^{-/-}$ mice: IR sensitivity and chromosomal abnormalities (Morales et al., 2003). Although this implies a role for 53BP1 in dsb repair, only a specific subset of repair reactions are affected in these mice.

Both gene conversion by HR and V(D)J recombination via NHEJ remain active but the NHEJ events required for class switch recombination are specifically defective. It has been suggested that these particular dsbs may be similar to the subset which cannot be repaired in ATM cells (discussed in more detail later) (Ward et al., 2004b).

BRCA1

BRCA1 was first identified as the product of the gene mutated in certain families predisposed to breast and ovarian cancer (Miki et al., 1994). More recently, the cancer-prone phenotype has been reproduced in BRCA1+/- p53-/- knockout mice, although homozygous BRCA1 knockouts show embryonic lethality (reviewed (Moynahan, 2002)). BRCA1 is another large BRCT-domain protein which is phosphorylated by ATM (Chen et al., 1996; Cortez et al., 1999) and is implicated as another adaptor in the ATM network. However, BRCA1 also has several other roles including transcriptional induction, ubiquitination, chromatin remodelling and DNA repair and it may act as central mediator for many disparate checkpoint-associated functions (reviewed (Parvin, 2001; Starita and Parvin, 2003; Venkitaraman, 2004)).

BRCA1 is cell cycle regulated, being expressed maximally in S/G2 (Chen et al., 1996), but it can recruit active ATM to IR-induced foci in all stages of the cell cycle (Kitagawa et al., 2004). This is required for phosphorylation of several ATM substrates (including those which have been reported elsewhere to be focus-independent): CHK1 (Yarden et al., 2002), NBS1, p53 and CHK2 (Foray et al., 2003). BRCA1 also binds to a number of other checkpoint-associated proteins: it binds BRCA2 and FANCA (discussed in more detail under FANCD2) (Folias et

al., 2002) and is constitutively associated with CHK2 – an interaction which is released after irradiation when activated CHK2 phosphorylates Ser988 of BRCA1 (Lee et al., 2000). This is important for CHK2's subsequent checkpoint signalling role and it also activates BRCA1 to function in HR (Zhang et al., 2004).

Another interaction which is disrupted after BRCA1's phosphorylation by ATM is BRCA1's constitutive binding to CtIP (CTBP-interacting protein, (Schaeper et al., 1998)). ATM phosphorylates CtIP and thus releases BRCA1, whereupon BRCA1 acts as a transcriptional inducer of several repair-related genes including GADD45 (Li et al., 2000), XPC and DDB2 (Hartman and Ford, 2002). This role in transcriptional induction may explain another constitutive binding partner of BRCA1, the RNA Pol2 holoenzyme (Anderson et al., 1998; Scully et al., 1997). Alternatively, BRCA1's association with the transcriptional machinery may facilitate a damage-scanning function (Starita and Parvin, 2003).

A second set of BRCA1 interactions are induced rather than released when cells are irradiated. BRCA1 associates with BARD1 in an IR-inducible manner (Wu et al., 1996), forming an E3 ubiquitin ligase (Hashizume et al., 2001; Kleiman and Manley, 2001) which is activated by its own self-poly-ubiquitination (Mallery et al., 2002). The targets of this E3 ligase are as yet unclear but it may act on the FANCD2 protein, which is known to form damage-induced foci with BRCA1 specifically when mono-ubiquitinated (Garcia-Higuera et al., 2001). BRCA1 also interacts with FANCA, a component of the complex that lies upstream of FANCD2, possibly targeting BRCA1 to FANCD2 (Folias et al., 2002). A second target of the BRCA1/BARD1 ligase may be the pol2 holoenzyme when DNA damage is encountered during transcription. This would promote degradation of the holoenzyme and allow BRCA1 to replace the

transcriptional machinery with repair factors. This model remains speculative but BRCA1 mutant cells *are* defective in transcription-coupled repair (Gowen et al., 1998) and BRCA1 does associate with the MSH2 and MSH6 mismatch repair proteins (Wang et al., 2000). BRCA1 also has non-transcription-coupled roles in promoting DNA repair: it can induce chromatin decondensation (Ye et al., 2001), interacting with a number of proteins which have chromatin remodelling roles including SWI/SNF (Bochar et al., 2000), the histone acetyltransferases p300 and CBP (Pao et al., 2000) and the helicase-motif protein BACH1 (Cantor et al., 2001). More directly, BRCA1 can also recruit Rad51 to IR-induced foci, promoting homologous recombination (Paull et al., 2001). BRCA1 is thought to inhibit NHEJ as well as promote HR, perhaps by controlling the amount of ssDNA generated at the dsb. BRCA1's binding to DNA can inhibit the nucleolytic activity of the MRN complex *in vitro* (Paull et al., 2001) and *in vivo,* HR is specifically defective and NHEJ is up-regulated in BRCA1 mutant cells (Moynahan, 2002; Snouwaert et al., 1999).

Finally, like the other adaptor proteins, BRCA1 contributes to checkpoint arrests in both S phase and G2 phase (Xu et al., 2001), probably acting as an adaptor for the checkpoint transducers. BRCA1 is essential, for example, for CHK1 phosphorylation and probably also for SMC1 phosphorylation (Kim et al., 2002b; Yazdi et al., 2002). In fact, separate phosphorylation events within BRCA1 are specifically linked to the S and G2 checkpoints (Ser1387 and Ser1423 respectively) (Xu et al., 2001; Xu et al., 2002a), so perhaps different phosphorylations are able to direct BRCA1 towards aiding the activation of cell cycle phase-specific targets.

TopBP1

TopBP1 was first reported as a binding partner of topoisomerase II (Yamane et al., 1997) but has subsequently been established as having a role in normal replication: it is the putative homolog of Dpb11 in *S.cerevisiae* (Cut5 in *S.pombe*) which is needed to load pol α and pol ε at origins (Masumoto et al., 2000). In addition, the *Xenopus* homolog of TopBP1, Mus101 is required for Cdc45 loading (Van Hatten et al., 2002). TopBP1 also acts in the response to DNA damage (Makiniemi et al., 2001): it is yet another BRCT-domain adaptor protein which forms foci co-localising with γ–H2AX after IR damage. These foci do not depend on phosphorylation of TopBP1 by ATM, instead they are mediated by one of the BRCT domains (Yamane et al., 2002). TopBP1 associates constitutively with BRCA1, presumably via a BRCT-BRCT interaction, and both proteins relocate to γ–H2AX foci in response to damage.

TopBP1 does not have any reported role in dsb repair but it is an important transducer of checkpoint signalling. TopBP1/BRCA1 foci play a crucial role in signalling to CHK1 (but not CHK2) to enforce the G2 checkpoint (Yamane et al., 2003) and although a similar role in the intra-S phase checkpoint remains speculative, TopBP1's normal replication function may allow it to scan DNA during S phase and then signal for the recruitment of checkpoint proteins that can activate CHK1. This is observed in *Xenopus* extracts treated with aphidicolin or etoposide, with TopBP1 recruiting ATR and RAD1 to chromatin. However, it is not clear whether the same is true of ATM recruitment at dsbs: CHK2 is not activated when DNA ends are added to such extracts (Parrilla-Castellar and Karnitz, 2003).

TopBP1 plays a separate role in the G1 checkpoint, inhibiting S phase entry altogether via repression of E2F1 (Liu et al., 2003a; Liu et al., 2004). This prevents the activation of S phase CDKs and also inhibits apoptosis, which explains the finding that TopBP1-knockdown cells die by apoptosis (Yamane et al., 2002).

In conclusion, the actual reason for TopBP1's phosphorylation by ATM is not yet known, since it is not required for any of TopBP1's reported functions. Possibly it simply contributes to the stabilization of the protein, which is normally rapidly degraded via ubiquitin-mediated proteolysis. ATM also acts on the E3 ligase responsible for TopBP1's turnover, hHYD (Honda et al., 2002) and an IR-induced association of TopBP1 with PML forms a second method of stabilization (Xu et al., 2003). TopBP1 therefore accumulates in response to IR damage, allowing the protein to facilitate checkpoint signalling.

MSH2

MSH2, one of the effectors of mismatch repair, has recently been reported as a specific adaptor for CHK2 activation (Brown et al., 2003). MSH2 binds to a second mismatch repair component MLH1 and since MSH2 also binds to CHK2 and MLH1 to ATM, this results in the targeting of CHK2 to ATM. The lack of these mismatch repair proteins leads to RDS, showing that they are functional in signalling for the S phase checkpoint. Uniquely, however, they play their adaptor role at IR-induced 8-oxoguanine adducts and not at dsbs.

Signal Transducers: CHK1, CHK2, SMC1 and c-Abl

CHK1

CHK1 is a conserved protein kinase which acts to arrest the cell cycle in response to activated PIKKs (Sanchez et al., 1997). It is cell cycle regulated, being maximally expressed in S/G2 (Kaneko et al., 1999) and regulates normal cell cycle progression as well as checkpoint arrests (Shimuta et al., 2002; Sorensen et al., 2003). Consistent with this constitutive role, CHK1 knockouts in mice or *Drosophila melanogaster* show embryonic lethality (Fogarty et al., 1997; Liu et al., 2000b; Takai et al., 2000), but knockouts made in certain adult somatic cells (chicken DT-40 cells and siRNA-knockdown human cells) can be viable, although they show high rates of apoptosis (Chen et al., 2003b; Zachos et al., 2003). This differential lethality is not because CHK1 is specifically required in the embryo, since a conditional knockout in the proliferating mammary gland of adult mice also leads to cell death by apoptosis, probably due to misregulated cell cycle progression and the absence of a proper checkpoint response to endogenous DNA damage (Lam et al., 2004). Possibly such damage is simply better tolerated in DT-40 cells, while siRNA knockdowns may be saved by residual levels of CHK1. Alternatively, CHK2 may be better able to substitute for CHK1 in some cells than in others. Nevertheless, CHK1 is generally regarded as an essential protein.

CHK1 is phosphorylated by ATM (and also ATR) on several C terminal residues including Ser 317 (Gatei et al., 2003) and Ser345 (Liu et al., 2000b). Efficient CHK1 activation after IR damage requires the adaptor proteins MDC1 (Stewart et al., 2003) and MRN (Gatei et al., 2003). Phosphorylation activates

CHK1 by increasing its kinase activity (Zhao and Piwnica-Worms, 2001), probably releasing an auto-inhibitory interaction between the C-terminal and catalytic domains of the protein (Chen et al., 2000b; Katsuragi and Sagata, 2004; Shann and Hsu, 2001). In addition, Ser345-phosphorylated CHK1 is retained in the nucleus and preferentially associated with 14-3-3 proteins (Jiang et al., 2003). Specific Ser345 mutants in CHK1 (Capasso et al., 2002; Lopez-Girona et al., 2001), as well as the lack of the whole CHK1 protein via knockout, knockdown or drug inhibition (Liu et al., 2000b; Sorensen et al., 2003; Zhao and Piwnica-Worms, 2001) all confer checkpoint deficiencies in S and G2. In *S.pombe*, a phosphatase Dis2 is specifically responsible for dephosphorylating Ser345 and turning the checkpoint response off (Latif et al., 2004), but it is not yet known whether this pathway is conserved in higher eukaryotes.

Activated CHK1 transduces the checkpoint signal by phosphorylating the phosphatases CDC25A and C (Sanchez et al., 1997). This prevents them from removing inhibitory phosphorylations on CDK2 and CDK1, thus preventing cell cycle progression (the G1/S transition and S phase progress in the case of CDK2 and the G2/M transition in the case of CDK1). Inhibition of CDC25A is achieved by ubiquitin-mediated degradation (Mailand et al., 2000; Mailand et al., 2002): CDC25A is phosphorylated on serines 123, 178, 278 and 292 (Sorensen et al., 2003) and this primes it for recognition by another unknown kinase which targets it for ubiquitation via $SCF^{B\text{-}TRCP}$ (Jin et al., 2003). CDC25A can act on both CDK1 and CDK2 and its inhibition can therefore arrest cells at G1/S, within S phase or in G2 (Mailand et al., 2002; Xiao et al., 2003; Zhao and Piwnica-Worms, 2001). For CDC25C, phosphorylation on Ser216 causes exclusion from the nucleus and inhibition via binding to 14-3-3 proteins (Dalal et al., 1999; Peng et

al., 1997). This prevents CDC25C from accessing CDK1, thus arresting cells in G2. The same nuclear exclusion mechanism also contributes to the inhibition of CDC25A at this late stage in the cell cycle (Chen et al., 2003a). The action of CHK1 on the same serine residues in CDC25A regulates CDC25A turnover during normal S phase progression as well as damage-induced arrest (Sorensen et al., 2003) whereas the block to CDC25C seems to be checkpoint-specific. In addition to all these mechanisms, CHK1 in *S.pombe* also acts to up-regulate the kinases Wee1 and Mik1 which actually phosphorylate CDK in the first place but it is not known whether a similar mechanism operates in higher eukaryotes (O'Connell et al., 1997). Finally, as well as promoting checkpoint arrest, CHK1 can also promote apoptosis by phosphorylating the p53-related transcription factor p73 (Gonzalez et al., 2003).

Transducing signals for arrest and/or apoptosis appears to be CHK1's principal role. It does not to promote DNA repair directly but may aid other repair mechanisms via the modification of chromatin structure. CHK1 phosphorylates and inhibits Tousled-Like Kinases (TLKs) - proteins that regulate chromatin assembly during S phase (Groth et al., 2003). Consistent with such a role, Rad53 in *S.cerevisiae* monitors free histone levels and promotes their degradation if DNA synthesis is arrested (Gunjan and Verreault, 2003). A deficiency in the *S.pombe* CHK1 homolog, meanwhile, can be suppressed by the overexpression of MSC1, a protein which modifies chromatin structure by recruiting HDACs (Ahmed et al., 2004). Finally, it has also been reported that CHK1 interacts with DNA-PK and stimulates its NHEJ activity *in vitro*, but this is not dependent on CHK1's kinase activity and the mechanism remains unclear (Goudelock et al., 2003).

CHK2

CHK2 is a second IR-activated kinase which has overlapping roles with CHK1. However, CHK2 deficiency gives rise to distinct phenotypes, suggesting only partial redundancy. CHK2 is not essential for viability either in embryogenesis or in adult cells (Hirao et al., 2000; Jack et al., 2002; Takai et al., 2002) nor is it required for normal cell cycle progression (Sorensen et al., 2003). It is, however, linked to the cancer-prone Li Fraumeni syndrome (Bell et al., 1999) and to sporadic breast cancers (Meijers-Heijboer et al., 2002; Vahteristo et al., 2002).

Like CHK1, CHK2 is activated by ATM (and also ATR) but the mechanism of activation is different (Ahn et al., 2000; Matsuoka et al., 2000; Melchionna et al., 2000). Phosphorylation on Thr68 causes CHK2 to oligomerise via its FHA domains, enhancing its autophosphorylation on Thr383 and 387. This relieves CHK2's auto-inhibition and therefore activates it towards its substrates (Ahn et al., 2002; Xu et al., 2002b). Like CHK1, the activating phosphorylation of CHK2 by ATM is at least partially dependent on the adaptors MRN and MDC1, as well as 53BP1, TopBP1/BRCA1 and MSH2. The subsequent positive feedback loop, however, is not dependent on adaptors (Ahn et al., 2002) which may explain why CHK2 activation requires adaptors only at low doses of dsbs: after higher doses, transient interactions with ATM may be sufficient to set the feedback loop in motion.

CHK2 acts on the same sites in CDC25A/C as CHK1 but it contributes only after IR damage and is not active in the normal cell cycle (Sorensen et al., 2003). Instead, CHK2 interacts constitutively with both PML and BRCA1 (Lee et

al., 2000; Yang et al., 2002) which presumably inhibit CHK2 oligomerisation. Only after phosphorylation by ATM does CHK2 phosphorylate both PML and BRCA1 and thus release itself (simultaneously activating BRCA1) (Zhang et al., 2004).

The role of CHK2 in actually enforcing the intra-S and G2 checkpoints remains obscure. If CHK2 is acutely knocked down with siRNA or a dominant negative CHK2, both checkpoints are defective (Falck et al., 2001; Falck et al., 2002) but in established CHK2 knockout cell lines, only the G2 checkpoint is lost (Hirao et al., 2000; Takai et al., 2002). This suggests that CHK1 can somehow evolve to compensate for CHK2's activity on CDC25A, though not on CDC25C. (Since the turnover of CDC25A is required for the normal cell cycle, there may be selective pressure for low CDC25A and/or high CHK1 activity in CHK2 knockout cells). Furthermore, if CHK1 is knocked down, both the S and G2 checkpoints are lost (Gatei et al., 2003; Zhao and Piwnica-Worms, 2001), suggesting that CHK2 cannot enforce either of them alone. Thus CHK2 may only really be needed to amplify checkpoint signalling.

Although CHK2's checkpoint roles overlap extensively with CHK1, CHK2 has non-overlapping targets in p53 and E2F1, both of which promote apoptosis after IR damage. This may explain the CHK2 cancer connection, since a failure to apoptose can lead to the propagation of mutated or unstable genomes. In the absence of BRCA1, for example, CHK2 is not constitutively inhibited and therefore promotes apoptosis in BRCA1 mutant cells (McPherson et al., 2004). If CHK2 was also absent, such cells might survive.

CHK2 acts on Ser 364 of E2F1 (Stevens et al., 2003) and Ser20 of p53 (Chehab et al., 2000; Shieh et al., 2000), although this latter event is very

controversial: activated CHK2 has also been reported *not* to act directly on p53 *in vitro* or *in vivo* (Ahn et al., 2003). Two independent CHK2 knockout mice show defects in p53-mediated apoptosis but one was reported as having normal p53 stabilisation and the other, defective stabilization (Hirao et al., 2000; Takai et al., 2002), while CHK2 knockout cells show normal p53 stabilisation and also normal apoptosis (Jallepalli et al., 2003). In any case, p53 is not required for the intra-S phase checkpoint in adult cells so if it is stabilized by CHK2, it must somehow be directed towards apoptosis rather than cell cycle arrest. In one study, Ser20 phosphorylation has indeed been reported to activate pre-existing p53 for apoptosis but not to actually stabilize p53 (Jack et al., 2002). Further work is certainly required to resolve this issue, as well as to clarify CHK2's apparently variable role in enforcing the S and G2 checkpoints.

SMC1

SMC1 (standing for Structural Maintainance of Chromosomes 1) belongs to an evolutionarily conserved family of proteins with roles in chromosome condensation, sister chromatid cohesion and recombination: functions which SMC1 carries out as a heterodimer with SMC3 (Michaelis et al., 1997) and, in the case of recombination, as part of the large RC-1 complex (Jessberger et al., 1996; Stursberg et al., 1999). SMC1, however, is also an ATM target: it is phosphorylated on Ser 957 and 966 after IR damage and this is required for the intra-S phase checkpoint (Kim et al., 2002b; Yazdi et al., 2002). The phosphorylation of SMC1 absolutely requires NBS1 and probably also BRCA1, although conflicting results have been published regarding BRCA1 (Kim et al., 2002b; Yazdi et al., 2002). This places SMC1 in the class of ATM substrates

which can only be phosphorylated via adaptors in chromatin-bound foci, which would be consistent with SMC1's location on chromosomes rather than in the nucleoplasm (Kitagawa et al., 2004). The mechanism by which phosphorylated SMC1 acts in the S phase checkpoint is not known, but it must have a specific role in preventing DNA synthesis since the G2 checkpoint is unaffected (Kim et al., 2002b). Possibly the phosphorylation of SMC1 modifies its cohesion function, and cohesion has been linked to the proper progress of replication (Toth et al., 1999). Regarding DNA repair, an exact role for SMC1 has again not been established but cells with mutant SMC1 are radiosensitive and show delayed repair of dsbs identical to that seen in AT cells (Kitagawa et al., 2004). Cohesion has previously been shown to be important for postreplicative dsb repair in yeast, so SMC1's repair function may also depend upon its role in cohesion (Sjogren and Nasmyth, 2001). Counter-intuitively, however, it has recently been reported that the *removal* of cohesion by separase is actually required for DNA repair in yeast (Nagao et al., 2004).

c-Abl

c-Abl is a non-receptor tyrosine kinase which associates constitutively with BRCA1 but is released after its phosphorylation by ATM (Foray et al., 2002; Shafman et al., 1997). c-Abl promotes damage-induced apoptosis via p73 (reviewed (Wang, 2000)) but alternatively it can also promote dsb repair by phosphorylating Rad51. This promotes Rad51's interaction with Rad52 and therefore promotes HR, thus c-Abl could be considered a specific transducer of ATM's role in DNA repair (Chen et al., 1999; Kitao and Yuan, 2002).

Effectors of dsb Repair: FANCD2 and BLM

FANCD2

FANCD2 is encoded by one of the seven genes which are linked to the inherited cancer-predisposition syndrome Fanconi anaemia (FA) (reviewed (D'Andrea and Grompe, 2003)). FANCD2 is the only FA determinant conserved through evolution (Liu et al., 2003b; Timmers et al., 2001) and it is the only FA protein established as an ATM target (Taniguchi et al., 2002).

FANCD2 may actually fall into the category of a transducer as well as an effector since it appears to have separable roles in both the S phase checkpoint and DNA repair, although neither role is currently well understood. The checkpoint role is directly dependent on phosphorylation of FANCD2 by ATM (and other PIKKs) on Ser222. The repair role is not, but it is nevertheless indirectly dependent on active PIKKs because FANCD2 must be mono-ubiquitinated in order to promote DNA repair and this requires at least one PIKK, ATR (Andreassen et al., 2004). This is probably because BRCA1/BARD1 – a complex which is dependent on ATM/ATR – is proposed as the ubiquitin ligase.

The checkpoint role of FANCD2 is demonstrated by RDS in cells either lacking FANCD2 or expressing a non-phosphorylatable form, but no actual mechanism has yet been established. The exact mechanism for FANCD2's repair role also remains unclear, but FANCD2 is implicated in the proper repair of both dsbs and DNA crosslinks (which may be repaired via dsb intermediates). FA cells are sensitive to crosslinking agents (D'Andrea and Grompe, 1997) and also, more modestly, to IR (Alter, 2002) and show increased chromosome breakage after exposure to either agent. The mono-ubiquitinated form of FANCD2 is able

to bind to chromatin and form foci at dsbs together with BRCA1 (Garcia-Higuera et al., 2001). This somehow facilitates error-free HR (Tutt and Ashworth, 2002; Tutt et al., 2001) while suppressing the inaccurate recombination and/or end-joining events which occur at high levels in FA cells (Thyagarajan and Campbell, 1997), giving rise to quadriradial chromosomes and deletion mutations (Laquerbe et al., 1999; Papadopoulo et al., 1990). As further circumstantial evidence for a specific role for FANCD2 in HR, BRCA2, which has a known HR function via its binding to Rad51, is mutated in some FA patients and FANCD2 cells show reduced formation of Rad51 foci (Digweed et al., 2002). Despite all this evidence, a clear picture of the interactions between BRCA1, BRCA2 and FANCD2 in dsb repair has yet to emerge.

BLM

BLM in the product of the gene mutated in Bloom's Syndrome, a cancer-prone inherited disease (reviewed (Bachrati and Hickson, 2003)). It is a member of the RecQ family of 3'-5' DNA helicases and is cell-cycle regulated with maximal expression in S/G2. BLM is phosphorylated by ATM on Thr 99 and 122 (Beamish et al., 2002) but this phosphorylation is not apparently required for BLM's helicase function or its recruitment to foci (Davies et al., 2004). Possibly the phosphorylation prevents some modest radiosensitivity seen in BLM cells and also in cells with non-phosphorylatable BLM (Beamish et al., 2002) but the basis of this radiosensitivity remains unclear and it is not reproduced in other reports. Alternatively, phosphorylation may simply stabilize the BLM protein, which accumulates to high levels after IR damage (Ababou et al., 2000).

BLM forms foci together with BRCA1 (Wang et al., 2000), Rad51 (Bischof et al., 2001) and p53 (Sengupta et al., 2003), both in response to DNA damage and also during normal S phases. Its role in these foci is thought to be the promotion of a specific HR pathway which resolves Holliday junctions without sister chromatid exchanges (SCEs), via BLM-mediated branch migration and TopoIIIα–mediated resolution (Wu and Hickson, 2003). As well as promoting this non-crossover repair pathway, BLM may indirectly suppress the competing Rad51-mediated HR reaction, since p53, which binds to BLM, suppresses Rad51-mediated strand exchange (Yoon et al., 2004). In the absence of the BLM pathway cells have very high levels of SCES, elevated numbers of Rad51 foci (Wu et al., 2001) and also increased error-prone NHEJ (Gaymes et al., 2002).

The role of BLM in dsb repair is probably an extension of its constitutive role in normal replication, resolving any DNA secondary structures and/or stalled replication forks. BLM co-localises with a subset of normal replication foci, especially in late S phase (Bischof et al., 2001), and the yeast homolog of BLM, Sgs1, is found by chromatin immunoprecipitation (ChIP) at normal forks (Cobb et al., 2003). Consistent with this, the absence of Xblm in Xenopus extracts leads to DNA breakage during normal replication and BLM cells have dramatic defects in recovering from fork stalling by hydroxyurea (Davies et al., 2004). Furthermore, overall replication timing and replication forks themselves are abnormally slow in BLM cells (Hand and German, 1975; Rassool et al., 2003). Confusingly, yeast Sgs1 mutants have replication forks that actually move *faster* than normal, yet they are still retarded in the rDNA where fork stalling is especially frequent (Versini et al., 2003).

Thus, the role of BLM in DNA damage repair is relatively well established but its role in cell cycle checkpoints is much less clear. BLM cells do have a defective G2 checkpoint after IR (Ababou et al., 2000) and, although no clear data is available on mammalian cells, yeast Sgs1 mutants or *C.elegans* mutants in the BLM homolog him-6 also have a defective intra-S phase checkpoint (Frei and Gasser, 2000; Wicky et al., 2004). Possibly the presence of BLM repair foci could contribute to checkpoint signalling via the association of BRCA1 with BLM foci, but no evidence for such a mechanism has yet been reported.

The end results of ATM pathway: checkpoint arrests and DNA repair

The ATM pathway controls two main aspects of the response to dsbs: arrest of the cell cycle (and/or apoptosis) and dsb repair. The two aspects are closely linked since the cell cycle need only be arrested until DNA repair is complete and apoptosis should probably only occur if damage cannot be repaired. The assembly of foci containing effectors of both arrest and repair – plus the fact that several of the proteins discussed above play both roles at once – is a logical way of ensuring the proper coordination of the whole response to DNA damage. Indeed, a constitutive complex containing many checkpoint and repair proteins, including proteins which are not actually PIKK targets, exists in cells even before the damage-induced foci are assembled (Wang et al., 2000). This BRCA1-associated complex (BASC) may promote the rapid assembly of foci or even the actual sensing of dsbs.

ATM-dependent checkpoints

The ATM pathway is essential for checkpoint arrests in all phases of the cell cycle. The transient ATM-dependent arrest within G2 depends on suppression of CDK1 activity, which prevents the onset of mitosis by inhibiting a complex network of CDK1 targets. Arrest within G1 centres on p53 and operates via inhibition of CDK2/CycE, suppressing the S phase-promoting transcriptional program. Checkpoint arrest within S phase, however, is signalled by a much more complicated network of proteins and the way in which they all collaborate to eventually reduce the rate of DNA synthesis has not yet been fully established.

The evidence already described for checkpoint-competent cells suggests that after IR damage, origin firing is blocked and ongoing forks are also somewhat slowed after very high doses of IR. AT cells do not show the origin firing suppression when examined by DNA size sedimentation (Painter and Young, 1980), nor in a more accurate 2D-gel analysis of origin firing kinetics (Larner et al., 1999). The reduced fork progression after very high IR doses is probably a result of direct fork blockage by ssbs or base damage and this does not apparently activate ATM, though it may activate ATR during S phase.

Downstream of ATM, many targets seem to contribute partially to reducing DNA synthesis. But does the whole ATM network converge on a single mechanism to prevent origin firing? What are the actual effectors of this mechanism? And is this the only way of suppressing replication or can ongoing forks as well as unfired origins be affected? At least two ATM targets, NBS1 and CHK2, have been shown to have partial, additive effects on RDS (Falck et al., 2002) but neither alkaline sucrose sedimentation nor 2D-gel analysis has been applied to replication in NBS1-deficient or CHK2-deficient cells to determine an origin-firing and/or fork progression mechanism.

The only established way in which the ATM network acts on the replication machinery is by preventing CDC45 from loading onto chromatin. ChIP for CDC45 indicated that this response is defective in AT cells (Falck et al., 2002) and the same CDC45 loading pathway has been reconstituted in *Xenopus* extracts, where it is dependent on ATM, CDC25A and CDK2/CycE (Costanzo et al., 2000). The prevention of CDC45 loading could potentially block both origin firing and fork progression since CDC45 acts on MCM2-7, the putative replicative helicase (Masuda et al., 2003), and both travel with the replication fork

(Katou et al., 2003). However, this mechanism has been interpreted so far as a block to origin firing alone. CDC45 was absent from one specific origin sequence in the cell lines tested by ChIP (Falck et al., 2002), while in the *Xenopus* experiments, replication was not actually ongoing at the time of checkpoint activation (Costanzo et al., 2000).

The prevention of CDC45 loading still occurs in MRN-deficient cells (Falck et al., 2002), so it has been proposed that the CHK2-CDC25A-CDK2 pathway specifically controls CDC45 while MRN suppresses DNA synthesis in a different way. However, all this data must be interpreted with caution given the extensive interconnectedness of the ATM network and the use of non-quantitative readouts such as RDS or ChIP in various incomplete knockout or dominant-negative situations. Partial RDS phenotypes may indicate incomplete activation of various components of the ATM network rather than actual separate pathways. In different cell lines, for example, the extent of RDS has been reported to depend on SV-40 transformation, and transformed cells actually have the same level of RDS whether they are NBS-deficient or not (Kraakman-van der Zwet et al., 2001). Therefore, it remains unclear whether MRN genuinely operates in a separate checkpoint mechanism, or whether it just promotes ATM's activation of the Chk kinases.

In mammalian cells, no good evidence exists for any other mechanism for the IR-induced S phase checkpoint besides CDC45 loading. In *S.cerevisiae*, however, another component of the replisome, polα-primase has also been implicated downstream of the CHK2 homolog Rad53 (Marini et al., 1997; Pellicioli et al., 1999) and it is possible that more components will turn out to be checkpoint targets. In addition, the second S phase promoting kinase,

CDC7/DBF4, is targeted by the ATR pathway and a similar role for CDC7/DBF4 downstream of ATM has yet to be rigorously tested.

ATM-dependent DNA repair

Many ATM substrates promote the repair of dsbs by HR and some actively suppress NHEJ, whereas a second PIKK, DNA-PK, has an established role in promoting NHEJ. Thus the two PIKKs may compete for DNA ends with ATM promoting the less error-prone pathway. In DT-40 knockout cells, for example, ATM is largely epistatic with the HR protein Rad54 but is not epistatic with DNA-PK (Morrison et al., 2000).

This division of labour does not, however, explain why AT cells are radiosensitive and retain around 10% of IR-induced dsbs long after control cells have repaired them (Cornforth and Bedford, 1985; Foray et al., 1997). If HR and NHEJ are entirely interchangeable then these breaks should simply be repaired via error-prone NHEJ in the absence of ATM. The unrepaired dsbs are seen even in non-cycling AT cells so they cannot be due to checkpoint failure simply allowing insufficient time for their repair. ATM must therefore have a more fundamental role in repairing certain dsbs and it has recently been shown that although NHEJ can efficiently repair the ends of restriction-digested plasmids, it cannot repair a subset of IR-induced breaks. Rather, the assembly of an ATM focus might be required to recruit DNA processing elements such as MRN to otherwise-irreparable dsbs (Kuhne et al., 2004). Thus the ATM pathway is actually vital for dsb repair as well as cell cycle checkpoints.

The S phase checkpoint: ATR pathway

ATR is the second PIKK with central importance in DNA damage checkpoints. Unlike ATM, ATR is not associated with a well-established disease syndrome and instead it was discovered by searching for genes encoding a catalytic domain similar to that of ATM (Cimprich et al., 1996). More recently, however, ATR knockout mice and cell lines have been characterised and a human disorder associated with reduced levels of ATR expression has been reported.

ATR is essential for viability and knockout mice die early in embryogenesis. Cells from these embryos live for only a few cell cycles before undergoing apoptosis due to widespread chromosome breakage (Brown and Baltimore, 2000) and conditional ATR knockouts made in adult cell lines show a similar phenotype (Cortez et al., 2001). Heterozygous knockout mice show only slightly decreased survival but are cancer-prone, and humans with reduced levels of ATR due to aberrant splicing exhibit a growth and mental retardation syndrome called Seckel syndrome (O'Driscoll et al., 2003). The marked difference between these phenotypes and those of AT patients suggests that ATR plays distinct roles in normal cell proliferation and/or in responding to endogenous DNA damage. In addition, ATR is the PIKK responsible for enforcing the G1 and intra-S phase checkpoints in response exogenous damage by UV or methylating agents (Heffernan, Simpson et al. 2002), but not in response to IR.

Like ATM, ATR is present throughout the cycle, although both ATR and ATM are down-regulated in quiescent cells (Fukao et al., 1999; Jones et al., 2004). ATR, however, exists as a monomer rather than a homodimer, weakly associated with a second protein, ATRIP (Cortez et al., 2001; Unsal-Kacmaz and Sancar, 2004). This ATR-ATRIP interaction is conserved from yeast to man with

ATRIP being the homolog of the yeast Rad3/Mec1 partner Rad26/Ddc2 (also called Lcd1 or Pie1 (Rouse and Jackson, 2000)). ATRIP and ATR are mutually essential for stability and ATRIP is itself a phosphorylation target of ATR (Cortez et al., 2001) but the two proteins do not dissociate in response to UV damage (Unsal-Kacmaz and Sancar, 2004). and the *in vitro* kinase activity of ATR is not increased after either UV or IR treatment so DNA damage must activate ATR differently to ATM. It appears that, like ATM, ATR is activated towards its substrates when bound to DNA (Guo et al., 2000; Hekmat-Nejad et al., 2000), although it remains unclear whether the process of DNA binding actually activates ATR or whether a pre-activated form selectively binds to DNA. Either way, ATR is also bound to chromatin in undamaged cells and the overall binding is not increased after UV damage (Bomgarden et al., 2004; Dart et al., 2004), so ATR activation must require more than just DNA binding. ATR also forms foci in response to UV, MMS and replication stalling (Tibbetts et al., 2000a) and these foci are probably the crucial factor in promoting ATR activity via tighter, more concentrated DNA binding and/or association with adaptor proteins. Thus there are clear parallels with ATM foci at dsbs, but ATR foci can form in response to apparently disparate DNA damaging agents.

Several recent studies suggest that single stranded DNA (ssDNA) is the common factor generated by all ATR-activating agents, and that the ssDNA binding protein RPA is essential for ATR recruitment. SsDNA, RPA and Rad51 can all be specifically detected within damage-induced ATR foci (Raderschall et al., 1999). A functional role for the RPA in these foci is supported by the fact that its absence abrogates focus formation after both UV and high-dose IR (Barr et al., 2003; Zou et al., 2003), and also abrogates ATR's loading onto chromatin in

Xenopus extracts treated with aphidicolin (You et al., 2002). A distinct form of UV-induced DNA binding that is stronger than ATR's association with undamaged DNA has been detected (Unsal-Kacmaz et al., 2002) and again, this depends on RPA. RPA appears to recruit ATR via an interaction with its binding partner ATRIP (Rouse and Jackson, 2002; Unsal-Kacmaz and Sancar, 2004; Zou and Elledge, 2003).

Conflicting data has been published on whether or not ongoing replication is actually required to activate ATR: the confusion may result from the fact that most forms of DNA damage only generate large amounts of ssDNA when they are processed via a replication fork. Thus UV and MMS damage only cause an S phase checkpoint arrest in the *Xenopus* system if DNA is allow to replicate (Lupardus et al., 2002; Stokes et al., 2002). Similarly, in human cells, UV lesions specifically induced in late-replicating DNA do not trigger the checkpoint until the progress of S phase reaches them (Hamlin, 1978). By contrast, etoposide-induced DNA breaks which can be processed nucleolytically into long stretches of ssDNA can activate the checkpoint in *Xenopus* extracts without replication being required (Costanzo et al., 2003). This situation may be unique to etoposide-induced dsbs because IR-induced ATR foci in human cells *are* largely S-phase dependent (Barr et al., 2003): perhaps IR-induced dsbs are not readily processed into sufficient amounts of ssDNA.

Even outside S phase, the repair of UV-induced lesions by NER does generate ssDNA patches of about 30 nucleotides but it is unclear whether these are enough to recruit ATR or not: ATR-dependent H2AX phosphorylation has been reported in G1 (O'Driscoll et al., 2003) but detectable ATR foci are not formed (Ward et al., 2004a). Possibly, like ATM, ATR can act to some extent via

transient associations with short patches of ssDNA, although it only becomes stably bound to larger stretches. The collision of replication forks with UV lesions during S phase is proposed to generate much larger stretches of ssDNA (Ward et al., 2004a).

Consistent with the idea that the sensing of ssDNA by ATR is dose-dependent, ATRIP recruitment to RPA-coated oligonucleotides is most efficient with 50-75nt oligomers binding at least two RPA molecules - possibly there is an element of cooperativity in ATR/ATRIP recruitment to RPA. Nevertheless, several reports suggest that the presence of ssDNA alone is not enough to recruit ATR since large quantities of ssDNA added to *Xenopus* extracts do not necessarily activate the checkpoint. Instead, the presence of replication bubbles in dsDNA, complete with RNA primers, is apparently required (Michael et al., 2000; Stokes et al., 2002). Further work is clearly still needed to elucidate the exact mechanism of ATR activation.

Accepting that ssDNA is the signal for ATR activation, RPA and ATRIP may play analogous roles to H2AX and MRN in ATM activation. In such a model, both ATR and ATM must collaborate with several sensor/adaptor proteins (which subsequently become PIKK targets themselves) in order to sense DNA damage and form functional checkpoint-activating foci.

The ATR pathway: substrates of ATR

A distinct 'ATR pathway' is difficult to define because ATR shares the same S/Q-T target motif as ATM (Kim et al., 1999) and accordingly acts on many of the same substrates. ATR has dedicated co-sensors in RAD17 and HUS1/RAD1/RAD9 (9-1-1), and it also has a specific adaptor protein, claspin. However, most of the other adaptors and downstream transducers of checkpoint signalling are shared by both PIKKs. In addition, although ATM responds principally to dsbs and ATR to replication-blocking damage, there is considerable cross-talk between ATR and ATM. Either form of DNA damage can be processed into a form that can activate the other kinase: blocked replication forks can generate dsbs (Furuta et al., 2003; Lundin et al., 2002; Strumberg et al., 2000) and dsbs may be processed into stretches of ssDNA (Costanzo et al., 2003; Maringele and Lydall, 2002). The resultant crosstalk between ATR and ATM has been examined in some detail after IR damage: since the G2 arrest which eventually occurs in an irradiated population is enforced by the two kinases with different kinetics, crosstalk can be dissected using the appropriate knockout cells (Xu et al., 2002a). IR damage *within* G2 only activates ATM, which only enforces a transient G2 arrest, (Beamish et al., 1996). IR damage during S phase, however, leads to an additional, delayed accumulation of cells in G2 and this is ATR/CHK1-dependent. This second G2 arrest is also dose-dependent and is further exacerbated by the lack of ATM, suggesting that in the absence of ATM, more dsbs get processed into ATR-activating forms and that ATR is more strongly activated the more DNA damage it encounters. Indeed, ATM-deficient cells accumulate in G2 with a hyper-activated ATR/CHK1 response (Wang et al., 2003a). Even in the presence of ATM, ATR retains its role in this G2 checkpoint

because a kinase-dead ATR can exert a dominant-negative effect on the G2 arrest in wild-type cells (Cliby et al., 1998). Crosstalk also operates in the other direction: when cells are damaged with UV, if RAD17 is knocked out so that ATR cannot be activated then CHK2 is phosphorylated at a much higher level suggesting that the UV damage is processed into ATM/CHK2-activating dsbs (Wang et al., 2003b).

In addition to this cooperation between ATR and ATM in the G2 checkpoint, ATR has been reported to contribute to the IR-induced intra-S phase checkpoint in ATM-/- cells as well (Cliby et al., 1998). Here, the lack of ATM presumably reveals ATR's activation within S phase in response to nucleolytically-processed dsbs and to the IR-induced base damage or ssbs that can lead to stalled forks and replication-associated breaks. It is less clear, however, whether ATR still contributes to the IR-induced intra-S phase checkpoint if ATM is *not* knocked out – perhaps in this situation, any acute activation of ATR is redundant and ATR only becomes important at later times when ATM has ceased to be active but residual DNA damage may remain. In conclusion, only within G1 does it seem relatively clear that ATR acts after UV damage and ATM after IR damage.

ATR-specific substrates: RPA, Rad17, the 9-1-1 complex and claspin

RPA

RPA has essential roles in replication and also in many forms of DNA repair, including HR, NER, BER and MMR (reviewed (Binz et al., 2004)). RPA is a heterotrimer of 70, 32 and 14kDa subunits. The 32kDa subunit is extensively phosphorylated by PIKKs after DNA damage (Carty et al., 1994; Zernik-Kobak et al., 1997), as well as by CDKs during G1/S and M phase of the normal cell cycle (Din et al., 1990; Dutta and Stillman, 1992; Fang and Newport, 1993). Damage-induced phosphorylation of RPA is probably carried out by ATR in response to IR, UV and stalled replication (Barr et al., 2003; Brush et al., 1996) and RPA is also phosphorylated by DNA-PK and ATM (Oakley et al., 2001; Shao et al., 1999). RPA phosphorylation is not apparently needed for checkpoint signalling via ATM (Morgan and Kastan, 1997) and it actually causes RPA to dissociate from ATM and DNA-PK (Oakley et al., 2003); by contrast, the phosphorylation clearly promotes ATR-induced checkpoint signalling.

As discussed above, RPA acts upstream of ATR in the initial recruitment of ATR to ssDNA. The subsequent phosphorylation of RPA then promotes focus formation and activates ATR towards its substrates: ATR's kinase activity is needed for the formation of visible RPA foci and also for ATR's own recruitment to these foci (Barr et al., 2003; Dart et al., 2004; Kobayashi et al., 2002b). Thus phosphorylated RPA probably acts like γ–H2AX in a positive feedback loop – RPA recruits ATR, gets phosphorylated and then recruits more ATR, and also more RPA, in an iterative process. RPA is also needed to recruit Rad17 and the

9-1-1 complex to ssDNA (Zou et al., 2003), further contributing to ATR activation.

In yeast, phosphorylated RPA is functionally implicated in checkpoint arrests in G1/S, intra-S and G2 (Lee et al., 1998; Longhese et al., 1996; Pellicioli et al., 2001) and an RPA mutant which is defective in Ddc2 recruitment to DNA is also specifically checkpoint-defective. In mammalian cells such mutants have not been reported but RPA *is* needed for CHK1 activation and therefore presumably for checkpoint signalling (Zou and Elledge, 2003). In addition, the phosphorylation of RPA directly inhibits its function in replication (Liu et al., 2000a; Wang et al., 1999) by reducing its association with replication proteins such as polα, its affinity for dsDNA and its visible co-localisation with replication foci *in vivo* (Oakley et al., 2003; Vassin et al., 2004). An RPA mutant mimicking hyper-phosphorylation does, however, still associate with DNA damage foci (Vassin et al., 2004). Thus, phosphorylation of RPA by PIKKs may alter the balance of RPA activity between replication and DNA repair.

RAD17

RAD17 is an evolutionarily conserved checkpoint protein (Rad24 in *S.cerevisiae*) which has homology to all five subunits of the RFC clamp-loader complex (Venclovas and Thelen, 2000). It replaces the large RFC1 subunit of this complex (Green et al., 2000; Griffith et al., 2002; Lindsey-Boltz et al., 2001), forming an alternative pentameric clamp-loader which interacts with the 9-1-1 complex in an analogous way to RFC/PCNA (Rauen et al., 2000). Functionally, it loads the 9-1-1 complex onto DNA both *in vitro* (Bermudez et al., 2003; Zou et al., 2003) and *in vivo,* in a damage-dependent manner (Zou et al., 2002). Unlike

RFC, which loads PCNA at the 3' primer-template junction, RAD17 can bind to gapped, nicked and primed DNA structures and it loads 9-1-1 at both 3' and 5' junctions – features that would be necessary for damage recognition.

RAD17 is present on DNA throughout the cell cycle, probably at sites of ssDNA since it is preferentially associated with replication foci and its binding to DNA is stimulated by RPA (Post et al., 2003; Zou et al., 2003). RAD17 is also phosphorylated to some degree in undamaged S phases but its phosphorylation (on Ser 635 and 645) is greatly increased after UV and IR damage (Bao et al., 2001; Post et al., 2001). Both ATR and ATM phosphorylate RAD17 but RAD17 has no detectable role in promoting ATM's activity (Ali et al., 2004; Jones et al., 2003) whereas it does enable ATR to act on CHK1. There is some confusion as to whether RAD17 must be phosphorylated by ATR before it can perform its function of recruiting 9-1-1: Bao et al. state that if the target residues ser635 and ser645 are mutated, then RAD17 no longer associates with 9-1-1 (Bao et al., 2001), however, this may be an indirect effect of mutating the RAD17 protein because Zou et al report that RAD17 can recruit 9-1-1 to DNA independently of ATR and in fact, 9-1-1 must already be present to enable ATR to phosphorylate RAD17 (Zou et al., 2002).

The essential function of RAD17 in checkpoint signalling is supported by the fact that knockout or dominant-negative RAD17 abrogates G2 arrest and CHK1 phosphorylation on Ser345. Conversely, RAD17 overexpression causes ectopic arrests in G1 and G2 (Bao et al., 1998; Li et al., 1999; Wang et al., 2003b). RAD17 is also essential for controlling endogenous DNA damage and/or normal replication because knockout cells develop multiple chromosome

aberrations and die within a few cell cycles (Wang et al., 2003b). Furthermore, they show partial endoreduplication but the reason for this is not yet clear.

HUS1/RAD9/RAD1

HUS1, RAD9 and RAD1 (Kostrub et al., 1998; Lieberman et al., 1996; Parker et al., 1998) form the PCNA-like complex (Burtelow et al., 2001) which is loaded onto damaged DNA by RAD17. Like RAD17, HUS1 associates constitutively with replicating DNA (You et al., 2002) during S phase but it is additionally recruited after UV or IR damage in any phase of the cell cycle (Komatsu et al., 2000b; Roos-Mattjus et al., 2002), co-localising with γ-H2AX foci (Greer et al., 2003).

RAD9 is then phosphorylated by both ATR and ATM on its C terminal tail and this allows the 9-1-1 complex to act as an adaptor for CHK1 activation by either PIKK kinase (Chen et al., 2001; St Onge et al., 2003). The actual mechanics of this are not known and in fact, the site in RAD9 which is crucial for CHK1 activation is not an S/Q-T site, suggesting that other kinases provide an extra layer of regulation (Roos-Mattjus et al., 2003). Furthermore, in *S.pombe*, a second adaptor Rad4 (TopBP1), is needed to bring CHK1 into contact with Rad3 (ATR) (Furuya et al., 2004). Thus, the general model of multiple co-dependent adaptors all facilitating PIKK action on downstream targets appears to apply to ATR as well as ATM. Consistent with a central role for 9-1-1 in this network, HUS1 knockout mice show embryonic lethality and, if cell death is prevented by knocking out p21, the surviving MEFs are sensitive to HU and UV and specifically defective in the S phase checkpoint response to blocked replication (Weiss et al., 2000; Weiss et al., 2003; Weiss et al., 2002).

In addition to checkpoint signalling, the 9-1-1 complex has roles in both apoptosis and DNA repair. Rad9 associates with and sequesters the anti-apoptotic proteins BCL2 and BCLx (Komatsu et al., 2000a) and when c-ABL is activated by DNA damage, c-ABL phosphorylates RAD9 and the sequestering of BCLx increases, promoting apoptosis (Yoshida et al., 2002).

With regard to DNA repair, a role for 9-1-1 in up-regulating translesion bypass has been reported in *S.pombe:* 9-1-1 up-regulates transcription of the translesion polymerase DinB and also recruits DinB to damaged DNA (Kai and Wang, 2003). Although this is not yet reproduced in higher eukaryotes, HUS1 knockout cells do show reduced progression of replication forks after very high doses of IR, which could be due to a failure to bypass IR-induced base damage (Wang et al., 2004). Finally, the RAD1 component of the 9-1-1 complex has 3'-5' exonuclease activity (Bessho and Sancar, 2000) and is required to process dsbs into ssDNA (Lydall and Weinert, 1995). Therefore it is implicated in both the production of the ATR signal and also potentially in dsb repair.

Claspin

Claspin was recently identified in *Xenopus laevis* as a CHK1 binding protein specifically required for CHK1, but not CHK2, activation (Jones et al., 2003; Kumagai and Dunphy, 2000). It is conserved in mammalian cells and is probably the homolog of yeast Mrc1 (Alcasabas et al., 2001). Claspin binds to both ATR and RAD9 and is then phosphorylated by ATR, an event absolutely required for CHK1 activation (Chini and Chen, 2003). Claspin probably acts as a physical mediator between ATR and CHK1 similarly to MDC1 between ATM and CHK2, since in *S.pombe*, Mrc1 recruits the Chk kinase via its FHA domain

(Tanaka and Russell, 2004). In addition, claspin binds to BRCA1 and facilitates BRCA1's phosphorylation by ATR as well: a second event required for CHK1 activation (Lin et al., 2004a). ATR, 9-1-1, claspin and BRCA1 are all recruited to DNA independently and checkpoint signalling only occurs if all four of them are present so this could ensure four-fold against inappropriate activation of the checkpoint. Although the same mediator role for claspin in CHK1 activation downstream of ATM has not been reported, siRNA knockdown of claspin causes RDS and cellular sensitivity to IR (Lin et al., 2004a) as well as UV (Chini and Chen, 2003), so claspin might overlap somewhat with the other adaptors that mediate CHK1 phosphorylation by ATM.

In contrast to ATR and 9-1-1, claspin is specifically associated with replication forks throughout normal replication: it is maximally expressed in S/G2 and is loaded onto DNA at the origin-firing stage (Lee et al., 2003; Osborn and Elledge, 2003). It then travels with the replisome and halts at sites of stalled replication, at least in the case of Mrc1 in yeast Katou et al., 2003(Kataoka et al., 1984; Osborn and Elledge, 2003). This signals to the checkpoint and simultaneously stabilizes the stalled replisome: without Mrc1 (and a second protein Tof1) CDC45 moves progressively onwards, perhaps indicating DNA unwinding that is uncoupled from actual replication. This replication 'monitoring' role has not been directly observed in higher organisms but human claspin has been purified as a ring-shaped molecule with a high affinity for branched DNA structures – appropriate features for a fork-associated protein (Sar et al., 2004).

Finally, claspin may be the key to turning the checkpoint off as well as on: once claspin becomes phosphorylated by ATR, a second kinase Plx1 begins to act

on a second site within the protein, causing claspin to dissociate from DNA and terminating the checkpoint response, apparently in a continuing aphidicolin arrest (Yoo et al., 2004). The *S.cerevisiae* Polo-like kinase, Cdc5, is also required to turn off the checkpoint although it has not been reported to do this by phosphorylating Mrc1 (Toczyski et al., 1997). Checkpoint turn-off when DNA damage cannot be repaired is termed adaptation in yeast but it remains unclear whether it really occurs in metazoans such as *Xenopus*. Plk in both *Xenopus* and human cells is normally down-regulated after DNA damage (Smits et al., 2000) and why it should start to act again in the presence of unrepaired damage is not known.

In addition to its role in checkpoint signalling, claspin may have a somewhat obscure role in promoting normal replication. The overexpression of human claspin increases cell proliferation and is detected in several cancer cell lines (Lin et al., 2004a) whereas yeast Mrc1 mutants have a slow S phase that is genetically separable from the checkpoint signalling role of Mrc1 (Osborn and Elledge, 2003). This would apparently conflict with reports that overexpression or inhibition of CHK1 results in exactly the opposite phenotypes - blocked or increased origin firing respectively (Heffernan et al., 2002; Shechter et al., 2004). Perhaps claspin has an independent role in promoting fork progression rather than origin firing and this might be the dominant effect when overall DNA synthesis is assayed. No change in the progression rate of early-fired forks was observed in ChIP experiments with yeast Mrc1 mutants (Katou et al., 2003), but perhaps such a change would only be seen later in S phase when forks might encounter more problems.

Shared substrates of ATM and ATR

p53

ATR activates the same p53-dependent G1 checkpoint as ATM (Hall-Jackson et al., 1999; Lakin et al., 1999; Tibbetts et al., 1999) although ATR does not apparently activate CHK2 so p53 is phosphorylated only on Ser15, not Ser20. How ATR is activated towards p53 in G1 remains unclear since there is conflicting evidence on the formation of UV-induced ATR foci in G1 cells. However, it possible that, like ATM, ATR can act on p53 without focus formation, via only transient interactions at the relatively short ssDNA patches generated during NER.

A second UV-induced method of G1 arrest has also been reported, operating exclusively after high UV doses (Bendjennat et al., 2003). In this method, ATR induces ubiquitin-mediated p21 degradation rather then p21 stabilization via p53, yet this still results in G1 arrest by inducing inhibitory phosphorylation of CDK2. It has been proposed that this second, UV-specific method of G1 arrest is necessary because p21 inhibits PCNA and PCNA is needed for the synthesis of NER repair patches. p21 must therefore be degraded rather than stabilised after UV damage in order to allow DNA repair by NER.

H2AX

H2AX is phosphorylated by ATR after UV damage, HU arrest and replication-induced dsbs (Furuta et al., 2003; Ward and Chen, 2001). This does not require the co-sensor 9-1-1 complex so H2AX phosphorylation may be an

early event promoting the formation of ATR foci as well as ATM foci. It is not clear, however, whether γ–H2AX is absolutely required for ATR foci: some reports show only limited ATR/γ–H2AX co-localisation (Barr et al., 2003) but on the other hand, ATR foci are not seen at all in H2AX knockout cells (Ward et al., 2004a) so γ–H2AX is probably one of the conserved signals for focus formation by PIKKs.

NBS1

NBS1 is phosphorylated by ATR in response to UV (O'Driscoll et al., 2003) and also DNA crosslinks (Pichierri and Rosselli, 2004). In contrast to its role in the ATM pathway, the MRN complex does not appear to act as a DNA damage sensor or activator of ATR (Carson et al., 2003; Pichierri and Rosselli, 2004), however, it does still act as an adaptor. Phosphorylated NBS1 is required for the S phase checkpoint in response to DNA crosslinks because the lack of NBS1 leads to a partial RDS-like phenotype. As was reported by Falck et al ('02) in their studies of the ATM pathway, this NBS1 pathway is additive with the reduction in DNA synthesis induced by the lack of the CHK kinases. The NBS1 branch of checkpoint signalling – at least in response to crosslinks - operates instead via FANCD2 with NBS1 acting as an adaptor for FANCD2 phosphorylation in ATR foci. Furthermore, ATR is directly required to activate FANCD2 for DNA repair by monoubiquitination (Andreassen et al., 2004). Unfortunately, the exact role of FANCD2 in the S phase checkpoint remains unknown.

MDC1

MDC1 phosphorylation occurs after UV and HU as well as after IR and, although ATR-dependent phosphorylation has not actually been demonstrated *in vivo*, ATR can act on MDC1 *in vitro* (Stewart et al., 2003). A functional role for MDC1 downstream of ATR has not been reported, but it might act like MRN as another adaptor in the ATR pathway.

BRCA1

BRCA1 is another adaptor protein shared by ATM and ATR: it is phosphorylated by ATR and forms ATR-co-localising foci (Tibbetts et al., 2000b). These are not required for the recruitment or phosphorylation of the co-sensors RAD17 and the 9-1-1 complex but they are essential for ATR to activate its downstream targets including CHK1 and probably also SMC1 (Kim et al., 2002b; Yarden et al., 2002).

TopBP1

TopBP1 is another shared adaptor protein which is phosphorylated independently of ATM and therefore presumably by ATR in response to blocked replication (Yamane et al., 2002). In *Xenopus,* TopBP1 is required as an adaptor to recruit both ATR and the 9-1-1 complex to chromatin – the recruitment of 9-1-1 probably occurring via TopBP1 binding to RAD9 (Greer et al., 2003). This implicates TopBP1 as an early sensor of DNA damage but by contrast, the *S.pombe* homolog of TopBP1, Rad4, only binds to Rad9 if Rad9 has first been phosphorylated by Rad3 (Furuya et al., 2004), so it is not altogether clear whether TopBP1 is recruited to DNA upstream or downstream of the 9-1-1 complex.

Nevertheless, stable assembly of ATR foci probably depends on interactions between all three proteins and the downstream result – CHK1 activation - requires TopBP1 (Parrilla-Castellar and Karnitz, 2003).

MSH2

MSH2 plays its sensor/adaptor role in the ATR pathway differently to the way it acts in the ATM pathway (Wang and Qin, 2003). MSH2 binds to ATR and could therefore target ATR to methylated DNA which requires mismatch repair. MSH2 thus mediates the phosphorylation of CHK1 and SMC1 by ATR in response to methylation damage. The functional importance of MSH2 as an adaptor is supported by an S phase checkpoint deficiency in MSH2-knockdown cells, although an alternative explanation for this could be that active MMR physically blocks the progress of DNA replication.

CHK1

ATR activates CHK1 as the principal transducer of the S and G2 checkpoints after UV damage or replication stalling (Feijoo et al., 2001; Guo et al., 2000; Hekmat-Nejad et al., 2000). Whilst CHK activation by ATM depends only partially on adaptor proteins, CHK1 activation by ATR absolutely requires RAD17, the 9-1-1 complex and claspin (Kumagai and Dunphy, 2000; Zou and Elledge, 2001). There may also be a sensor or adaptor role for the pre-RC component CDC6, since CDC6 is required for CHK1 activation at stalled forks both in *S.pombe* and in mammalian cells (Murakami et al., 2002; Oehlmann et al., 2004). Furthermore, overexpression of CDC6 can enforce a G2 arrest via CHK1 independently of ATR, although how it does this remains unclear (Clay-Farrace et

al., 2003). In addition to its role in checkpoint signalling, CHK1 may be important for maintaining replication fork stability when replication is stalled (Feijoo et al., 2001; Zachos et al., 2003) (discussed in more detail later).

CHK2

CHK2 is activated independently of ATM in response to UV or stalled replication, so ATR is presumably responsible (Feijoo et al., 2001; Matsuoka et al., 2000). However, the functional significance of this activation is unclear. CHK1 alone can enforce the S phase checkpoint and consistent with this, the down-regulation of CHK1 activity correlates with the resumption of DNA synthesis, whereas CHK2 remains active after the checkpoint arrest has ceased (Feijoo et al., 2001). This suggests that CHK2 is not actually functional in the S phase checkpoint response to stalled replication.

SMC1

SMC1 is phosphorylated by ATR in response to methylation damage and uniquely, this is independent of RAD17 and RPA. This suggests that the adaptor requirements for the ATR-SMC1 interaction are different to the requirements for CHK1 activation. SMC1 is constitutively chromatin-bound and ATR can be targeted to methylation-damaged DNA via MSH2 – therefore RPA on ssDNA may not be required to sense this particular form of DNA damage and recruit either ATR or SMC1. The same would not apply to CHK1 activation after methylation damage because CHK1 is not DNA-bound, and indeed this still requires RAD17 and RPA. This supports a model in which ATR/substrate co-localisation alone determines ATR activity (Wang and Qin, 2003).

As to the actual function of SMC1, it is unclear whether SMC1 plays the same role in the S phase checkpoint downstream of ATR which it has been attributed downstream of ATM. SMC1 is, however, required for cell survival, suggesting an as-yet-unknown role in DNA repair (Wang and Qin, 2003).

BLM

BLM is phosphorylated by ATR in a RAD17-dependent manner when replication is stalled by HU (Davies et al., 2004). This facilitates the recovery of cells from an HU arrest, perhaps by allowing stalled forks to restart or by processing the stalled replication structures so that S phase can be properly finished. Without BLM, bulk DNA synthesis is still completed but cells arrest in G2, suggesting that some unreplicated DNA or aberrant structures remain in the genome. It is not clear how phosphorylation by ATR activates BLM but it is not simply by recruiting the helicase to stalled forks because a phosphorylation-site mutant of BLM is still recruited to γ-H2AX foci. In contrast to the ATM-dependent S and G2 checkpoints, BLM has not been reported to act in ATR-dependent checkpoint arrest. However, it clearly has important ATR-dependent roles in DNA repair and/or fork stability.

The end results of ATR pathway: Checkpoint arrests, DNA repair and fork stabilisation

ATR-dependent checkpoints

The ATR pathway enforces cell cycle checkpoints using very similar tranducers to those used by the ATM pathway. Some of the effector mechanisms are probably also shared but there is evidence for additional, specific effectors downstream of ATR.

The mechanisms already described for the G1 and G2 checkpoints apply to both PIKKs. (In addition, ATR may activate a second distinct mechanism for the G1 checkpoint after UV damage, as discussed above (Bendjennat et al., 2003).) As for the S phase checkpoint, suppression of DNA synthesis clearly occurs downstream of ATR as well as ATM. Early evidence for reduced DNA synthesis after UV damage came from alkaline sucrose sedimentation of UV- or MMS-damaged DNA (Painter, 1977; Painter, 1985b), showing that origin firing is suppressed and fork elongation is also reduced. These reports suggested that both phenomena depended on ATM but subsequent more detailed analyses implicated ATR and CHK1 but not ATM or NBS1 (Heffernan et al., 2002). In a different approach, immunofluorescent labelling of early and late replication patterns in CHO cells showed that if replication at early sites is stalled by aphidicolin, later sites do not synthesise DNA, i.e. origin firing is blocked, but the inactivation of ATR or CHK1 allowed late replication foci to appear (Dimitrova and Gilbert, 2000b). ATR and CHK1 therefore clearly enforce a block to origin firing as part of the S phase checkpoint.

Any role for ATR in the slowing of fork progression is less clear. In yeast, fork slowing by MMS damage is independent of Mec1 and Rad53 (Tercero and Diffley, 2001), while in mammalian cells any dependence has not been rigorously tested. Heffernan et al (2002) showed that origin firing is blocked at a lower level of UV damage than that which slows fork movement, suggesting at least that the thresholds for the two phenomena are different. This study also showed that fork progression depended on functional NER of UV lesions. A DNA fibre analysis of replication-slowing through UV or cisplatin lesions demonstrated that the HR proteins Rad51 and XRCC3 also modulate fork rate - unlike NER, HR actually slows forks down – but again, any ATR/CHK1 involvement in fork slowing was not assessed (Henry-Mowatt et al., 2003).

Returning to a mechanism for the established block to origin firing, the modulation of CDC45 loading remains a conserved mechanism for enforcing this block but it may be executed differently downstream of ATR and ATM. In the *Xenopus* extract system, ATR activation causes the second S phase promoting kinase, CDC7, to dissociate from DBF4 and this too prevents CDC45 loading (Costanzo et al., 2003). CDC7/DBF4 is required for replication throughout S phase as well as for S phase onset and is proposed to act on origins in a sequential way to enforce the temporal program of origin firing (Bousset and Diffley, 1998; Donaldson et al., 1998a; Walter, 2000). Thus it makes sense that this kinase should be targeted in order to prevent late origin from firing when S phase is already ongoing. It is not yet known which of the checkpoint proteins actually causes DBF4 to dissociate from CDC7 but DBF4 gets phosphorylated both in yeast, by Rad53, and also in *Xenopus* (Furukohri et al., 2003), where CHK1 is probably responsible.

A second DBF4-like protein DRF1 has recently been discovered in higher eukaryotes and this may take the place of DBF4 upon checkpoint activation, perhaps actively inhibiting CDC7 as well as displacing the activating subunit DBF4 (Yanow et al., 2003). This active-inhibition model remains speculative, but it might explain why adding extra CDK2 to arrested chromatin does not override the CDC7-induced checkpoint (Costanzo et al., 2000)). Indeed, the whole balance between the two checkpoint-enforcing pathways CDK2/CycE and CDC7/DBF4 remains an unresolved issue. In *Xenopus* extracts, dsbs, which activate ATM, lead to only CDK2 inhibition (Costanzo et al., 2000) whereas etoposide treatment, activating ATR, acts exclusively through CDC7/DBF4 (Costanzo and Gautier, 2003). One possibility is that in the *Xenopus* system, ATM-activated CHK2 acts only on CDK2/CycE whereas ATR-activated CHK1 acts only on CDC7/DBF4. In mammalian cells, however, crosstalk between the CHK kinases is apparently more extensive than it is in *Xenopus* (Sorensen et al., 2003). Alternatively, the diffusible tranducer CHK2 may act most efficiently on diffusible nucleoplasmic targets like CDC25A and CDK2 whereas CHK1, bound within ATR foci, may only act efficiently on the chromatin-associated CDC7/DBF4. Again, this is unlikely to be the full explanation since CHK1 must act *in trans* at least to some degree to suppress unfired origins. None of these data have yet been reproduced in mammalian cells and nor has the effect of CDC45 on replication elongation been investigated. However, it seems likely that the control of CDC45 loading is the mechanism for blocking origin firing, at least in *Xenopus* extracts.

A second mechanism for the ATR-induced S phase checkpoint involves direct phosphorylation of the MCMs. It is again unclear whether this affects origin firing, fork progression or both, but in addition to the activating

phosphorylation of MCM2 by CDC7/DBF4, MCM4 in human cells is phosphorylated in an ATR-CHK1-CDK2 dependent manner after replication stalling. This phosphorylation inhibits MCM4's helicase activity (Ishimi et al., 2003). It has also been proposed that when CDC7 becomes bound to DRF1 it could inhibit MCM2-7 either by inhibitory phosphorylations or by the loss of the activating ones (Yanow et al., 2003). The direct action of both ATR and ATM on MCMs 2 and 3 has also been reported (Cortez et al., 2004).

Finally, a few reports have been published of S phase checkpoints that are independent of both ATR and ATM, although mechanisms for most of these are lacking. For example, primary cell lines exhibit a caffeine-insensitive (PIKK-independent) G2 arrest via decreased transcription of cyclin B (Florensa et al., 2003). CDC6 appears to monitor replication throughout S phase in order to activate CHK1 at stalled forks independently of ATR, providing a possible mechanism for a caffeine-insensitive checkpoint (Clay-Farrace et al., 2003). How CDC6 actually activates CHK1, however, remains unclear. Most importantly, Brown and Baltimore reported a functional S phase checkpoint in ATR/ATM double-knockout cells. This may be explained by the recent discovery of a third checkpoint-activating PIKK, hSMG-1 (Brumbaugh et al., 2004). hSMG-1 shares the S/T-Q target motif of ATR and ATM and contributes to p53 stabilisation after IR damage, although it does not act on CHK2, suggesting only partially overlapping substrate specificities. hSMG-1 also has a second role in activating hUlf1, a component of the nonsense-mediated mRNA decay machinery (NMD). This activation of hUlf1 is IR-induced and ATM also contributes to it but the significance of NMD in the response to IR damage has yet to be fully investigated.

ATR-dependent DNA repair and fork restart

With regard to DNA repair, ATR has been implicated in certain repair mechanisms but there is less evidence for a crucial role for ATR in repairing UV or alkylation damage than there is for ATM in dsb repair.

ATR recruits and phosphorylates FANCD2 at DNA crosslinks, suppressing radiosensitivity and presumably promoting crosslink repair (Pichierri and Rosselli, 2004). ATR may also indirectly promote NER by causing p21 degradation (Bendjennat et al., 2003) and may promote the bypass of UV lesions by translesion polymerases (Yamada et al., 2003), probably via the activation of the 9-1-1 complex. Much stronger data exists, however, on the requirement for ATR in the resumption of DNA synthesis after fork stalling. This could be considered to prevent the generation of DNA damage before it occurs.

Studies in yeast have led to a model in which Mec1/Rad53 signalling stabilises stalled forks and allows them to restart when the block to replication is released. Stalled replication forks have actually been visualised in the yeast genome (Sogo et al., 2002) and in the absence of Mec1/Rad53, they degenerate into aberrant structures containing large amounts of ssDNA and regressed 'chicken feet'. These events are proposed to render forks terminally stalled. By contrast, the 'stable' forks observed in the presence of Mec1/Rad53 must be able to resume replication because new origin firing is not needed to complete S phase after HU release (Tercero et al., 2003). Indeed, specific stalled fork structures seen on 2D gels do disappear after HU release, coincident with the deactivation of Rad53, strongly suggesting that these forks do resume replication (Lopes et al., 2001a).

In mammalian cells, evidence for a similar role for ATR/CHK1 is still circumstantial: stalled forks have not been directly observed and fork restart cannot be guaranteed since early origins are not able to replicate the whole genome (Kim et al., 2002a). However, ATR-dependent fork restart has been inferred because when cells are released from an aphidicolin arrest, BrdU incorporation resumes at the same replication foci and this is dependent on ATR and CHK1 (Dimitrova and Gilbert, 2000a; Zachos et al., 2003).

The checkpoint-mediated factor which actually stabilises stalled forks remains elusive but Mrc1 (claspin) and the yeast protein Tof1 seem to mediate stable fork stalling, at least in yeast. In their absence, the replisome moves progressively away from the site of stalling, perhaps indicating DNA unwinding that is uncoupled from actual replication (Katou et al., 2003). Similarly, replisome-associated proteins RPA, PCNA and MCM2 are progressively lost from early replication foci when replication is arrested in CHO cells treated with caffeine to inhibit ATR (Dimitrova and Gilbert, 2000b). Mrc1 and Tof1 both associate with the MCM activating factor CDC45 and the MCMs are a logical ultimate target for fork stabilisation (reviewed (Bailis and Forsburg, 2004)). In addition to preventing origin firing, the inhibition of MCM2-7 could prevent excessive DNA unwinding ahead of a stalled polymerase – unwinding that might otherwise allow DNA breakage, disassembly of the replisome or aberrant recombination events. BLM, as outlined above, may also have a role in preventing aberrant recombination at stalled forks and BRCA2 has a similar role although this does not seem to be directly checkpoint-mediated. (Like BLM cells, BRCA2 mutants still complete bulk DNA synthesis after HU release but 2D-gel analysis shows that stalled forks remain in these cells and visibly disintegrate over

time without being properly resolved (Lomonosov et al., 2003).) In addition to modulating recombination, the BLM homolog in yeast, Sgs1, is actually required to stabilise the replisome by keeping DNA polymerases in place at stalled forks (Cobb et al., 2003), although how Sgs1 achieves this is unclear.

In conclusion, although all the details of ATR-mediated fork stabilisation are not yet known, the general picture seems to be of a primary Mrc1/Tof1 mediated mechanism to prevent excessive DNA unwinding at stalled forks and to keep all the replisome components poised to restart. If this fails, a backup mechanism involving RecQ helicase activity and/or BRCA2 mediates recombination, perhaps within a chicken foot structure, allowing replication to resume without generating SCEs or mutations.

A role for ATR in normal S phase progression

The role of ATR in arresting S phase and stabilizing stalled forks may in fact be a hyper-activated form of a constitutive ATR pathway for modulating normal replication dynamics. The lethal effects of ATR, RAD17 or CHK1 deficiency (Brown and Baltimore, 2000; Wang et al., 2003b; Zachos et al., 2003) and the chromosome fragility observed in ATR or RAD17 mutants even in the absence of replication stress (Casper et al., 2002; Wang et al., 2003b) suggest that ATR plays a vital constitutive role in the cell cycle. Furthermore, the recent report that spontaneous DNA damage occurs in h-SMG1 knockdown cells suggests that this role may extend to h-SMG1 as well (Brumbaugh et al., 2004). That the loss of the third PIKK, ATM, does not cause these phenotypes may reflect the fact that physiologically-occurring dsbs are a rare, cell type-specific event compared to stalled or impaired replication forks. Nonetheless, dsbs do

occur during meiosis and in immune cell development and ATM may have evolved specifically to respond to problems with physiological dsbs. In fact, the roles of ATM and ATR may have been much closer at earlier stages in evolution, since a *Drosophila melanogaster* ATM homolog shows serious defects in normal development, high levels of apoptosis and an aberrant response to endogenous DNA damage – phenotypes reminiscent of ATR in higher eukaryotes (Song et al., 2004).

In addition to this circumstantial evidence, two recent reports state that ATR directly imposes the temporal program of origin firing (Marheineke and Hyrien, 2004; Shechter et al., 2004). CHK1 is activated throughout S phase in *Xenopus* extracts, to an extent directly correlated with the amount of RPA-bound ssDNA. This in turn correlates with the inhibition of new origin firing. Conversely, if ATR is inhibited or if its effector CDC7 is up-regulated, origin firing occurs more rapidly and synchronously than usual. ATM is also transiently activated during S phase and inhibiting ATM, or bypassing it by up-regulating its effector CDK2, also promotes origin firing, although to a lesser extent than for ATR. This suggests that some dsbs do occur in a normal S phase and that ATM modulates origin firing in response to these dsbs. Interestingly, in *Xenopus,* CHK1 activation during normal S phases only begins at the MBT when origin firing starts to space out – thus the MBT may actually occur because maternal replication factors are diluted sufficiently to impede replication progression and activate a 'physiological checkpoint' (Shimuta et al., 2002). Consistent with this idea, DNA fibre labelling shows that when ATR is inhibited by caffeine, allowing an aberrant excess of origin firing, ongoing forks are forced to slow down (Marheineke and Hyrien, 2004). It remains possible, however, that this is a

secondary effect of caffeine treatment. None of these results has been directly reproduced in mammalian cells so they may be specific to the *Xenopus* extract system. However, CHK1 inhibition by the drug UCN-01 has been reported to reduce origin firing (as seen by alkaline sucrose sedimentation) in mammalian cells as well (Heffernan et al., 2002).

In summary, the model proposed is that ATR monitors all active replication forks via its association with RPA. Excessive ssDNA may occur at stalled forks or sites of damage, but ssDNA must also be present transiently at normal forks. Therefore, ATR will become activated dose dependently as more origins fire, and will suppress subsequent origin firing accordingly. At the same time, ATR senses forks that become stalled by DNA secondary structures, base damage or other impediments and acts (probably via claspin) to stabilize the replisome and perhaps recruit helicases or recombination proteins such as BLM, MRN and FANCD2. Such 'physiological' fork stalling at chromatin-bound proteins had been shown to activate Mec1/Rad53 during normal cell cycles in *S.cerevisiae* (Ivessa et al., 2003) but in normal replication this may be a limited, local response to individual stalling events and it may only be seen throughout the genome when all forks are abnormally stalled by drug treatment. Thus the whole PIKK-mediated checkpoint system may coordinate both normal replication and, by the same means, the response to DNA damage.

Conclusion

An understanding of the S phase checkpoint is particularly important with regard to cancer therapy because many chemotherapeutic agents act by damaging DNA or interfering with DNA replication. These agents may promote the death of tumour cells more effectively than normal cells because tumour cells already have impaired checkpoints, making them less able to tolerate DNA damage via cell cycle arrest and appropriate repair. However, chemotherapy is rarely entirely specific for cancer cells and because it damages DNA non-selectively it may increase the risk of further mutations in both normal and cancer cells (Bignami et al., 2003). A better understanding of exactly how DNA damage affects replication dynamics might lead to the development of better drugs or combinations of drugs which could trigger cell death more effectively in cancer cells whilst minimising mutagenesis or death of normal cells. Therefore, in order to integrate all the different pieces of information about the S phase checkpoint using a single experimental system, a DNA fibre-labelling technique has been developed in which all the various parameters determining DNA synthesis during S phase can be assessed individually and on the level of single replication forks as opposed to whole cell populations. This method measures DNA synthesis across the entire genome, independently of sequence or structure; it is quantitative and the results can be statistically analysed. The technique has been used in a systematic investigation of both the immediate and longer-term changes to replication dynamics which occur after a variety of DNA-damaging and replication-stalling stimuli. Cells with specific checkpoint deficiencies have then been examined for any change in their replication response to DNA damage.

Chapter 2: Materials and Methods

Cell culture and synchronisation

HeLa cells, IMR-90 fibroblasts, AT (GM03487) fibroblasts and NBS-ILB-I cells were obtained from CR-UK cell services and grown as monolayers in DMEM+10% FCS, incubated in 10% CO_2. HCT116, HCT116:$ATR^{flox/-}$ (Cortez et al., 2001) and HCT116:$CHK2^{-/-}$ (Jallepalli et al., 2003) cells were grown in McCoy's medium+10% FCS, 10% CO_2.

Synchronisation of HeLa cells by mitotic shakeoff was carried out by adding 0.17μM nocodazole to cells plated at 50-70% confluence 24hrs previously. After 4-5hrs, rounded mitotic cells were shaken off into pre-warmed PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EDTA, 2mM $MgCl_2$, pH 6.9), collected with minimal centrifugation (~130g 5mins) and re-plated in fresh medium. DNA damaging treatments were applied 15-16hrs after re-plating, when the majority of cells were in early S phase. For additional synchronisation using aphidicolin (APH), 5μg/ml APH was added to the re-plated cells 3hrs after mitotic shakeoff and then washed out 13hrs later when the cells had all accumulated at the S phase border. Stock solutions were made in DMSO and stored at –20°C: 3.4mM nocodazole (Sigma) and 1mg/ml APH (Sigma).

For synchronisation of siRNA-transfected cells, HeLa cells were plated at ~50% confluence on 150mm plates and transfected 24hrs later. 16hrs post-transfection, 0.17μM nocodazole was added for 8hrs, then mitotic shakeoff was performed as before. 15-16hrs after re-plating (early S phase), DNA damaging treatments were carried out. At this point cells were 39-40hrs post-transfection and siRNA-mediated knockdown was usually at its maximum.

DNA damaging treatments

MMS (100% solution, Sigma) was added directly to the culture medium at final concentrations of 0.005%-0.03% (0.59-3.54mM). After 20min treatments the MMS was removed and cells were washed twice with MMS-free medium before incubating in further fresh medium.

IR exposures were carried out at between 1 and 10 Grays (~25-250s). Control cells were removed from the incubator for the same time period.

UV exposures of 5-20J/m^2 (~2.5-10s) were carried out by placing cells under a 254nm UV lamp. Cells were washed in PBS and directly exposed to UV under a minimal quantity of PBS, then returned to fresh medium.

HU (Sigma) was dissolved in water and added to the culture medium at 20μM-2mM (stock solution 0.5M, stored at –20^{0}C).

Caffeine (Sigma) was added to the culture medium 1hr before any DNA damaging treatment (aqueous stock solution 100mM, final concentration 2mM).

Flow cytometry

Samples were prepared from 30mm plates of cells, at least 25% confluent. Cells were trypsinised, washed in cold PBS and fixed for at least 2hrs in 70% ethanol at 4°C. Cells were then spun down, washed again in complete PBS and incubated for 30mins in 0.5ml complete PBS containing 40μg/ml propidium iodide and 0.5mg/ml RNase A. All washes were carried out in round-bottomed tubes with minimal centrifugation (~130g 5mins) to avoid cell breakage and aggregation. Flow cytometry was carried out using a Becton Dickenson FACScan.

Modified RDS assay

HeLa cells were plated at ~50% confluence on 8x150mm plates. ~16hrs after plating, 4 of the plates were labelled for 24hrs with [methyl-^{14}C]Thymidine (Amersham) (50μCi/ml stock, final concentration 40nCi/ml and 0.72μMthymidine). The same amount of cold thymidine was added to the remaining 4 plates. ^{14}C-labelled cells were subsequently used for scintillation counting while the unlabelled cells were treated exactly in parallel but fixed for flow cytometry to monitor the cell cycle. After 24hrs the [methyl-^{14}C]Thymidine was washed out and mitotic shakeoff synchronisation was carried out on both labelled and unlabelled cells, re-plating to 60mm plates.

15hrs post-shakeoff, a FACs sample was fixed to confirm that the cells were in early S phase and DNA damaging treatments were applied: 5Gy IR, 0.0005% MMS or no damage. At the same time, [methyl-^{3}H]Thymidine (Amersham) was added to the ^{14}C-labelled cells (1mCi/ml stock, final concentration 25nCi/ml and 1nM thymidine). 6hrs later, 0.17μM nocodazole was added to all plates to prevent cells from entering another cell cycle. FACs and scintillation samples were then fixed in parallel at 24, 39 and 63hrs post-shakeoff.

For scintillation counting, all the cells were collected (both rounded/floating and trypsinised populations), washed in PBS, then re-suspended in 100μl lysis buffer (10% SDS, 1mM EDTA). 900μl ice-cold 10% trichloro-acetic acid (TCA) was added and the samples were held on ice for 15mins. Lysates were transferred to TCA-soaked 2.5cm glass microfibre filters (Whatman) and the filters were washed three times with TCA and then twice with ethanol using vacuum-filtration. Filters were air-dried, placed in scintillation vials with 5ml scintillation fluid and counted for ^{3}H/ ^{14}C.

Whole-cell fixation and indirect immunofluorescence

Cells were grown on 13mm coverslips, sterilised by baking. All fixation and washing steps were carried out in a 24-well plate. Cells on coverslips were washed with PBS, fixed for 10mins in cold 4% formaldehyde/PBS (Sigma), washed twice with PBS, then blocked for 10mins in blocking buffer (1mg/ml BSA, 0.1% Tween-20 in PBS). For methanol fixation, the formaldehyde was replaced with 15mins in ice-cold methanol.

All staining steps were carried out with the coverslips placed on parafilm in a humidified chamber: 25μl of antibody diluted in blocking buffer was used per coverslip and three 5min washes in PBS were carried out after each staining step. After staining, coverslips were mounted in 50:50 PBS/glycerol, sealed with nail varnish and examined using a Zeiss LSM Meta 510 confocal microscope.

Replication foci by BrdU or PCNA

Cells were labelled with 50μM BrdU for 10-20mins (50mM aqueous stock solution), then permeablised for 10mins in ice-cold 0.5% TritonX-100/PBS before formaldehyde fixation. BrdU was detected with mouse anti-BrdU/nuclease (RPN202 Amersham), 1.5hrs at 37°C. Alternatively, cells were methanol-fixed and stained for 1hr at R.T. with 1:5000 mouse anti-PCNA (PCIO). Both primary antibodies were detected with 1:2000 Cy3 anti-mouse IgG (C-2181 Sigma) for 1hr at R.T.

Phosphorylated Chk1 (Ser317)

Methanol-fixed cells were stained overnight at 4°C with 1:2000 anti-P-Chk1 (2344s Cell Signalling Technology), then 1hr at R.T with 1:5000 Cy3 anti-rabbit (C-2306 Sigma).

Replication labelling and DNA fibre spreads

Cells were plated in 24-well plates or 30mm dishes (a 24-well dish of cells is sufficient but individual 30mm dishes are compatible with the gamma irradiator and with UV exposures).

For single-labelled fibres, cells were labelled with 50μM IdU for 10-60mins. For double-labelling, 10μMIdU was added for 10mins, any DNA damage being applied during the last few minutes of this label. IdU was then washed out with fresh medium and replaced with 100μM CldU for 20 or 25mins. For experiments using HCT116 cells and for all experiments using HU, 20μM IdU and 200μM CldU were used. For origin-firing recovery experiments, cells were pulsed with 20μM IdU for 10mins directly before DNA damage, then incubated with 50μM thymidine for 15mins to chase out the IdU, then kept in fresh medium before double-labelling 1.5-4.5hrs later.

DNA spreads were made as in (Jackson and Pombo, 1998), with certain modifications. Slides were made in duplicate for each experiment.

Cells were trypsinised and re-suspended in ice-cold PBS at $2.5x10^5$ cells/ml. (Cells can then be held on ice for several hours before spreading.) Labelled cells were diluted 1:5 or 1:10 in unlabelled cells, then 2.5μl of the cell suspension was pipetted onto the end of a glass slide. 7.5μl of spreading buffer (0.5% SDS, 200mM Tris/HCl pH 7.4, 50mM EDTA) was added to the cells, mixed lightly with the pipette tip and allowed to lyse for 7-10mins. (Depending on the environment, up to 10μl spreading buffer may be used if the room is very warm, or over 10mins of lysis may be allowed if it is very cold. The mixture must not dry out too much, but it must have time for the DNA to be properly released).

The slides were propped up on the edge of a lid from a 6-well culture dish, allowing the DNA to run smoothly but fairly slowly for the whole length of the slide. The resulting DNA spreads were air-dried and fixed in 3:1 methanol/acetic acid, until there was no visible residue of SDS on the glass (~10mins). Slides were air-dried and stored overnight or longer at 4°C.

Immuno-labelling of DNA fibre spreads

All staining steps were carried out with the slides on parafilm in a humidified Tupperware box. The slides must be kept level at all times and not allowed to dry out. After each staining step, slides were rinsed in PBS using a slide bath, then laid out on damp paper towel and washed three times for 20mins in 1.5ml blocking buffer for each wash (1% BSA, 0.1% Tween in PBS).

Slides were first rinsed in water and treated with 2.5M HCl for 1.5-2hrs, then washed several times in PBS and blocked in blocking buffer for ~1hr. Staining steps were as follows:

1) Overnight in 0.5ml 1:1000 rat anti-BrdU (detects CldU) (OBT0030 ImmunologicalsDirect).
2) 2hrs in 0.5ml 1:1000 Alexafluor 633-conjugated anti-rat (A-21094 Molecular Probes).
3) 2hrs in 0.25ml 1:500 mouse anti-BrdU (detects IdU) (MD5100 Caltag).
4) 1.5-2hrs in 0.25ml 1:1000 Cy3-conjugated anti-mouse (C-2181 Sigma).
5) Counterstain for 15mins with 1:20,000 YOYO-1 in PBS (Molecular Probes).
6) Wash through 3 changes of PBS, mount in PBS/glycerol and seal thoroughly with nail varnish. It is very important not to jog the coverslip during mounting as this can smear and break the DNA fibres.

Microscopy was carried out using a Zeiss LSM Meta 510 confocal microscope and only clearly-isolated tracks on unbroken DNA fibres were measured.

Adenovirus preparation

E1-deleted Ad-Cre and Ad-GFP were gifts from Dr Robert Weiss, Cornell University. The virus was amplified in 293 cells grown in 175mm flasks in DMEM+10%FCS at 5% CO_2. Infections were carried out in DMEM with only 2% FCS. Caesium Chloride (CsCl), TSG and dialysis solutions were prepared as follows:

CsCl solutions

Solution density (g/ml)	Wt/vol CsCl (g/100ml)
1.25	36.16
1.35	51.20
1.4	62.00

TD diluent for CsCl solutions

	Weight (g/l)	Final conc (mM)
NaCl	0.40	14
KCl	0.19	5
Na_2HPO_4	0.05	0.7
Tris Base	1.50	25

TSG

Solution A	Solution B	Combine 700ml A with 3.5ml B, add 300ml glycerol, then filter-sterilise.
900ml ddH_2O	100ml ddH_2O	
8.0g NaCl	2.0g $MgCl_2$	
0.1 g Na_2HPO_4 (dibasic)	2.0g $CaCl_2$	
0.3g KCl		

Dialysis Solution

Stock solution	Vol. stock (ml/l)	Final conc (mM)
1M Tris/HCl pH7.4	10	10
5M NaCl	30	150
1M $MgCl_2$	1	1
Glycerol	100	10%
ddH_2O	859	

For virus expansion, a single flask of cells at 70-80% confluence was infected with 30μl adenovirus in 30ml DMEM+2%FCS. After 48hrs, or at 100% cytopathic effect (CPE), the cell suspension was freeze-thawed three times (-70/37°C). 25 flasks of cells were then re-infected with the lysate and after a further 48hrs, cells were harvested by centrifugation at 2000rpm for 10mins at 4°C. Pellets were washed in 15ml cold PBS, centrifuged again at 1000rpm for 10mins at 4°C, then re-suspended in 12ml cold 10mM Tris/HCL pH 8.0. Freeze-thawing was repeated and the virus suspension was centrifuged at 6000rpm for 10mins at R.T. before loading the supernatant onto a Caesium Chloride (CsCl) gradient.

10ml 1.25g/ml CsCl was placed into each of two ultracentrifuge tubes and underlaid with 7.6ml 1.4g/ml CsCl. The virus was split between the two tubes which were then balanced with PBS and centrifuged at 25,000rpm for 2hrs at 15°C in an Optima LE-80K ultracentrifuge. The lowest of the three visible bands, representing encapsulated virus particles, was removed via a syringe, piercing the tube just below the band. The virus was again layered onto 2.5ml 1.35g/ml CsCl in each of two ultracentrifuge tubes, balanced and spun at 40,000rpm for 15hrs at 15°C. The lowest band was removed and made up to 12ml in TSG.

Virus/TSG was injected into a slide-a-lyzer using an 18G needle. Excess air was removed through a second needle, the slide-a-lyzer was placed in a float and immersed in 2l dialysis solution which was then stirred at 4°C for 24hrs. The dialysed virus was removed and stored at -70°C

Virus titre by $TCID_{50}$ (Tissue Culture Infectious Dose 50)

293 cells were plated in DMEM+2%FCS in 96-well plates at 10^4/well. 24hrs later, they were infected with serial dilutions of virus as follows (prepared in duplicate for each virus):

- Initial 1:10 dilution = 0.1ml virus stock in 0.9ml medium.
- 0.2ml of the 1:10 dilution added to 1.8ml medium; repeated to generate serial dilutions from $1:10^3$ to $1:10^{10}$.
- 100μl of the $1:10^3$ dilution dispensed into wells 1 to 10 of a 96-well plate, then 100μl fresh medium into wells 11 and 12 as negative controls. Repeated for each dilution in all 8 rows of the 96-well plate.
- Incubate for 10days, then count all wells showing CPE. The highest dilution must yield 100% CPE-positive wells and the lowest, none.

The ratio of positive/negative wells for each dilution was noted and the titre as $TCID_{50}$ was calculated using the KÄRBER method:

$T = 10^{1+d(S-0.5)}$ when d = log10 of dilution and S = sum of ratios (starting from a ratio of 1 for the 1:10 dilution, even if this was not actually plated).

$TCID_{50}$ is 0.7log higher than the titre determined by a standard plaque assay (G. Vassaux), therefore the titre in PFU/ml = $1x10^{T-0.7}$.

Infection of conditional knockout cells with Ad-Cre

HCT116-ATR$^{flox/-}$ cells were plated at $4x10^5$ cells/60mm plate. After 24hrs, cells were infected with 10μl Ad-Cre (stock solution 10^9 PFU/ml): a multiplicity of infection (MOI) of ~25 if infection was 100% efficient. The medium was replaced after allowing 8hrs for infection.

48hrs after infection, cells were split to 30mm plates or 24-well dishes as required and experiments were carried out at 72hrs post-infection. The efficiency with which [ATR] was reduced was checked by Western blotting with a sample of cells from every experiment. The Cre-mediated excision of the ATR allele could also checked by PCR on genomic DNA.

Preparation of genomic DNA and PCR for cre-mediated excision

30mm plates of cells, at least 50% confluent, were trypsinised, washed with cold PBS and re-suspended in 50μl TE pH 8.0. 500μl lysis buffer was added ((0.5% SDS, 10mM Tris/HCl pH 8.0, 0.1M EDTA pH 8.0, 20μg/ml RNase) and the suspension was incubated at 37^{C}C for 1hr. 100μg/ml Proteinase K was added (20mg/ml aqueous stock solution) and incubated at 50 ^{C}C for 3hrs. Phenol/chloroform extraction was carried out twice and the DNA was then ethanol-precipitated (0.2vol 10M ammonium acetate, 2vol 100% ethanol). DNA was collected by centrifugation, washed in 70% ethanol, then re-suspended in 20μl ddH_2O and quantified using a spectrophotometer.

PCR was carried out with primers: 5' gtc tac cac tgg cat aac agc 3'

5' cag cgg gag cag gca ttt cc 3'

25μl reactions (27 cycles, 60^{0}C, 1.5mins extension): 2.5μl 10x buffer

0.2μl Ex taq (Takara)

0.2μl 25nM dNTPs

1.0μl each primer, 100ng/μl

1.0μl DNA, or approx 500ng

19.1μl ddH_2O

PCR products were run on standard 0.8% agarose gels in TAE, stained with ethidium bromide. The constitutively-disrupted allele yields a PCR product of ~2kb, the floxable allele, ~1.1kb and the floxable allele after Cre-mediated excision, a product of only ~100-200bp.

siRNA: sequences and transfection protocols

siRNAs were obtained from Dharmacom, made up as 20μM duplexes as per Dharmacom instructions and stored at –20^{0}C. The anti-sense sequences were:

ATM: 3' dTdT cgu ggu cag guc aua acc g

ATR: 3' dTdT gcu cug aag acg ccu aac g

NBS1: 3' dTdT cgu caa guc agg uuc uuc g

CHK1: 3' dTdT agc acu cgc aaa caa cuu g

pRb: 3' dTdT gac acc ccu uag aca uag a

CHK2: SmartPool (Dharmacom)

Cells were transfected with siRNA at ~50% confluence in DMEM+5%FCS without antibiotics. 1ml was used for 30mm dishes, 2ml for 60mm dishes and 8ml for 150mm dishes.

To each ml tranfection medium, siRNA was added as follows:

- 6μl 20μM duplex + 100μl Opti-MEM (Invitrogen), held for 10mins at R.T.
- 6μl Oliofectamine (Invitrogen) + 25μl Opti-MEM, held for 10mins at R.T.
- RNA and Oligofectamine mixed together, incubated for 20mins R.T, then added to cells.
- In the case of CHK2 SmartPool siRNA, 10μl instead of 6μl was transfected per ml of DMEM.

After 24hrs, cells were split as necessary and experiments were carried out between 40 and 48hrs post-transfection. The efficiency of reduction of the target protein was checked by Western blotting.

SDS-PAGE

Extracts were made from 30mm plates of cells by washing the monolayers with cold PBS, removing all residual PBS, then adding 50μl cold lysis buffer (50mM Tris/HCl pH 7.4, 1% Triton X-100, 0.27M sucrose, 0.1% β-mercaptoethanol). Cells were held on ice for 5mins, collected by scraping, transferred to eppendorffs and held on ice for a further 5mins. The suspensions were then centrifuged for 3mins at 13,000rpm in a bench top centrifuge at 4^{0}C. Supernatants were mixed with 3x Lammeli's buffer and boiled for 1min. 1μl of each supernatant was retained for a Bradford assay.

5% acrylamide gels were made for ATR and ATM or 10% acrylamide for CHK1, CHK2 and NBS1. Equal amounts of transfected and control extracts were loaded and electrophoresis was carried out at 75mA for ~1.5hrs for 10% gels and 3hrs for 5% gels. (5% gels must be run until the 216kDa kaleidoscope marker (BioRad) is at least halfway down the gel to ensure that ATR/M enter the resolving gel.)

Western blotting for ATR, ATM, CHK2, CHK1 and NBS1

Gels were blotted onto HyBond membrane (Amersham) at 15V for 30mins using a Panther™ Semidry electroblotter; (Autogen Bioclear). Membranes were Ponceau-stained to verify equal loading, destained, blocked in 5% milk in TBST for 1hr, then stained with the following primary antibodies:

- Mouse anti-PCNA (PC10): 1:10,000 1hr R.T.
- Rabbit anti-ATR (PC538 Oncogene): 1:2000 overnight 4^0C
- Mouse anti-ATM (ab78 Abcam): 1:1000 overnight 4^0C
- Mouse anti-phospho-ATM (05-740 Upstate Ltd): 1:1000 overnight 4^0C
- Rabbit anti-NBS1 (PC269 Oncogene): 1:500 2hrs R.T.
- Mouse anti-CHK2 (gift from Dr Niels Mailand): 1:20,000 1hr R.T.
- Rabbit anti-CHK1 (FL-476 Santa Cruz): 1:200 2hrs R.T.
- Rabbit anti-phospho CHK1 Ser317 (2344s Cell Signalling Technology)

  N.B 1:500 in 5% BSA *not* milk, overnight 4^0C

After 3xTBST washes, all primary antibodies were detected with secondary antibodies at 1:5000 in 5% milk for 1-2hrs at R.T. HRP-protein A (NA9120 Amersham) was used for rabbit primary antibodies or HRP-anti-mouse IgG (PI-2000 vector labs) for mouse antibodies. Membranes were washed again in TBST and developed using ECL detection reagents (Amersham).

Chapter 3:

Preliminary studies of S phase parameters in response to DNA damage

3a) S phase progression by Flow Cytometry: S phase is slowed by IR, MMS and HU

DNA damaging treatments which activate the ATM or ATR checkpoint pathways are known to cause reduced DNA synthesis in asynchronous populations of cells (Painter, 1977; Painter and Young, 1980). This is thought to be due to a reduced rate of DNA synthesis within S phase as well as to the arrest in G1 of cells which would otherwise be entering S phase (Larner et al., 1997; Larner et al., 1999). In *S.cerevisiae*, the intra-S phase checkpoint can be measured by flow cytometry as a dose-dependent slowing of overall S phase progression induced by either MMS or IR damage (King et al., 2003; Paulovich and Hartwell, 1995). In order to confirm that the same response occurs in mammalian cells, populations of HeLa cells were synchronised so that the intra-S phase checkpoint could be separated from any arrest of cells in G1. (HeLa cells lack functional p53 so a transcription-induced G1 arrest should not occur but the prevention of S phase entry at the G1/S border via CDK2 inhibition is probably retained (Larner et al., 1997).) Carrying out all experiments in synchronised cells within S phase should mean that only the genuine effects of the intra-S phase checkpoint are seen. It also means that the defect in the p53-dependent G1 checkpoint in HeLa cells compared to primary cells should not be relevant. HeLa cells have been previously shown to down-regulate their DNA synthesis in

response to both IR and MMS damage (as measured by reduced ^{3}H thymidine incorporation in asynchronous cells) so they apparently retain a functional intra-S phase checkpoint (Goldberg et al., 2003; Painter, 1977).

Many of the techniques used to synchronise cells in S phase, such as aphidicolin, mimosine or double thymidine blocks interfere with replication forks and are likely to activate DNA damage responses even before any further DNA damage is applied (Jackson, 1995; Krude, 1995). Therefore, in this study, HeLa cells were synchronised by nocodazole arrest, mitotic shakeoff and release for 16 hours – at which point most cells are in early S phase. This method does not directly interfere with S phase entry so it should not activate any DNA damage checkpoint pathways.

Firstly, cells were treated with 20 minute pulses of 0.001%-0.03% MMS, the MMS was removed and S phase progression was followed by flow cytometry over the next 12hrs. Fig.1a shows that S phase was slowed in a dose-dependent manner, ranging from a mild effect after 0.005% MMS to near-complete arrest over 12 hours after the 0.03% MMS treatment. $20J/m^2$ of UV-C radiation had a similar effect to a high dose of MMS (see chap.3, Fig.21): UV is a second agent which is usually classed together with alkylating agents as an ATR-activating form of DNA damage. Secondly, the progress of S phase was followed after exposure to 1 Gy or 5 Gy of IR. 1 Gy did not cause a detectable slowing of S phase but 5 Gy did result in moderate slowing (Fig.1b). This response was only slightly increased after as much as 10 Gy of IR (data not shown). Thirdly, 5-100μM HU was added to the cells in early S phase. Again, a dose-dependent slowing of S phase was observed (Fig1c), 5μM HU having little effect, 20μM

causing a significant slowing of S phase and 100μM leading to arrest with a near-2C DNA content.

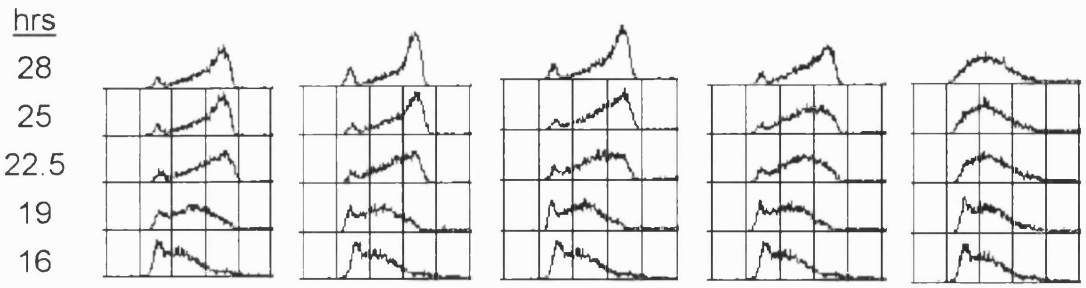
Figure 1: S phase progression is slowed by IR, MMS and HU

a) Cells were synchronised by mitotic shakeoff and treated in early S phase with 0.005%-0.03% MMS for 20mins. Cell cycle progression was followed over the next 12hrs by flow cytometry.

b) Cells as in A, exposed to 1-5Gy IR in early S phase.

c) Cells as in A, with 5-100μM HU added to the medium in early S phase.

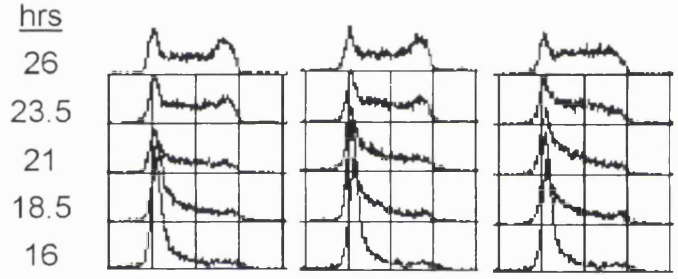
a



CONTROL 0.001% 0.005% 0.01% 0.03%

----------MMS (20min pulse at 16hrs)----------

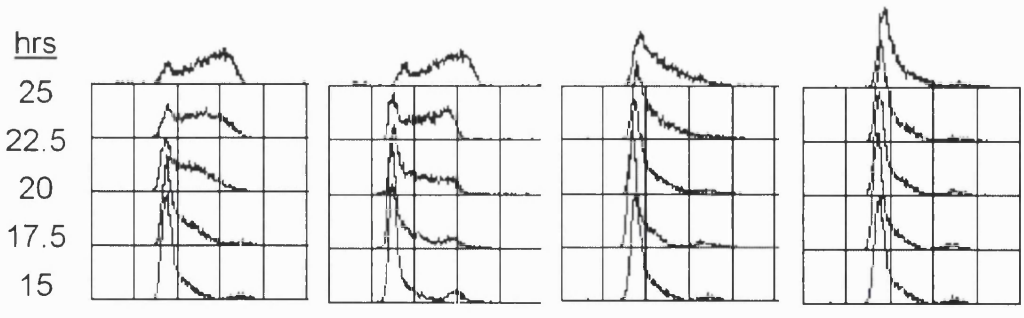
b



CONTROL 1 Gy 5Gy

--------IR at 16hrs---------

c



CONTROL 5μM 20μM 100μM

----------HU at 15hrs----------

3b) S phase progression by labelling replication foci:

The appearance of late replication patterns is delayed by MMS

Flow cytometry reveals only the bulk progression of S phase in a large population of loosely-synchronised cells. To gain more detailed information about the reduction in DNA synthesis over the course of S phase, individual cells were examined by BrdU labelling of replication foci. Replication foci show a well-defined series of patterns from early to late S phase (O'Keefe et al., 1992) and the late patterns do not appear until earlier replication has been completed (Dimitrova and Gilbert, 2000a; Jackson, 1995) so the appearance of successive patterns of foci is a relatively sensitive indicator of S phase progress. In *S.cerevisiae*, studies of specific origin sequences have revealed an MMS-induced block to the firing of late origins (Tercero and Diffley, 2001). The focus-labelling assay aims to give similar information about origin firing kinetics in mammalian cells (Dimitrova and Gilbert, 2000a), although at best it probably only assesses origin firing at the level of origin clusters rather than individual sequence-defined origins.

Synchronised cells were treated with MMS in early S phase and S phase progression was then assessed by counting the relative numbers of early, intermediate and late focal patterns over the next 10hrs (Fig.2a). For these experiments, cells were synchronised by mitotic shakeoff and then further synchronised at the S phase border with aphidicolin (APH): although loosely-synchronised cells were adequate for flow cytometry which counts many thousands of cells, a more tightly-synchronised population was needed to collect meaningful data by counting the replication patterns in a relatively small number

of individual cells. (Attempts were also made to carry out the same experiment in primary fibroblasts synchronised by serum starvation but here, imperfect cell synchrony as well as the difficulties of distinguishing damaged patterns from 'late' patterns in these cells led to inconclusive results.) The use of APH is not ideal because it stalls replication forks at the S phase border and therefore acts as an additional checkpoint stimulus. However, HeLa cells are reported to recover normal replication kinetics relatively soon after release from APH (Jackson 95), and since both control cells and MMS-damaged cells were treated in this way, any differences in their replication patterns should be attributable to the MMS damage.

In undamaged cells synchronised by this method, focal patterns progressed from exclusively 'early' to mostly 'late' in about 10hrs (Fig.2b). Treatment with 0.005% MMS for 1hr in early S phase delayed progression by about 4hrs (Fig.2c) and treatment with 0.01% MMS resulted in a more severe delay, with very few late patterns appearing at all within 10hrs (Fig.2d).

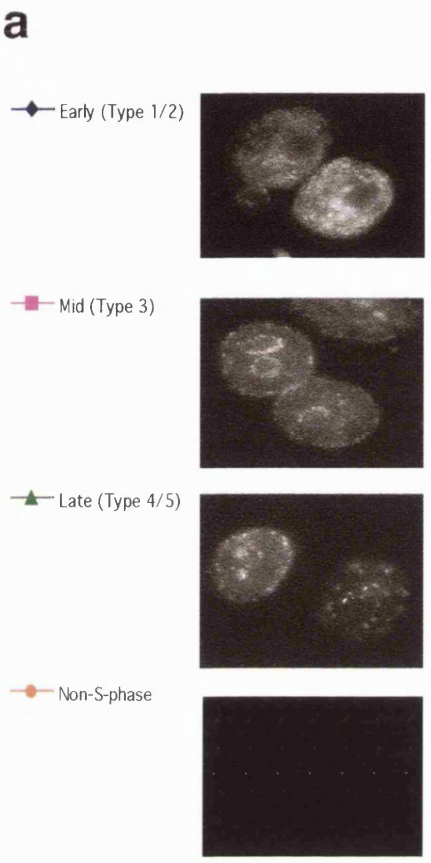
Figure 2: The progress of replication focal patterns is slowed by MMS

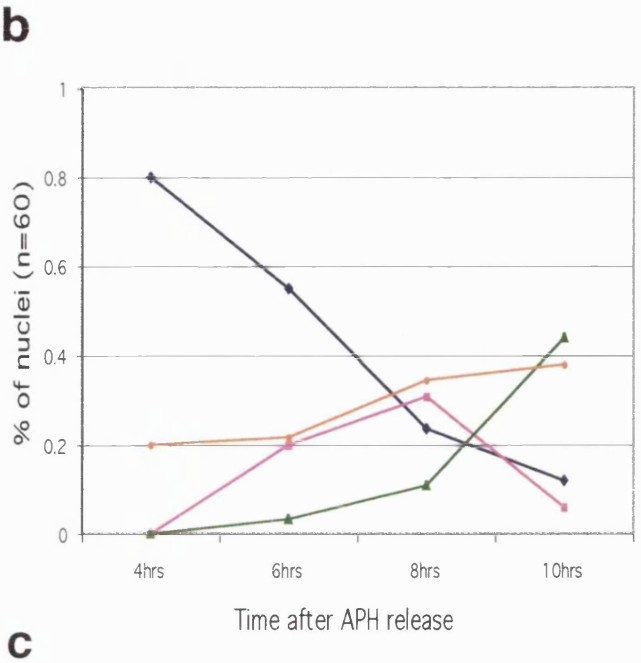
a) Typical early, mid and late S phase patterns in BrdU pulse-labelled cells.

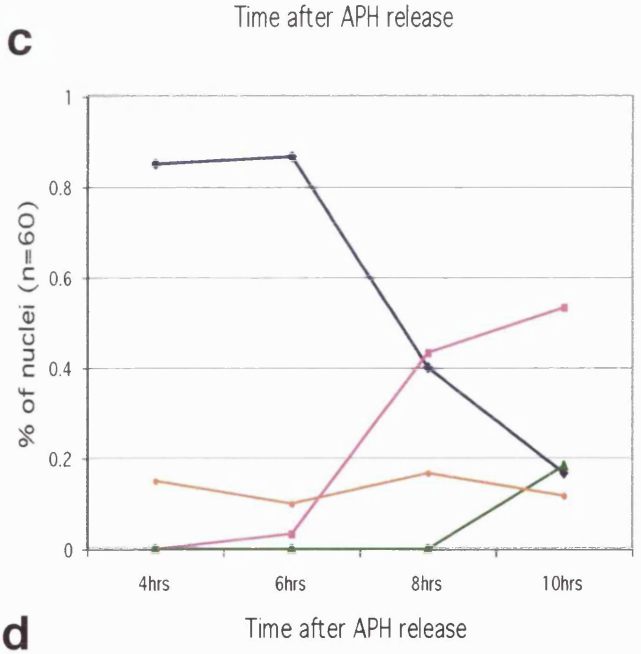
b) Proportions of nuclei showing each replication pattern in undamaged cells over the 10hrs following APH release (n=60 for each timepoint).

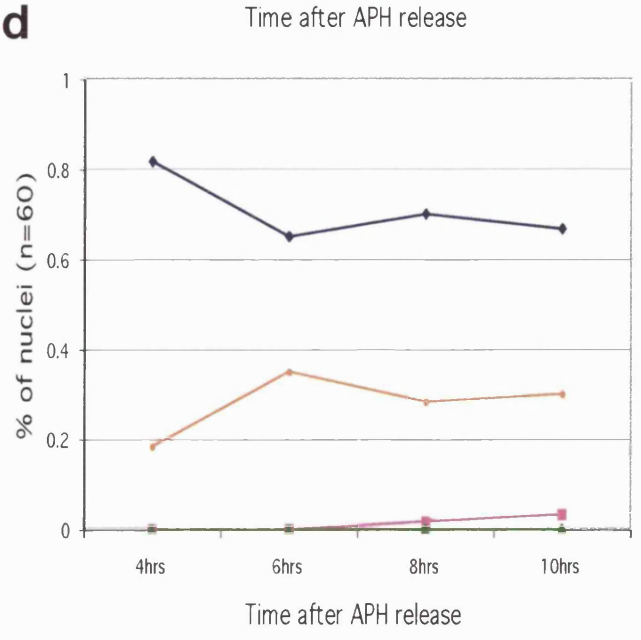
c) Cells as in b), treated with 0.005% MMS for 1hr after APH release.

d) Cells as in b), treated with 0.01% MMS for 1hr after APH release.


a
Early (Type 1/2)
Mid (Type 3)
Late (Type 4/5)
Non-S-phase


b
% of nuclei (n=60)
1
0.8
0.6
0.4
0.2
0
4hrs
6hrs
8hrs
10hrs
Time after APH release


c
% of nuclei (n=60)
1
0.8
0.6
0.4
0.2
0
4hrs
6hrs
8hrs
10hrs
Time after APH release


d
% of nuclei (n=60)
1
0.8
0.6
0.4
0.2
0
4hrs
6hrs
8hrs
10hrs
Time after APH release

3c) dNTP levels in damaged and undamaged cells:

The balance between nucleoside synthesis and salvage does not change after DNA damage

One recently-discovered aspect of the S phase checkpoint in *S.cerevisiae* is a 6 to 8-fold up-regulation of intracellular dNTP levels (Chabes et al., 2003). It is not known whether this is conserved in mammalian cells but the experiments described above suggested that something similar might be occurring. In addition to the delay in focal patterns, cells showed a distinct reduction in the brightness of labelled foci during the first few hours after MMS treatment. There are several possible explanations for this: it could be that fewer origins fire within each focus, or that ongoing forks move more slowly after MMS damage, but it could also be partly due to an increase in the intracellular synthesis of dNTPs. Mammalian cells derive dNTPs from *de novo* synthesis and also from a salvage pathway which accounts for their ability to use nucleoside analogues from the culture medium: nucleosides are taken into the cell via both equilibrative and concentrative transporters and then converted into nucleotides. If *de novo* synthesis within cells was up-regulated after DNA damage, the equilibrium promoting nucleoside uptake might be shifted so that less BrdU would be transported into the cell. The observation was, however, difficult to quantify using a non-linear technique like immunofluorescence so instead a modified form of the RDS assay was developed.

In the RDS assay, the amount of ^{3}H in DNA after a pulse-label with [methyl-^{3}H]thymidine is taken as a measure of the rate of DNA synthesis during the labelling period. In fact, the technique only measures the proportion of DNA synthesis which actually used nucleotides derived from the salvage pathway, and

this could change after DNA damage. However, if cells are labelled with ^{3}H throughout an entire S phase then whatever the rate of that S phase, the amount of ^{3}H incorporated once the whole genome is replicated should indicate the overall proportion of nucleotides derived from the salvage pathway during that S phase. If cells salvage fewer nucleotides during a DNA-damaged S phase, this would strongly suggest that *de novo* synthesis is up-regulated as part of the cell's response to damage.

HeLa cells were pre-labelled for 24hrs with ^{14}C so that measurements of ^{3}H could be normalised to the total amount of DNA in each sample, then the cells were synchronised by mitotic shakeoff. In early S phase, [methyl-^{3}H]thymidine was added to the medium together with 0.0005% MMS. In these experiments, MMS was left in the medium throughout S phase to maintain the DNA damaging stimulus and therefore, hopefully, the checkpoint response. (MMS has a half-life in culture medium of 8.5hrs (Jensen et al., 1977)), so it should continue to alkylate DNA for most of S phase). The effect of 5Gy IR was also tested but since irradiation could not be maintained at a constant low level, cells were simply irradiated once, 16hrs after mitotic shakeoff. Nocodazole was used to trap cells again in the subsequent mitosis: mitotic cells are guaranteed to have completed S phase and they could theoretically be shaken off and isolated for scintillation counting. However, it proved difficult to collect sufficient cells by this method, particularly in damaged populations where S phase was apparently followed by a long G2 arrest before mitosis. Furthermore, in damaged populations, rounding-up of cells could represent apoptosis as well as mitosis. (0.0005% MMS was chosen as a dose that allowed cells to complete S phase without showing massive apoptosis but nevertheless, damaged cells probably underwent more apoptosis

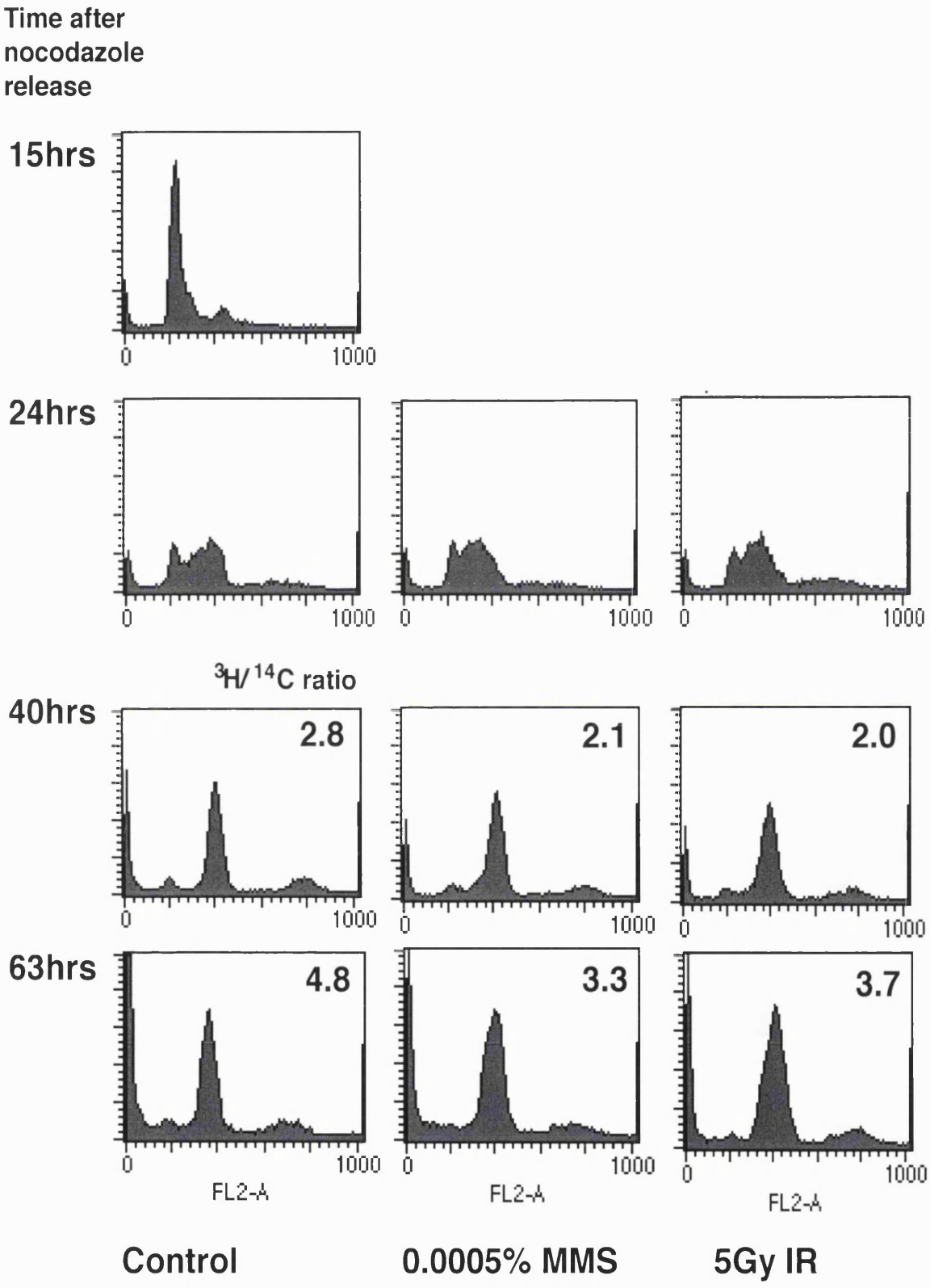
than undamaged cells.) Therefore the protocol was modified, waiting until the whole population had completed S phase and then collecting all the cells together by trypsinisation. Flow cytometry was performed in parallel to ensure that even if the cells were not actually in mitosis, they had at least completed bulk DNA synthesis.

Fig.3 shows that 24hrs into the first cell cycle, the S phase progress of MMS or IR-treated cells was significantly delayed compared to control cells but by 40hrs, all populations had apparently reached 4C. At this point, there was only a slight difference in the $^{3}H/^{14}C$ ratios for the three populations. By 63hrs, all the ratios had increased but this could be due to continued synthesis of mitochondrial DNA and also to the cells gradually escaping from the very long nocodazole arrest. The *difference* between the ratios for damaged and undamaged populations did not increase over time. Thus, at least in HeLa cells, no evidence was found for a many-fold increase in intracellular dNTP levels after DNA damage comparable to that observed in *S.cerevisiae.*

Figure 3: ^{3}H-Thymidine incorporation does not decrease significantly during a damaged S phase

Cell cycle profiles and $^{3}H/^{14}C$ ratios for cells treated 16hrs after mitotic shakeoff with 0.0005% MMS, 5Gy IR or no damage.

The $^{3}H/^{14}C$ ratios represent the average of two independent experiments.


Time after nocodazole release
15hrs
24hrs
40hrs
63hrs
$^{3}H/^{14}C$ ratio
2.8
2.1
2.0
4.8
3.3
3.7
0
1000
FL2-A
Control
0.0005% MMS
5Gy IR

Chapter 3: Discussion

Flow cytometry with mammalian cells reproduced the S phase-slowing phenomenon that is seen in *S.cerevisiae* in response to a range of DNA damaging agents. More detailed experiments examining the patterns of replication foci in these cells suggested that the slowing of S phase is at least partly due to delayed firing of late origins. These experiments did not, however, exclude other changes to fork progression or fork stalling as well. The severity of the response to different DNA-damaging agents is interesting because IR appears to slow S phase only moderately after doses of up to 10Gy, whereas 0.03% MMS or 20J/m^2 UV block DNA synthesis almost completely for many hours. It is difficult to equate the absolute amounts of DNA damage caused by different agents – IR, for example, is estimated to cause approximately 36 dsbs per Gy (Rothkamm and Lobrich, 2003) but estimates for the associated ssbs and other base damage vary widely (Lett et al., 1967; Veatch and Okada, 1969). MMS damage has been quantitated using *in vitro* studies of alkylated plasmids (Karran et al., 1993) but these cannot be applied to the *in vivo* situation when whole cells are exposed to MMS. Particular alkylated bases can be quantitated in genomic DNA by HPLC (Horton et al., 2003), but MMS induces a wide variety of different alkylations and these have not all be quantitated simultaneously. Nevertheless, the results in Fig.1 do suggest that levels of MMS and UV commonly used in experimental protocols can have more serious and sustained effects on replication than commonly-used doses of IR. This is probably because UV and MMS generate lesions in DNA which physically block the movement of replication forks. For example, UV-induced pyrimidine dimers and the 3-methyladenine induced by MMS have both been shown to block polymerases in *in vitro* replication (Larson et al., 1985;

Moore and Strauss, 1979), an effect which has also been observed *in vivo* by density substitution (Tercero and Diffley, 2001) and by the detection of ssbs in the daughter strand of DNA which is replicated from an alkylated template (Schwartz, 1989). In addition to their physical effect on replication fork movement, it is possible that alkylated bases are repaired more slowly than dsbs, generating a more sustained checkpoint signal than is generated by IR. The *in vivo* half-lives of alkylated bases range from 29hrs for 7-meG (Horton et al., 2003) to only 3hrs for 3-meA (Margison et al., 1973), suggesting that repair rates do vary a great deal for different lesions. However, it is not clear that IR-induced damage is fundamentally any less persistent than MMS damage, in fact, recent studies of the persistence of H2AX foci suggest that at least a subset dsbs can remain unrepaired for several days after irradiation (Rothkamm et al., 2003), and in my experiments, the exposure of HeLa cells to 5Gy IR in early S phase resulted in a G2 arrest in most cells up to 40 hours later (data not shown). A second – and not mutually exclusive – hypothesis is therefore that the checkpoint signal during S phase is 'turned off' at different rates after IR and MMS damage, perhaps because ATM ceases to respond to persistent dsbs faster than ATR becomes insensitive to any persistent MMS-induced lesions. Thirdly, whereas dsbs are detected and responded to all at once by ATM (Bakkenist and Kastan, 2003), fork-blocking lesions may be continuously processed throughout S phase into newly checkpoint-activating forms of DNA damage. This would be consistent with the observations that MMS damage in yeast and BrdU/UV-induced crosslinks in mammalian cells only activate the checkpoint when they are encountered by a replication fork (Hamlin, 1978; Tercero and Diffley, 2001).

These theories regarding the relative persistence of checkpoint responses will be tested in more detail in Chapter 4.

The experiments in Fig.3 suggest that mammalian cells do not show the same up-regulation of dNTP synthesis after DNA damage that is seen in *S.cerevisiae*. The evidence is indirect, so it remains possible that both *de novo* synthesis and the activities of concentrative nucleoside transporters are simultaneously up-regulated, leading to the same proportion of exogenously-derived nucleotides being incorporated into DNA. (Yeast, by contrast, do not salvage nucleosides in the same way so *de novo* synthesis is the only relevant pathway.) Alternatively, it is possible that nucleotide synthesis *is* specifically up-regulated in mammalian cells, but via a different mechanism to yeast: a mechanism that is not seen in HeLa cells. In yeast, up-regulation occurs via Mec1/Rad53, which modulates the activity of the tetrameric enzyme ribonucleotide reductase (RNR) - the rate-limiting factor in dNTP synthesis. Firstly, two subunits of RNR, RNR2 and 3, are transcriptionally up-regulated via Mec1/Rad53/Dun1-mediated inhibition of the Crt1 repressor (Huang and Elledge, 1997a). Secondly, the two small subunits of the tetramer, RNR2/4 are relocated to the cytoplasm where the large subunit RNR1 resides, presumably promoting the formation of a functional enzyme (Yao et al., 2003). Thirdly, RNR is post-transcriptionally de-repressed by phosphorylation and degradation of the Sml1 repressor (Zhao et al., 2000).

A mammalian Sml1 orthologue has not yet been identified and nor does increased transcription of mammalian RNR lead directly to increased dNTP levels because the enzyme is strictly feedback-inhibited by its product, dATP (Akerblom

et al., 1981; Eriksson et al., 1979). (De-repression and transcriptional up-regulation are only effective in *S.cerevisiae* because of the uniquely relaxed feedback inhibition of the yeast RNR enzyme (Domkin et al., 2002).) Nonetheless, mammalian RNR may still be up-regulated by relocalisation and/or damage-induced transcription of alternative subunits with higher thresholds for allosteric inhibition. The constitutive mammalian RNR is a tetramer formed from two homodimers of hRMM1 and hRMM2 but there is accumulating evidence for RNR up-regulation centring on a third subunit, p53R2. The gene encoding p53R2 is transcriptionally induced via p53 after DNA damage (Tanaka et al., 2000) and p53R2 forms a functional enzyme with hRMM1 (Guittet et al., 2001). Although this was not actually proven to increase intracellular dNTP levels, the inhibition of p53R2 did reduce the overall activity of RNR after DNA damage, so hRRM1/p53R2 probably does have higher activity than the constitutive enzyme. Simultaneously, a second enzyme involved in nucleotide metabolism, adenosine deaminase (ADA), is up-regulated by p73 (Tullo et al., 2003) and this may help to reduce the allosteric inhibition of RNR by converting deoxyadenosine to deoxyinosine. Without ADA, more deoxyadenosine is converted to dATP instead: dATP inhibits RNR and a deficit of ADA accordingly leads to inhibition of both DNA replication and repair (Bemi et al., 1998; Camici et al., 1995). In a second p53-dependent mechanism, p53R2 and hRRM2 are both constitutively bound to p53 but released upon DNA damage, allowing them to bind to the large subunit hRRM1 (Xue et al., 2003). Finally, the whole enzyme translocates to the nucleus after DNA damage (Tanaka et al., 2000; Xue et al., 2003), but it remains unclear whether this is checkpoint-regulated or has any impact on RNR activity.

Since most of these potential mechanisms for RNR up-regulation are p53 dependent, they would not be seen in p53-deficient HeLa cells. Any further investigation of dNTP regulation as part of the S phase checkpoint should be carried out in p53-positive cells, but to reproduce the experiment outlined above in primary cells would require good synchronisation by serum starvation or alternative non-checkpoint-activating methods.

In *S.cerevisiae,* up-regulation of dNTP synthesis facilitates DNA repair and the survival of DNA damage but it also increases mutation rates - even during normal replication in undamaged cells if RNR is falsely up-regulated (Chabes et al., 2003). Conversely, Mec1 mutant cells which have lower-than-normal dNTP levels because Sml1 is not degraded during S phase have problems replicating through RSZs. Thus is appears that the optimal level of dNTPs for a cell is a trade-off between efficient DNA synthesis and mutagenesis. For repair synthesis, this optimal level is raised - perhaps because translesion polymerases have higher K_m values for translesion bypass than replicative polymerases have for normal replication (Minko et al., 2003). Furthermore, polη, for example, also has a higher K_m for correct than for incorrect lesion bypass (Johnson et al., 2001). Therefore, raising dNTP levels when DNA is damaged would promote efficient and accurate repair and this may outweigh the risk of mismatches being inserted by replicative polymerases or by the inappropriate action of translesion polymerases. Whether the same balance of risks holds true for metazoans remains to be seen. It is possible that mutagenic replication would carry too great a risk of cancer-causing genomic instability for dNTP levels to be elevated further than normal during S phase. p53R2 would still be needed for the survival of DNA damage, but only to promote efficient DNA repair outside S phase, when dNTP

levels are normally very low (Tanaka et al., 2000). For example, global NER after UV damage has already been shown to require functional p53 (Ford and Hanawalt, 1997) and the expression of ectopic p53R2 can reduce the damage-sensitivity of $p53^{-/-}$ cells with RRM2 knocked down (Lin et al., 2004b). Within S phase, however, when dNTP levels are normally elevated anyway, p53R2 may not raise levels any further as part of the intra-S phase checkpoint. Detailed experiments in synchronised primary cells both in G1 and within S phase would be needed to clarify this issue.

Chapter 4:

DNA fibre studies of S phase parameters in response to DNA damage

The results in chapter 3 show that DNA synthesis *is* reduced as part of the intra-S phase checkpoint in mammalian cells, but they do not address all the possible mechanisms for this response. To investigate this in more detail, the DNA fibre labelling (DIRVISH) technique (Jackson and Pombo, 1998) has been adapted such that two distinguishable modified nucleotides, IdU and CldU (Aten et al., 1992), could be used to label replication within a single S phase. In this technique (itself adapted from the classical DNA fibre autoradiography technique (Huberman and Riggs, 1968) in which newly replicated DNA is labelled with tritiated thymidine), cells are pulse-labelled with halogenated nucleotides, then collected and lysed on a glass slide. By tipping the slide, DNA from the cells is spread out primarily in the form of single fibres. This DNA is subsequently fixed, denatured and immuno-labelled to detect the halogenated nucleotides. In these experiments, all DNA was then counterstained in a third colour with YOYO-1 DNA dye, allowing the exclusion of any broken or tangled fibres. Consecutive pulse-labelling of S phase cells with IdU and then CldU yields double-fluorescently-labelled tracks on the DNA which can be interpreted unambiguously as either ongoing forks, newly-fired origins, terminations or fork stalling events, as shown in Fig.4. The length of any track after a given labelling period is proportional to its fork rate, while counting the relative numbers of different track forms can determine changes in the rates of origin firing or fork stalling after DNA damage.

DNA fibre assays were carried out after each of the three DNA damaging treatments examined by flow cytometry in Fig.1 in order to establish which parameter(s) of DNA synthesis contributed to the overall slowing of S phase. All of these experiments were performed in HeLa cells synchronised in early to mid S phase. This should exclude the cells which are constantly entering S phase in an asynchronous population, and which might skew the data by appearing as a large number of newly-fired origins. The use of cells at a fairly early stage in S phase should, however, give the maximum possibility of seeing a checkpoint-mediated suppression of later origins. With regard to fork movement, it is important to note that fork rates are not constant throughout S phase: they have previously been reported to increase from early to late S phase in a variety of mammalian cell lines (Housman and Huberman, 1975; Painter and Schaefer, 1971) and this was reproduced in the HeLa cells used here, as shown in Fig.5. However, so long as control and DNA-damaged cells are examined at the same stage in S phase, fork rates before and after damage should be comparable.

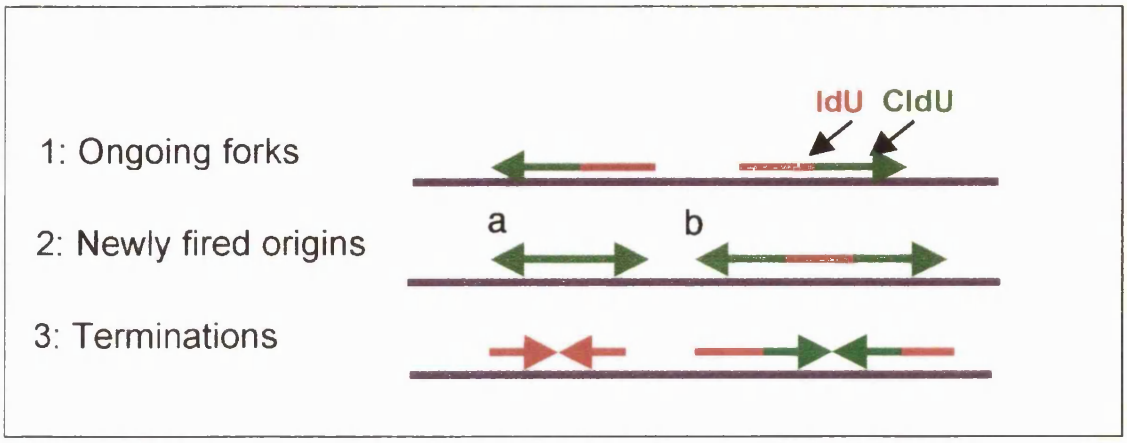
Figure 4: Replication track forms visualised by fibre labelling

a) Schematic of double-labelled replication tracks, IdU followed by CldU pulse-labels.

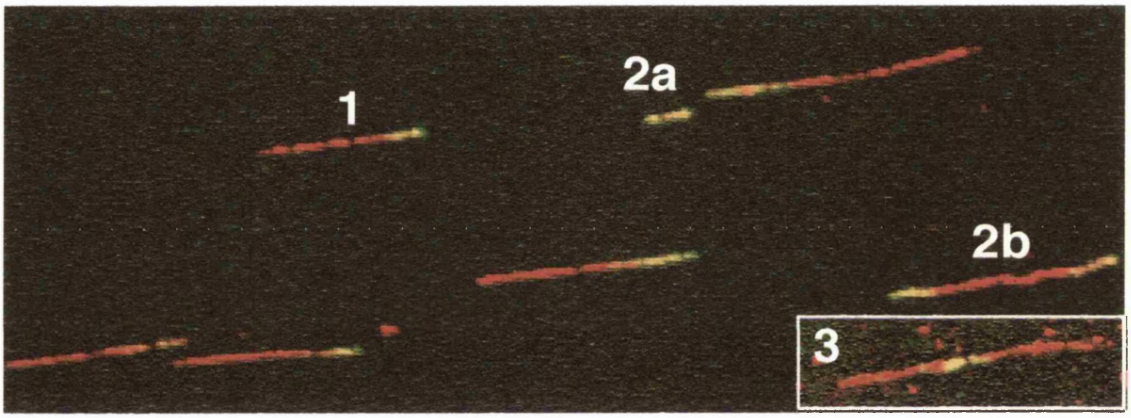
b) Example of labelled replication tracks on DNA fibres.

c) Schematic of alterations to replication tracks as a result of DNA damage.

a


IdU CldU
1: Ongoing forks
2: Newly fired origins
a
b
3: Terminations

b


1
2a
2b
3

c

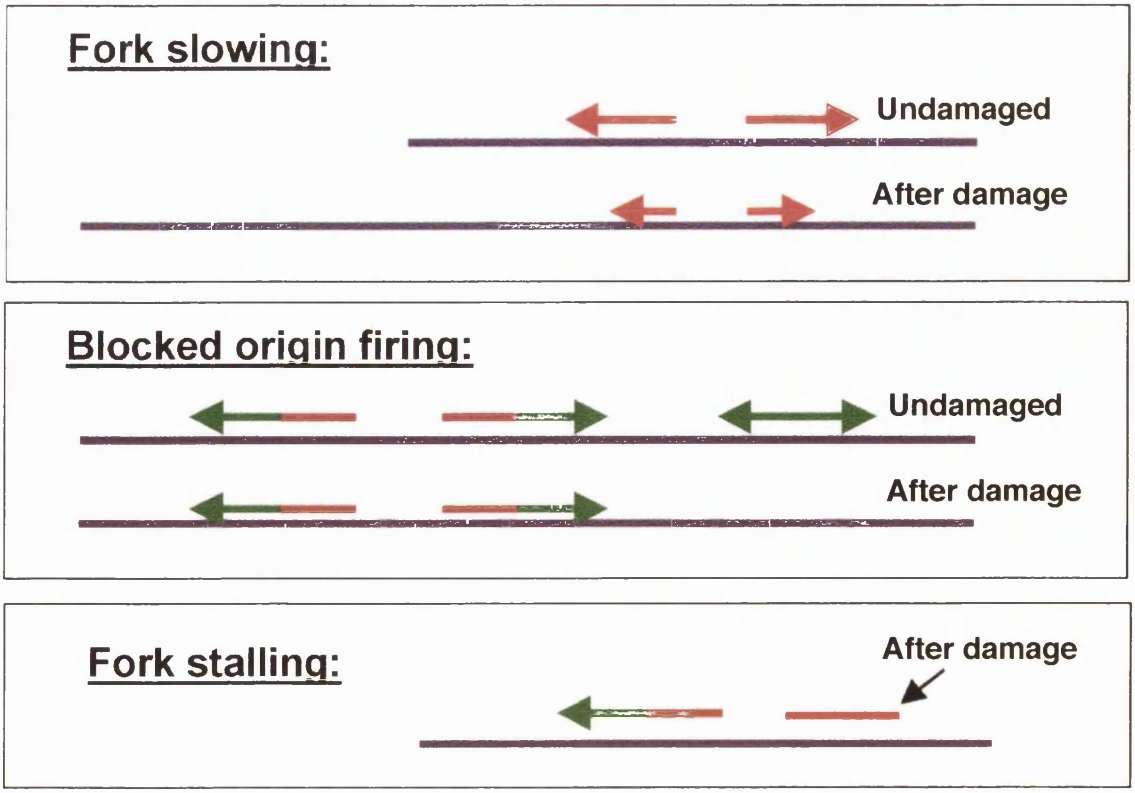
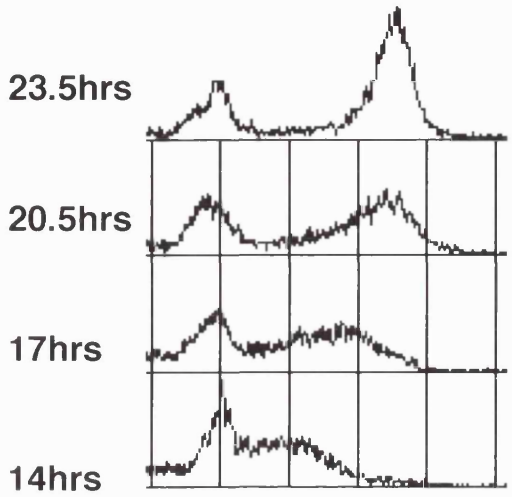

Fork slowing:
Undamaged
After damage
Blocked origin firing:
Undamaged
After damage
Fork stalling:
After damage

Figure 5: Replication fork rate increases from early to late S phase

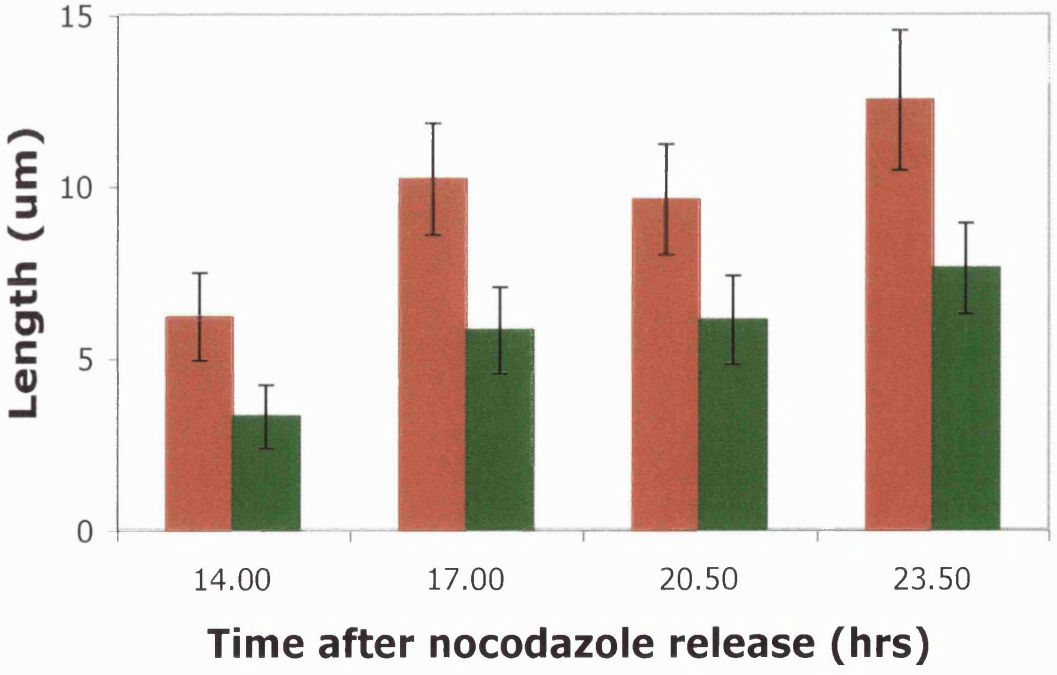
a) Flow cytometry showing progression of synchronised cells from early to late S phase.

b) Lengths of ongoing replication tracks at each of the timepoints shown in a), labelled for 10mins with 10μM IdU, then 20mins with 100μM CldU. The mean length of at least 50 replication tracks is plotted for each timepoint.

a


23.5hrs
20.5hrs
17hrs
14hrs

b


15
10
5
0
Length (um)
14.00
17.00
20.50
23.50
Time after nocodazole release (hrs)

4a) Rate of movement of ongoing forks:

Fork movement is reduced by MMS and HU but not by IR damage

In order to quantify any change in fork rates after DNA damage, cells were exposed to 20min pulses of MMS (0.005-0.03%) and then, after removal of the MMS, immediately labelled with IdU for 10-60mins before preparing DNA fibre spreads. Fig.6a shows examples of the tracks seen after a 20min labelling period with or without MMS damage. The mean length of at least 50 IdU-labelled tracks was then calculated for each labelling period, using Zeiss LSM measurement software, as shown in Fig.6b. Control experiments indicated that IdU equilibrates very rapidly with the intracellular pool of dNTPs - pulses as short as 1min resulted in efficient track labelling – so the lengths of IdU-containing tracks should be directly proportional to fork rate. The graphs in Fig.7 are therefore plotted from time 0. However, the collection of samples at the end of the labelling period may give rise to a small but consistent inaccuracy since trypsinisation and chilling of cells takes about 3mins at the end of any labelling period and replication may not be efficiently stopped until the cells are on ice.

Fig.7a shows that fork rates were reduced for at least 60 minutes after more severe MMS treatments. The severity of slowing was correlated with the MMS dose but slowing was only observed above ~0.01% MMS. Fig. 7b shows that reduction of cellular dNTP pools by treatment with HU also slows replication forks in a dose-dependent manner. When cells were treated with sufficiently high levels of HU (above ~100μM), forks were essentially stalled and very little progression occurred over several hours (data not shown). By contrast to MMS

and HU, IR did not cause detectable fork-slowing, even at doses which do cause a delay in overall S phase progression. Fig.7c shows no significant change in the mean lengths of tracks labelled for 20mins after IR exposures of up to 5Gy.

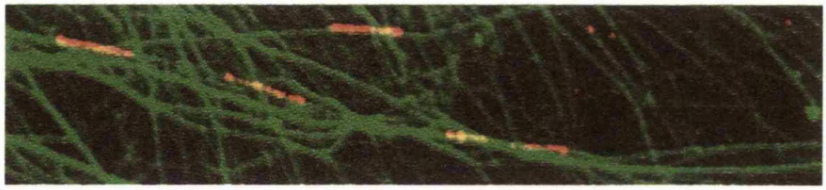
Figure 6: Track lengths are reduced by MMS treatment

a) Examples of single-labelled tracks after MMS damage at the levels indicated (labelling = 20min pulse labels with IdU).

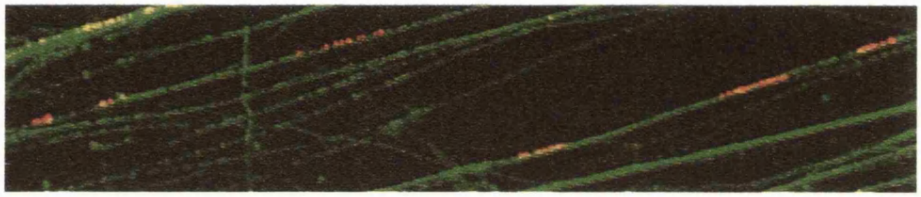
b) Measurement of track lengths using Zeiss software.

a

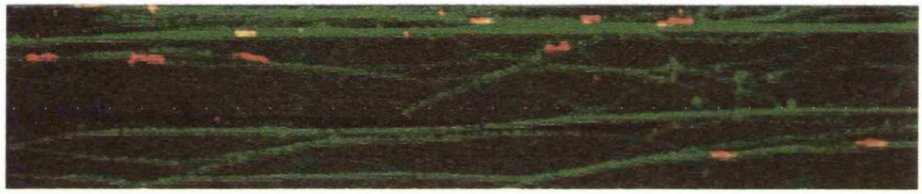
Control:
20mins IdU



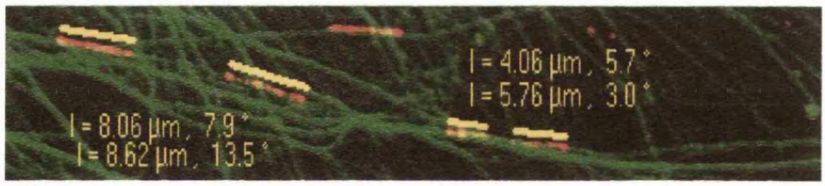
0.005% MMS:
20mins IdU

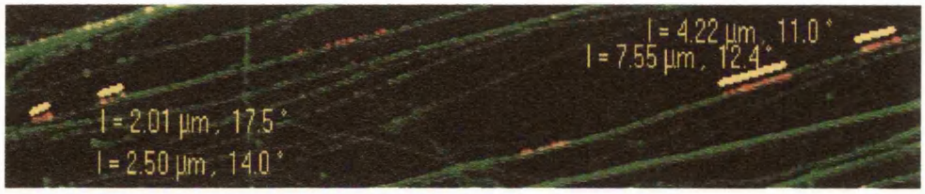


0.02% MMS:
20mins IdU



b





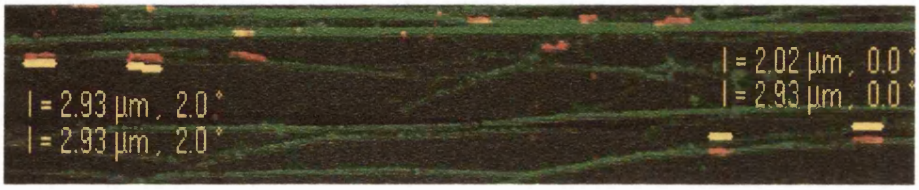
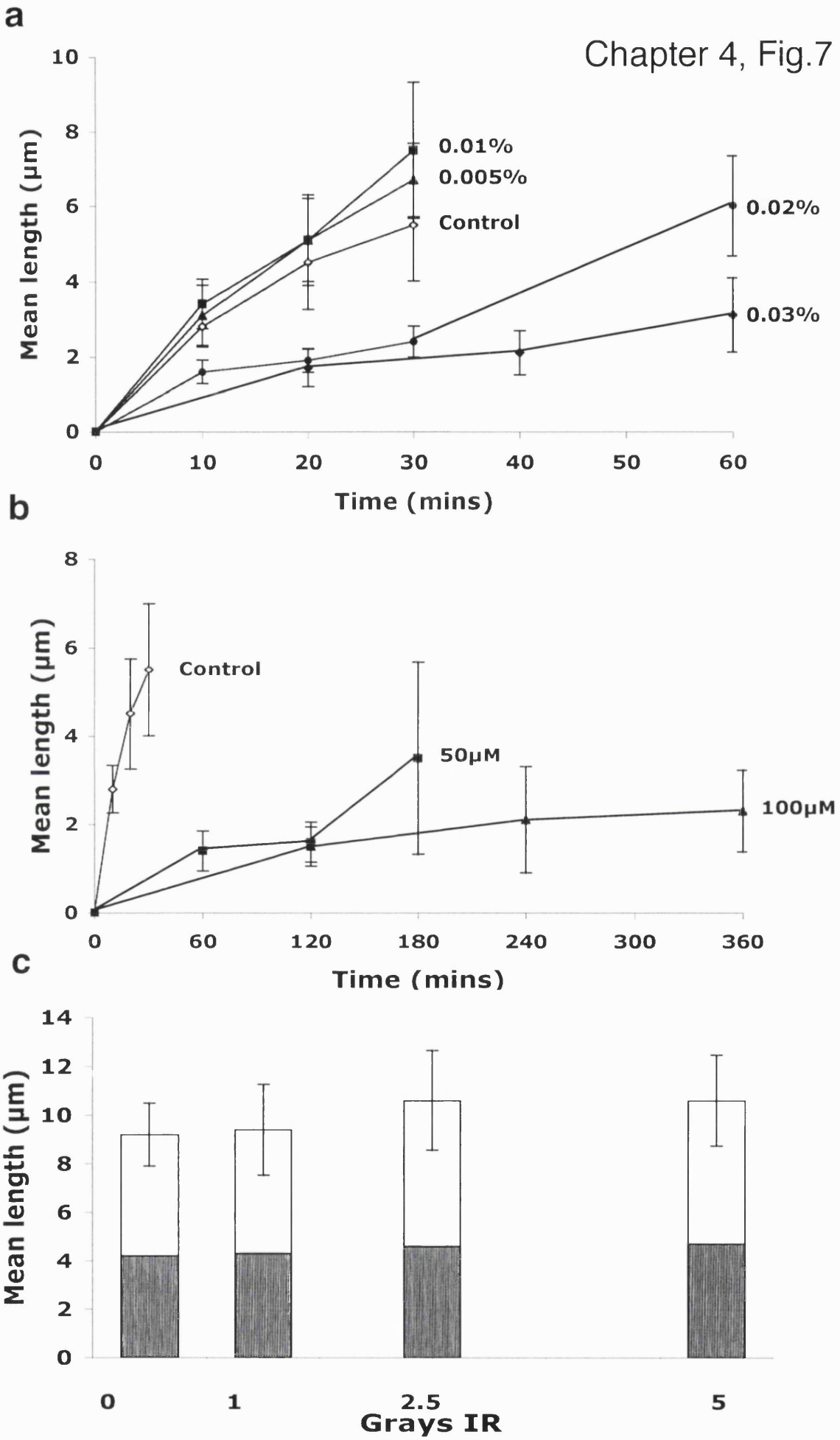


Figure 7: Reduced fork progression after DNA damage

a) Cells were treated with 0.005%-0.03% MMS for 20mins, then MMS was removed and cells were labelled with 50μM IdU for 10-60mins before preparing DNA fibres. The mean length of at least 50 replication tracks is plotted for each timepoint.

b) Synchronised cells in early S phase were labelled with 10μM IdU for 10mins, then 50-100μM HU was added and the IdU was replaced with 100 μM CldU. The mean length of track extension in CldU over the subsequent 6hrs is plotted for each HU concentration.

c) Synchronised cells in early S phase were labelled with 10μM IdU for 10mins, exposed to 1-5Gy IR, then labelled with 100μM CldU for 20mins. The mean total lengths of at least 50 unidirectional (red-then-green) tracks are plotted with the white portions of each bar representing the CldU-labelled length (replicated after IR exposure).


a
Mean length (µm)
Time (mins)
0.01%
0.005%
Control
0.02%
0.03%
b
Mean length (µm)
Time (mins)
Control
50µM
100µM
c
Mean length (µm)
Grays IR

4b) Suppression of origin firing:

Origin firing is rapidly inhibited after IR, MMS or HU

DNA fibre labelling can be used to distinguish newly-fired origins from ongoing forks using the double-labelling protocol outlined in Fig.8a. Active replication forks prior to damage were labelled with IdU (red), cells were then treated with damage and the IdU was replaced with CldU (green). During the subsequent 20 minutes, any newly-fired origins will generate tracks labelled along their entire length with CldU and these can be counted against the number of double-labelled (ongoing) forks which were tagged with IdU prior to damage. (The changeover between IdU and CldU in double-labelling experiments is not instantaneous – presumably because it takes some time for the second nucleotide to replace the first in the intracellular pool of dNTPs. Control experiments showed that a very short (1min) pulse with IdU can give detectable labelling for 20mins or more if it is not chased by CldU. However, using ten times more CldU than IdU forced a fairly sharp changeover between the red and green immuno-labelled tracks which appear on the DNA fibres.)

Fig. 8b shows that origin firing was inhibited in response to MMS and that the severity of inhibition was dose dependent over the entire range tested (20min pulses of MMS at 0.005%-0.02%). Exposure to IR also inhibited origin firing but unlike the response to MMS, this may show a threshold between 1 and 2.5 Grays (Fig.8c). No further decrease in origin firing was then seen after IR exposures up to 10Gy (data not shown). The damage-insensitive subset of initiation events - seen after the maximum doses of both IR and MMS damage - may represent the proportion of the total origins which are already committed to fire within this 20

minute labelling period at the time of damage. Alternatively, checkpoint-mediated suppression of origin firing may never be 100% efficient: in DNA size sedimentation, some small DNA fragments always remain at any time after a DNA damaging treatment (Heffernan et al., 2002; Painter and Young, 1980), although it is not clear whether these actually all represent new replicons.

The response of cells to HU was also tested in this origin-blocking assay, since nucleotide depletion has been shown to inhibit origin firing via the S phase checkpoint in *S.cerevisiae* (Santocanale and Diffley, 1998a; Shirahige et al., 1998; Tercero and Diffley, 2001). In higher eukaryotes the S phase checkpoint response to HU had not been tested but aphidicolin, which similarly stalls replication by inhibiting DNA polymerases, does inhibit the appearance of late S-phase foci in CHO cells (Dimitrova and Gilbert, 2000a).

Replication forks were pre-labelled for 10mins with IdU as before, then the IdU was replaced with CldU together with 250μM HU. The accumulation of new (CldU-labelled) origins was then counted against the IdU-tagged ongoing tracks over the subsequent 2-6hrs. Fig.8d shows that origin firing is greatly reduced in the presence of HU, such that it takes 6 hours to accumulate the same number of origin firing events which occur in control cells in less than 1 hour. It is unlikely that many new origins *did* fire but were simply not labelled due to nucleotide depletion because most existing forks were able to progress, incorporating CldU, for a further 1-2μm over the 6hrs of HU arrest. In order to confirm this, the experiment was repeated using only 50μM HU – a concentration which allows existing forks to elongate more extensively, growing by 3-4μm over 3hrs. As before, new origin firing was severely inhibited (Fig.8d). Because of a recent report indicating an increase in origin firing after treatment of a modified

hamster fibroblast cell line with HU (Anglana et al., 2003), this experiment was repeated using primary human fibroblasts instead of HeLa cells and a similar inhibition of new origin firing was observed (Fig.8e).

Figure 8: Inhibition of origin firing after DNA damage

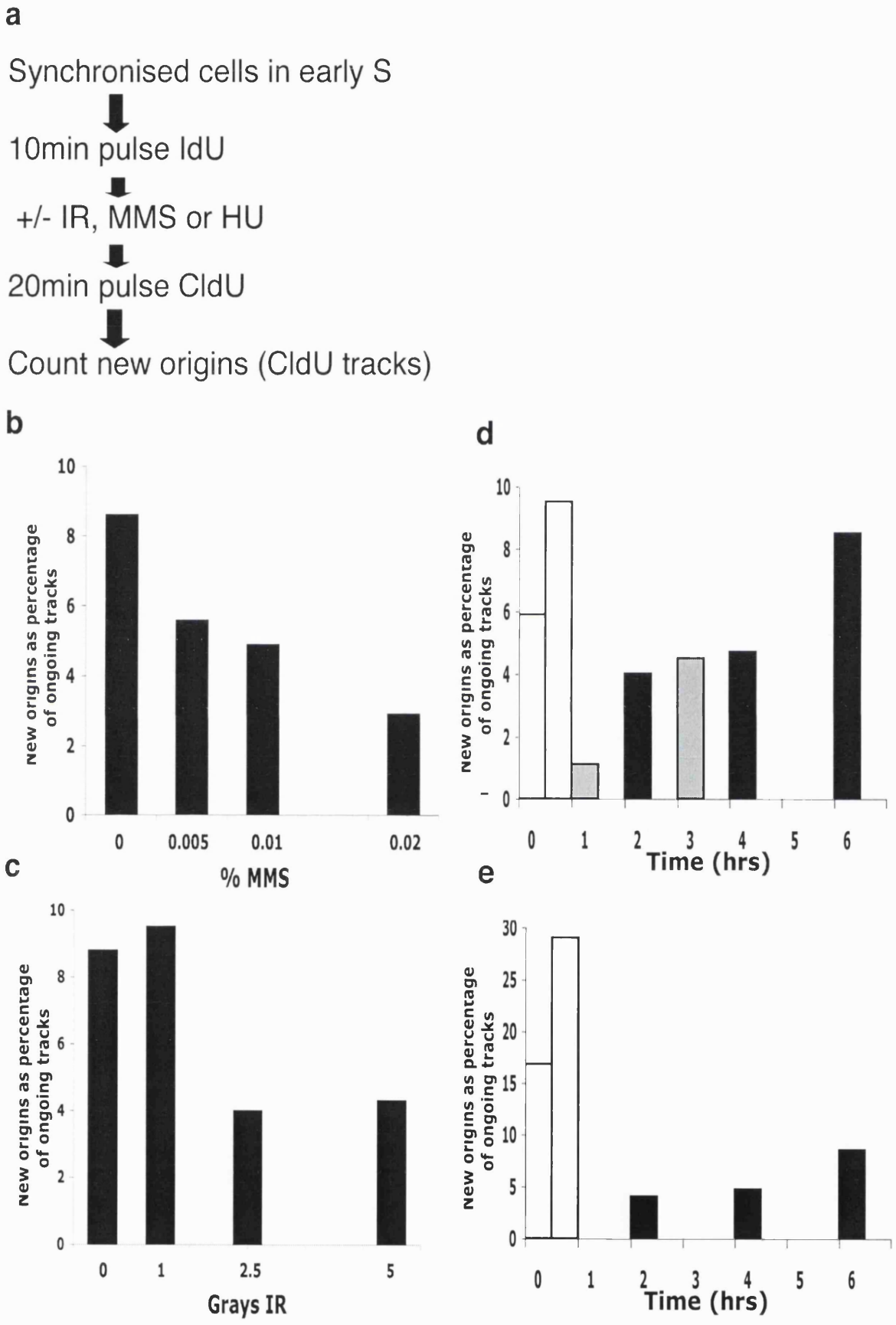
a) Outline of protocol for measuring origin firing within 20mins of DNA damage.

b) The protocol in A was used to quantify origin firing after 20min pulses of MMS (0.005%-0.02%). The graph shows pooled data from two independent experiments; at least 100 replication tracks were counted in each experiment for each MMS level.

c) As in B, using 1-5Gy IR instead of MMS.

d) As in B, pre-labelling with 20μM not 10μM IdU and adding 50 or 250μM HU together with the CldU directly after this pre-label. Newly fired (CldU-labelled) origins were then allowed to accumulate for 30-50mins (control: white bars), 1-3hrs (50μM HU: grey bars or 2-6hrs (250μM HU: black bars) and were quantified, as before, as a percentage of ongoing tracks.

e) As in D, using unsynchronised IMR90 cells instead of HeLa cells.


a
Synchronised cells in early S
10min pulse IdU
+/- IR, MMS or HU
20min pulse CldU
Count new origins (CldU tracks)
b
New origins as percentage of ongoing tracks
% MMS
0
0.005
0.01
0.02
c
Grays IR
0
1
2.5
5
d
Time (hrs)
e
Time (hrs)

4c) Recovery in origin firing after damage removal:

Origin firing recovers at different rates after IR, MMS and HU

The experiments in Fig.8 only address the changes to origin firing within 20mins of DNA damage. This is unlikely to measure the full extent of the checkpoint response, since in DNA size sedimentation experiments, the small DNA fragments assumed to represent new replicons do not reappear until at least 90mins after either IR or UV damage (Heffernan et al., 2002). Furthermore, in the first 20mins directly after damage, a significant proportion of origins may already be committed to firing - RDS assays, by contrast, are usually carried out at least 30mins after DNA damage is applied. However, in order to make a valid count of all newly-fired origins from the moment of DNA damage, it was necessary to fix cells after about 20mins, before these new replicons began to join up, either with each other or with ongoing unidirectional forks. The mixed tracks generated by merger of several replicons cannot be interpreted unambiguously and are excluded during counting. Therefore, a modified version of the assay was developed to assess the persistence of the block to origin firing over longer periods, and the rate at which replication recovers after different forms of DNA damage.

In the initial experimental design, synchronised cells were labelled briefly with IdU in early S phase to tag all the replication forks operating pre-damage, then damage was carried out and CldU pulse-labels were added 1, 2 or 4hrs later. The number of CldU-labelled tracks was counted and expressed as a proportion of the (theoretically constant) number of pre-damage IdU-labelled tracks. However, this method was not entirely valid after treatments with MMS or HU which cause

fork-slowing as well as affecting origin firing. After 0.03% MMS, for example, the additional fork-slowing phenomenon could cause serious differences in the S phase dynamics of damaged cells compared to control cells. MMS-treated cells will, for example, have a higher proportion of slowly-progressing tracks which still get double-labelled with IdU *and* CldU even if the two pulse-labels are separated by several hours. One way of avoiding this problem is to maintain the IdU label throughout the DNA damage and subsequent periods, such that all ongoing replication gets labelled with IdU *and,* subsequently, CldU at the growing ends. New origins which fired during the CldU pulse would be exclusively single-labelled and could be counted as a proportion of ongoing (double-labelled) stretches of DNA. However, these experiments proved very difficult to interpret due to the very small number of origins which fire during the 20min CldU pulse-label compared to the very large amounts of IdU-labelling which accumulate in undamaged cells.

The protocol was therefore modified once again, as outlined in Fig.9a. Active replication forks were tagged with a pulse of IdU prior to DNA damage (Fig.9a, tracks labelled 'a'), then the IdU was washed out before MMS or IR were applied. This generates exclusively-IdU-labelled tracks representing the number of active replication forks before DNA damage. At timepoints from 1.5 to 4.5hrs later, cells were then double-labelled with consecutive pulses of IdU (red) and CldU (green). This protocol distinguishes any new origins actually firing at each timepoint (exclusively green or green at both ends: labelled 'c' in Fig.9a) from ongoing replication forks (red-then-green: 'b' in Fig.9a). These new origins were then counted against the exclusively-red tracks which form an internal control since they had been tagged identically in all the cells before any DNA damage.

(The number of red tracks representing termination events during the second IdU label should only be a very small proportion of the total.)

Fig.9b shows that a 20min pulse of 0.01% MMS elicited a sustained block to origin firing when compared to the origin firing occurring in undamaged cells: origin firing recovered to only a very limited extent during at least 4.5hrs after the MMS treatment. By comparison, 5Gy IR (Fig.9c) caused a much more transient block to origin firing with significant recovery after only 1.5hrs. By 3hrs post-IR exposure, origin firing had returned to normal levels.

The efficiency of origin firing recovery was also assessed after release from an HU arrest. As before, replication forks were pre-labelled with IdU, then completely arrested by adding a high level of HU for 1-4hrs. Upon release from HU, the IdU was replaced with CldU and new origins fired within 1hr were counted against the pre-labelled tracks. By comparison to either IR or MMS damage, origin firing recovered relatively well after a brief (1hr) HU arrest, but recovery became progressively less efficient after longer periods (2-4hrs) (Fig 9e). This is unlikely to be an artefact due to under-detection of CldU-labelled tracks after HU release, since the nucleotide balance within the cells recovered sufficiently fast to allow the origins which did fire to elongate by ~3μm within 30mins and 6μm within 60mins (data not shown).

Figure 9: Recovery of origin firing after DNA damage

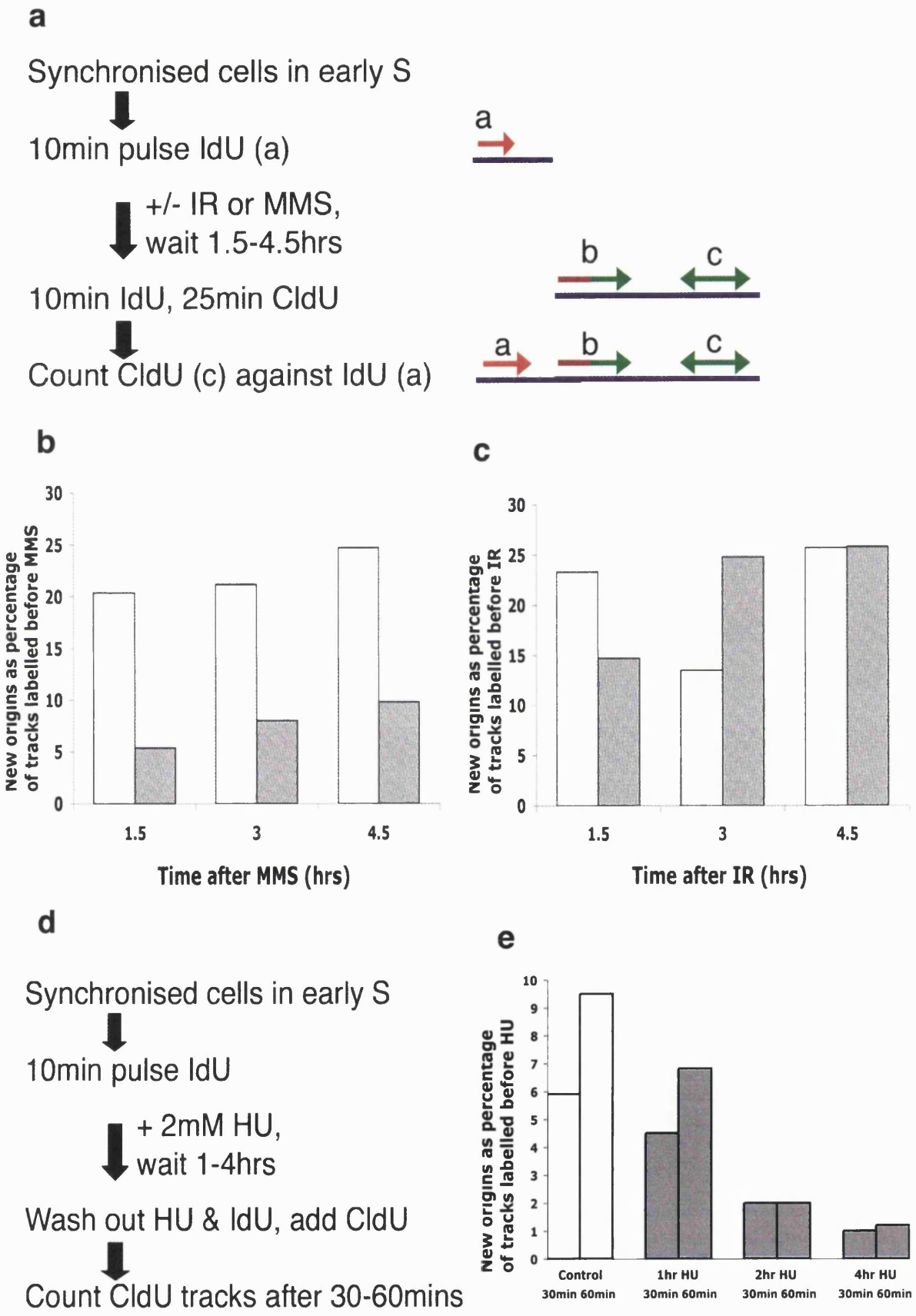
a) Outline of protocol to measure origin firing at timepoints up to 4.5hrs after DNA damage. New origins are counted as a percentage of tracks pre-labelled with IdU before damage.

b) The protocol in A was used to quantify origin firing 1.5-4.5hrs after a 20min pulse of 0.01% MMS (grey bars), and also in undamaged cells (white bars). The graph shows pooled data from two independent experiments; at least 100 replication tracks were counted in each experiment for each MMS level.

c) As in B, using 5Gy IR instead of MMS.

d) Outline of protocol to assess recovery of origin firing after 1-4hrs of HU arrest.

e) The protocol in D was used to quantify origin firing within 30 or 60mins of release from 2mM HU. The graph shows pooled data from two independent experiments; at least 100 replication tracks were counted in each experiment for each MMS level.


a
Synchronised cells in early S
10min pulse IdU (a)
+/- IR or MMS,
wait 1.5-4.5hrs
10min IdU, 25min CldU
Count CldU (c) against IdU (a)
a
b
c
a
b
c
b
New origins as percentage of tracks labelled before MMS
30
25
20
15
10
5
0
1.5
3
4.5
Time after MMS (hrs)
c
New origins as percentage of tracks labelled before IR
30
25
20
15
10
5
0
1.5
3
4.5
Time after IR (hrs)
d
Synchronised cells in early S
10min pulse IdU
+ 2mM HU,
wait 1-4hrs
Wash out HU & IdU, add CldU
Count CldU tracks after 30-60mins
e
New origins as percentage of tracks labelled before HU
10
9
8
7
6
5
4
3
2
1
0
Control
30min 60min
1hr HU
30min 60min
2hr HU
30min 60min
4hr HU
30min 60min

4d) Fork stalling:

Replication forks stall at a high rate after MMS and HU but not IR

The slowing of replication forks after MMS damage which was documented in Fig.7 could result from at least two distinct modes of altered fork progression. DNA damage may provoke a pan-nuclear change to a slower mode of replication, for example by modification of all replication forks or a change to a different DNA polymerase. Alternatively, there could simply be a series of transient stalling events at each fork in isolation as it encounters successive DNA lesions. If such fork stalling does occur within the timeframe of a double-labelling experiment (see Fig 8a), it should be detectable in the form of IdU-labelled tracks which fail to incorporate the subsequent 20-minute pulse of CldU because they are currently stalled. These events will therefore appear as an elevated number of red-only tracks (Fig.10a).

When the percentage of these red-only tracks was counted, a significant level of fork stalling was indeed found after higher MMS treatments (Fig.10b), supporting the hypothesis that fork slowing occurs via stochastic stalling events. By contrast, IR did not cause significant fork stalling, consistent with the lack of overall fork slowing after IR damage (Fig.10c).

In the case of HU treatment, all forks are essentially stalled by sufficiently high levels of HU. In lower levels of HU, however, replication does proceed at reduced speed (Fig 7b) and in this situation there is elevated fork stalling, detectable in as little as 5μM HU and increasing in a dose-dependent fashion to very high levels when S phase cells are subjected to 20 or 50μM HU (Fig 10d).

Figure 10: Fork stalling after DNA damage

a) Example of a red-only track (stalled fork).

b) From the labelling protocol outlined in Fig.4A, tracks labelled with IdU-only were counted as a percentage of the total number of tracks after MMS damage. (A proportion of these will represent terminations (see Fig.1) but any significant increase over the control percentage is taken as evidence of fork stalling.)

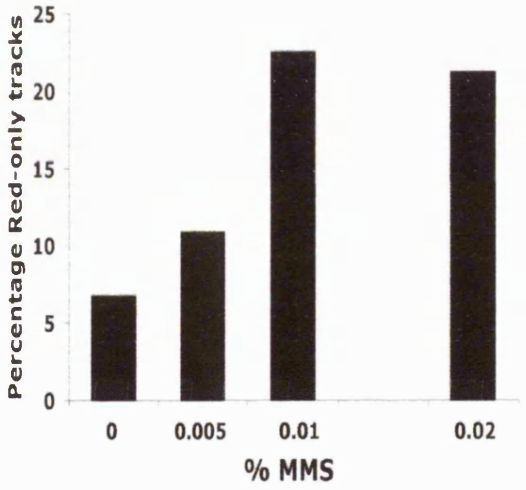
c) As in A, using 1-5Gy IR instead of MMS.

c) As in A, labelling cells after 3hrs of replication in low levels of HU.

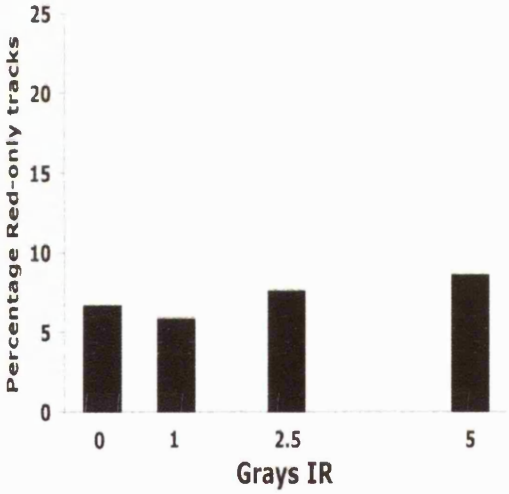
a


Stalled
fork

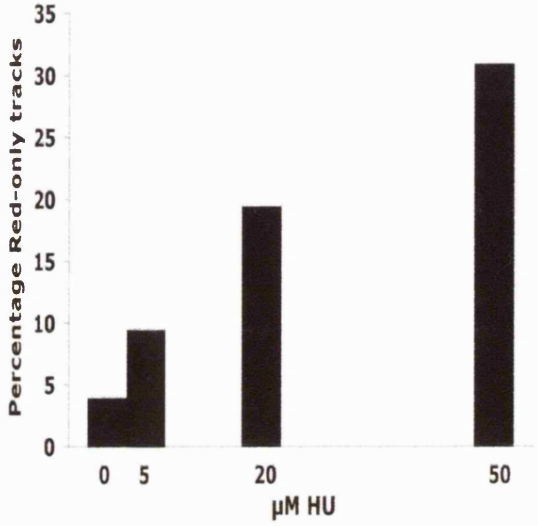
b


Percentage Red-only tracks
25
20
15
10
5
0
0
0.005
0.01
0.02
% MMS

c


Percentage Red-only tracks
25
20
15
10
5
0
0
1
2.5
5
Grays IR

d


Percentage Red-only tracks
35
30
25
20
15
10
5
0
0
5
20
50
μM HU

Chapter 4: Discussion

These DNA fibre labelling assays confirm and extend the results obtained from flow cytometry and the examination of replication foci. The work comprises the first systematic investigation of all the various parameters which determine the rate of DNA synthesis in mammalian cells during S phase and the ways in which these parameters are affected by DNA damage. The fibre labelling technique developed here is an improvement on other methods that have been used to investigate the S phase checkpoint because it unambiguously separates changes in the rate of origin firing from changes in the rates of fork movement and fork stalling. Using this technique, each of these parameters can be examined quantitatively and under comparable conditions - using the same experimental method throughout. (Labelling cells with short pulses of modified nucleotides does not in itself perturb S phase (Hamlin, 1978) or activate the S phase checkpoint in yeast (Vernis et al., 2003), so the technique should measure only changes in DNA synthesis which are induced by IR, MMS or HU.) Fibre labelling also offers the advantage of revealing replication dynamics on the level of individual forks rather than as an average of an entire cell population. It does not allow any analysis of replicon clustering in relation to higher-order chromatin or nuclear structure, but it does allow subtle yet potentially important effects on a minority of individual forks to be detected and quantified.

The effects of IR, MMS and HU on replication dynamics:

Mammalian cells compared to *S.cerevisiae.*

This data shows that different forms of DNA damage affect replication in different ways. Flow cytometry showed that moderate levels of ionising radiation, alkylation by MMS or nucleotide depletion by HU can all slow down the overall progression of S phase. DNA fibre labelling, however, reveals that this occurs differently after different forms of DNA damage. In the case of IR, S phase slowing appears to be entirely due to a rapid but fairly transient block to origin firing. Alkylation by MMS elicits a similar block to origin firing but this persists for much longer after the removal of the drug than does the block to origin firing after IR. MMS also causes additional changes to replication: a general dose-dependent slowing of fork movement and the stalling of many forks for significant periods, phenomena that are not observed after levels of IR which block origin firing to a similar extent. This explains the observation from flow cytometry that MMS can cause a much more severe delay in S phase progression than IR. The continual stalling of forks probably slows DNA synthesis in itself and also provides a continuous stimulus for checkpoint-mediated suppression of origin firing.

The changes to replication observed here in HeLa cells are essentially similar to the responses observed in checkpoint-competent *S.cerevisiae.* Exposure of synchronised yeast cells to IR during S phase results in an extension of S phase, probably due to reduced origin firing (King et al., 2003). The response of yeast to MMS or HU involves reduced fork movement and fork stalling as well as blocked origin firing (Shirahige et al., 1998; Sogo et al., 2002; Tercero and Diffley, 2001): all the same phenomena as are observed here in MMS or HU-

treated mammalian cells. In *S.cerevisiae*, the relative checkpoint dependence of each of these phenomena has been established: blocked origin firing depends on the Mec1 and Rad53 checkpoint kinases (Santocanale and Diffley, 1998a; Shirahige et al., 1998) and the same proteins are responsible for maintaining stalled forks in a stable state (Lopes et al., 2001b; Sogo et al., 2002; Tercero and Diffley, 2001). By contrast, the slowing of fork movement is independent of Mec1/Rad53 and has been proposed to be a direct physical result of replisomes encountering alkylated bases or their repair intermediates on DNA (Tercero and Diffley, 2001). A degree of ambiguity remains, however, as to which of the same replication phenomena are actually dependent on the Mec1 homologs ATM and ATR in human cells, and this issue is addressed in chapter 5.

Further insights into replication dynamics obtained from DNA fibre labelling

The fibre labelling technique offers a quantitative assessment of replication dynamics with detailed time resolution: it therefore lends itself to the analysis of both dose-dependent and time-dependent effects. This has revealed several aspects of the S phase response to DNA damage which were not apparent from RDS experiments, nor from the population studies previously carried out in *S.cerevisiae*.

The slowing of replication forks after MMS treatment, for example, has a non-linear dose-dependence. It is possible that this is due to a thresholded checkpoint response which acts *in trans* to slow down all ongoing forks once a critical level of DNA damage is detected; however, a threshold at comparable levels of MMS was not detected in the origin-blocking response (suggesting that if a checkpoint *is* responsible for both origin-blocking and fork-slowing, the two

must at least be differently thresholded). Instead, it is likely that fork-slowing is a direct result of replication forks encountering DNA lesions (Tercero and Diffley, 2001). After lower levels of MMS damage, these lesions may be cleared by methods such as base excision repair sufficiently fast that they are not detected by the subsequent fibre-labelling assay, although Fig.8 would suggest that they do generate a sufficient checkpoint signal to inhibit origin firing. At higher levels of MMS, however, repair may become saturated and alkylated lesions and/or repair intermediates may therefore accumulate on the DNA. This would be consistent with the slight recovery in fork rates seen at later times after intermediate MMS treatments (Fig.7a), since the accumulated fork-blocking lesions would presumably be progressively removed over time. A similar phenomenon has been observed when the replication products from UV-damaged DNA are separated by size sedimentation (Heffernan et al., 2002). This showed that origin firing was somewhat blocked even after very low UV doses ($1J/m^2$) whereas the elongation of longer fragments (representing ongoing forks) was only reduced after a higher dose of UV. However, elongation was severely reduced even after $1J/m^2$ in cells lacking the NER pathway, suggesting that fork progression depends on efficient DNA repair.

Ionising radiation differs from MMS in that no fork-slowing was detected after IR doses of up to 5Gy. This is again consistent with previous studies using DNA size sedimentation which showed that low doses of IR reduced the number of small DNA fragments (newly-fired replicons) without altering the sizes of larger fragments (ongoing forks) (Heffernan et al., 2002; Watanabe, 1974). If approximately 35 double-strand breaks are induced per Gy (Rothkamm and Lobrich, 2003) then these would be far too infrequent to be detected as fork-

blocking lesions, and broken DNA strands are in any case excluded when the data is collected. However, IR is also thought to cause many single-stranded breaks and other more minor DNA lesions. In striking contrast to the persistent lesions caused by MMS, it would appear that any single-strand lesions induced by IR are either too sparse to be detected, *or* that they do not impede fork movement for any significant length of time, *or* that they are repaired extremely rapidly. If ATM and/or ATR genuinely do have roles in promoting specific DNA damage repair pathways, as well as reducing origin firing, then this situation may change in cells lacking specific checkpoint pathways, as discussed in Chapter 5.

Regarding origin firing, it is notable that initiation events are blocked in a dose-dependent manner after both IR and MMS damage, but the response to IR appears to be thresholded between 1 and 2.5 Gy whereas the response to MMS increases linearly over the entire range tested. This may be due to the fact that IR damage is transduced via ATM and MMS damage via ATR (Abraham, 2001). ATM is activated via a rapid and sensitive signalling cascade involving intermolecular autophosphorylation and dissociation of ATM dimers after a global change in chromatin structure. ATM then acts on its checkpoint-signalling substrates such as CHK2 in a manner that is only weakly dependent on the number of dsbs in the cell. By contrast, ATR is probably activated stochastically at stretches of ssDNA which do not induce any global changes to chromatin, so an all-or-nothing checkpoint response involving ATR would not be expected. Experiments in yeast indicate that the activation of Rad53 in response to HU and MMS requires some threshold number of forks (Shimada et al., 2002) but that above this threshold, there is capacity for differential activation of Rad53

depending on the number of stalled replication forks (Tercero et al., 2003). The fact that ATR seems to be similarly involved in sensing the level of ssDNA in replicating *Xenopus* DNA (Marheineke and Hyrien, 2004; Shechter et al., 2004) would suggest that this capacity is conserved in higher organisms, and this too is investigated in mammalian cells in Chapter 5.

Finally, when cells are treated with HU, fork rates are reduced and forks stall at an elevated rate - as might be expected because HU inhibits RNR and therefore depletes the cell of dNTPs (Adams and Lindsay, 1967). However, it is unlikely that forks stall because they simply run out of dNTPs. It has been shown in yeast that HU-arrested cells are not completely dNTP-depleted, rather, ongoing forks are stalled and the checkpoint is activated before dNTPs pools drop to G1 levels (Koc et al., 2004). Compensatory salvage makes it difficult to carry out the same analysis conclusively in mammalian cells, but there is some evidence that basal dNTP pools are preserved in mammalian cells as well (Snyder, 1984). There are several possible explanations for this. Firstly, as dNTP levels fall and the equilibrium promoting the forward movement of polymerases shifts, helicase activity ahead of the replication fork may not slow down in concert with slowed polymerases. Excess ssDNA would be therefore exposed ahead of the fork, activating the ssDNA/ATR-dependent checkpoint. Secondly, slow-moving forks might stall stochastically, particularly at RSZs or DNA secondary structures. This too would activate the checkpoint to stabilise the stalled forks and also to prevent further origin firing. Alternatively, the cell may actually sense suboptimal dNTP levels and actively arrest DNA synthesis at ongoing forks, simultaneously activating the checkpoint. The fact that *rad53* mutants still preserve dNTP levels

identically to wild-type yeast supports the existence of an active, checkpoint-independent mechanism for stalling replication forks (Koc et al., 2004), although it remains possible that forks stall passively at merely suboptimal dNTP levels, and that origins then fail to fire, also passively, regardless of checkpoint activity. If an active fork-arresting mechanism does exist, it must respond to dNTP levels themselves, rather than simply to the presence of HU, because cells arrested in G1 do not show checkpoint activation when treated with HU (Murakami et al., 2002) and if extra dNTPs are accumulated during a G1/S arrest, they can delay the subsequent stalling of forks in S phase despite the presence of HU (Koc et al., 2004). Thus a novel, PIKK-independent method of matching replication progression to dNTP levels may have evolved to coordinate normal S phases, just as ATR may represent a constitutive controller of the origin firing parameter.

Whether nucleotide depletion is sensed actively or passively, the experiments described above show that it does result in fork stalling and concomitantly, blocked origin firing. However, unlike the block to origin firing elicited by MMS, the response induced by HU is relatively rapidly reversible: there is good recovery of initiation events within 1hr after a brief HU arrest, although the efficiency of recovery declines after longer arrests. Recovery from a short HU arrest may be rapid because the transient depletion of nucleotides causes little actual DNA damage at stalled forks and the stimulus for checkpoint signalling would therefore be removed as soon as dNTPs were restored and stalled forks were able to restart. After progressively more time in HU, stalled replisomes may begin to lose their integrity, meaning that they cannot restart directly and must be processed via recombinational repair. Severe HU treatment has been shown to result in replication-dependent dsbs requiring repair by both

HR and NHEJ (Lundin et al., 2002), although HR using BLM may actually be the preferred mechanism (Davies et al., 2004). Once the stalled forks are committed to recombinational processing, they probably restart relatively slowly - HR proteins have been reported to be directly responsible for preventing fork progression through DNA containing UV lesions or crosslinks (Henry-Mowatt et al., 2003). Furthermore, ongoing recombinational repair may in itself be sensed as DNA damage, resulting in a more persistent checkpoint signal to suppress origin firing.

The results presented here differ markedly from recent findings regarding the response to HU in Chinese hamster lung fibroblast cells (Anglana et al., 2003). In these cells, origin firing was not simply inhibited by HU: the firing of a particular 'dominant' origin became less efficient but this was accompanied by the activation of normally dormant origins and an increase in the overall density of origin firing. By contrast, in both of the cell types tested here, Hela cells and primary fibroblasts, origin firing throughout the genome was severely inhibited, even by 20-fold lower HU than the amount used by Anglana et al. (50μM compared to 1mM). This difference may be explained by the fact that the Chinese hamster cells had been selected for resistance to coformycin, an inhibitor of adenylate deaminase2 (AMPD2), and this may have selected for cells with adaptations to reduced nucleotide concentration – either direct adaptations in dNTP metabolism or adaptations in the sensitivity of S phase checkpoint pathway. As discussed in chapter 3, cells lacking adenosine deaminase are known to accumulate dATP, so the inhibition of AMPD2 with coformycin may well result in a similar imbalance in nucleotide pools. As well as selecting for amplification of the AMPD2 gene, coformycin might therefore select for mutated forms of RNR

with higher thresholds for allosteric inhibition or inherently higher activities, and such mutations might render the Chinese hamster cells relatively insensitive to HU. Consistent with this, fork-slowing exhibited in response to HU was also less severe in the hamster cells: in HeLa or IMR90 cells, 1mM HU is more than enough to completely arrest cells in early S phase with little or no fork progression. The Chinese hamster cells, however, were reported to show significant S phase progression and great heterogeneity in replication track lengths when subjected to 1mM HU; fork rates also varied a lot between different coformycin-selected lines, perhaps because the different lines had accumulated different adaptive mutations. Thus, an HU treatment that stalls forks severely and activates the S phase checkpoint in HeLa or primary cells may not stall forks and/or may fail to activate the checkpoint in the Chinese hamster cells. (In an attempt to reproduce this 'adaptation' phenomenon in HeLa cells, cells were grown for several weeks in sublethal levels of HU; however, growth rates remained slow and the threshold for checkpoint activation – as assessed by CHK1 phosphorylation and DNA fibre analysis – did not change. This suggests that the Chinese hamster cells have been selected for actual checkpoint mutations and/or RNR mutations, rather than for some reversible modification to their checkpoint system.) In addition to such selected mutations, in the Chinese hamster cells, it is also possible that the origins in the artificially amplified locus examined by Anglana et al are regulated differently from the majority of genomic replication origins. The DNA fibre labelling technique surveys all origins throughout the genome so it should provide a better overview of the normal response to HU.

In conclusion, a comprehensive picture of the replication response to DNA damage in HeLa cells has now been established. In chapter 5, the investigation is extended to try to establish which checkpoint proteins are actually responsible for the changes to replication outlined here.

Chapter 5:

Damage-induced changes to replication in checkpoint-deficient cells

5a) S phase length is unaltered but replication fork rates are reduced in caffeine-treated cells

Caffeine is well known to sensitise cells to a variety of DNA damaging agents, including IR, UV and alkylating agents (Das et al., 1982; Domon and Rauth, 1969). Caffeine acts by inhibiting the activities of PIKKs (Sarkaria et al., 1999; Zhou et al., 2000) and cells are sensitised because caffeine therefore abrogates cell cycle checkpoints, including the intra-S phase checkpoint (Lehmann and Kirk-Bell, 1974; Murnane et al., 1980; Tolmach et al., 1977). The drug is therefore widely used as a means of investigating the checkpoints controlled by ATM and ATR. Accordingly, some of the experiments in chapters 3 and 4 were repeated in the presence of caffeine to try to establish whether the changes in replication caused by DNA damage are actually due to ATM and ATR.

Initially, the effect of caffeine on bulk S phase progression was tested by treating synchronised cells with 2mM caffeine in early S phase, followed 1hr later by 0.0025% MMS throughout S phase. Caffeine's IC_{50} values for ATM, ATR, and DNA-PK are 0.2, 1.1 and 10mM respectively, and CHK1 is also affected with an IC_{50} of ~5mM (Sarkaria et al., 1999). Therefore, 2mM caffeine should substantially inhibit ATR and ATM, but have minimal effects on the downstream kinase CHK1 and on the repair activity of DNA-PK. Flow cytometry revealed no

significant or reproducible effect of caffeine on bulk S phase kinetics in undamaged cells, nor was there any significant change in the extent of S phase slowing caused by MMS (Fig.11a). Caffeine did, however, cause cells to accumulate with 2C DNA content as control cells began to enter the next cell cycle (data not shown). Similarly, serum-starved primary cells treated with caffeine also failed to re-enter the cell cycle upon serum stimulation (data not shown). This suggested that caffeine does not affect S phase progression, either with or without DNA damage, but that it does affect the G1/S and G0/G1 transitions.

Despite the lack of any obvious change to S phase kinetics in caffeine-treated cells, preliminary DNA fibre analysis was still carried out to detect any subtle effects of caffeine on individual replication forks. Precedents do exist for DNA fibre analysis revealing significant changes in the pattern of replication which are not revealed by flow cytometry, either in yeast or in mammalian cells (Anglana et al., 2003; Shimada et al., 2002). Fibre analysis revealed that caffeine causes a significant reduction in mean fork rate even in the absence of DNA damage: after 1hr of pre-treatment with 2mM caffeine, track lengths were reduced by at least 50% (Fig. 11b). The extent of the reduction varied somewhat between experiments and was generally more severe when cells were pre-treated with caffeine for longer periods, despite the fact that the intracellular concentration of caffeine equilibrates very rapidly with the culture medium (Rowley et al., 1988). The shortened track lengths probably represent a genuine change in the rate of replication rather than simply a transient delay in the uptake of halogenated nucleotides because track lengths were consistently reduced over at least 40 minutes. The result is consistent with an earlier study using density substitution

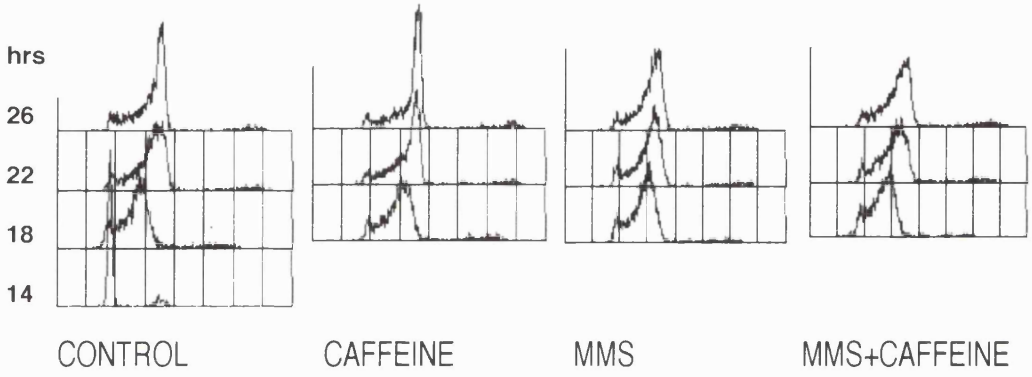
and DNA size sedimentation, which also concluded that cells treated with 10mM caffeine have slow replication rates (Tatsumi and Strauss, 1979). A second study reported that 1mM caffeine had no such effect, but an alternative interpretation of the size sedimentation data might suggest otherwise (Murnane et al., 1980). It was not clear whether the results in Fig.11 indicated that one or more of the PIKKs has a direct effect on replication fork rate or whether this was merely an indirect effect of caffeine on some other aspect of cellular metabolism, so it would be difficult to draw definitive conclusions about any further change to fork progression in the presence of DNA damage. Therefore, no further experiments were carried out using caffeine as a means of inhibiting PIKKs and each kinase was instead specifically inhibited by targeted knockdown methods.

Figure 11: Effects of caffeine on replication

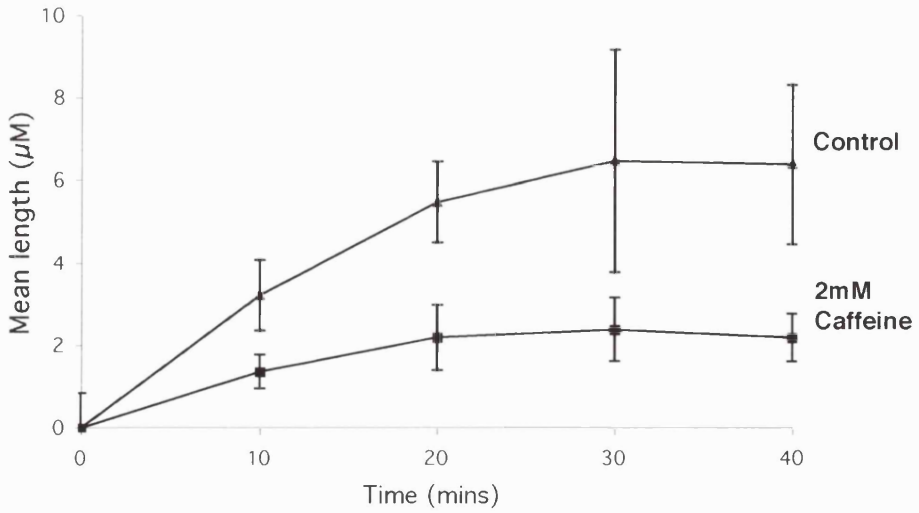
a) Cells were synchronised by mitotic shakeoff and treated in early S phase with 2mM caffeine. 1hr later, 0.0025% MMS was added and cell cycle progression was followed over the next 8hrs by flow cytometry.

b) Cells were treated with 2mM caffeine for 1hr in early S phase, then labelled with 50μM IdU for 10-40mins before preparing DNA fibres. The mean length of at least 50 replication tracks is plotted for each timepoint.

a


hrs
26
22
18
14
CONTROL
CAFFEINE
MMS
MMS+CAFFEINE

b


Mean length (μM)
10
8
6
4
2
0
0
10
20
30
40
Time (mins)
Control
2mM
Caffeine

5b) Replication in checkpoint protein-deficient cell lines

In order to avoid possible off-target effects of caffeine and to examine individual checkpoint proteins in isolation, DNA fibre analysis was carried out in various cell lines lacking particular checkpoint proteins. ATM-deficient and NBS1-deficient cells are available from AT and NBS patients, and a CHK2-deficient cell line has also been created by the targeted disruption of both CHK2 alleles in the HCT116 colon cancer cell line (Jallepalli et al., 2003). ATM, NBS1 and CHK2 have all been implicated in the RDS phenomenon (Falck et al., 2002), so these three cell lines would all be expected to lack some aspects of the intra-S phase checkpoint response to IR damage. Their replication responses (origin firing and fork progression) were therefore tested using the DNA fibre labelling protocol outlined in Fig. 8a.

Both the AT (GM03487) and the NBS (NBS-ILB-I) cell lines are fibroblasts which cannot be synchronised by mitotic shakeoff. In addition, both cell lines are immortalised and did not respond well to serum starvation. Therefore, the experiments could not be carried out in synchronised cells inearly S phase and were instead performed in asynchronous populations. Matched ATM-positive or NBS1-positive cell lines were not available, so IMR-90 primary fibroblasts were used as the control cell line.

Fig.12a shows that ATM-deficient cells lacked the dose-dependent suppression of origin firing which was seen in IMR-90 control cells within 20mins of exposure to 1-5Gy IR. (Origin firing did decrease in the AT cells after 1Gy of IR but the response was clearly absent after higher doses. This data was not averaged over several independent experiments, so statistical anomalies are

quite likely.) The results from NBS1-deficient cells (Fig.12b) were unclear: basal origin firing was much lower in undamaged NBS cells than in control cells but it showed no clear downward trend after IR damage. Fork movement was not significantly affected by IR damage in either AT or NBS cells (Fig.12c and 12d), although the replication tracks were generally slightly longer in the AT cells and significantly longer in the NBS cells compared to IMR-90 controls. It is possible that the disease cell lines have constitutively higher replication rates than IMR-90, since significant variation between fork rates in different cell lines has previously been reported (Painter and Schaefer, 1969). Therefore, it is not possible to draw any firm conclusions about the involvement of these checkpoint proteins in controlling fork progression. Since the lack of matched cell lines and of effective synchronisation protocols clearly made it difficult to carry out well-controlled investigations using these disparate cell lines, all further experiments with ATM and NBS1 were carried out using siRNA-mediated knockdown in HeLa cells.

Figure 12: Response to IR in AT and NBS cells

a) The protocol in Fig.8a was used to quantify origin firing after 1-5Gy IR. The graph shows data from a single experiment: at least 100 replication tracks were counted for each dose of IR.

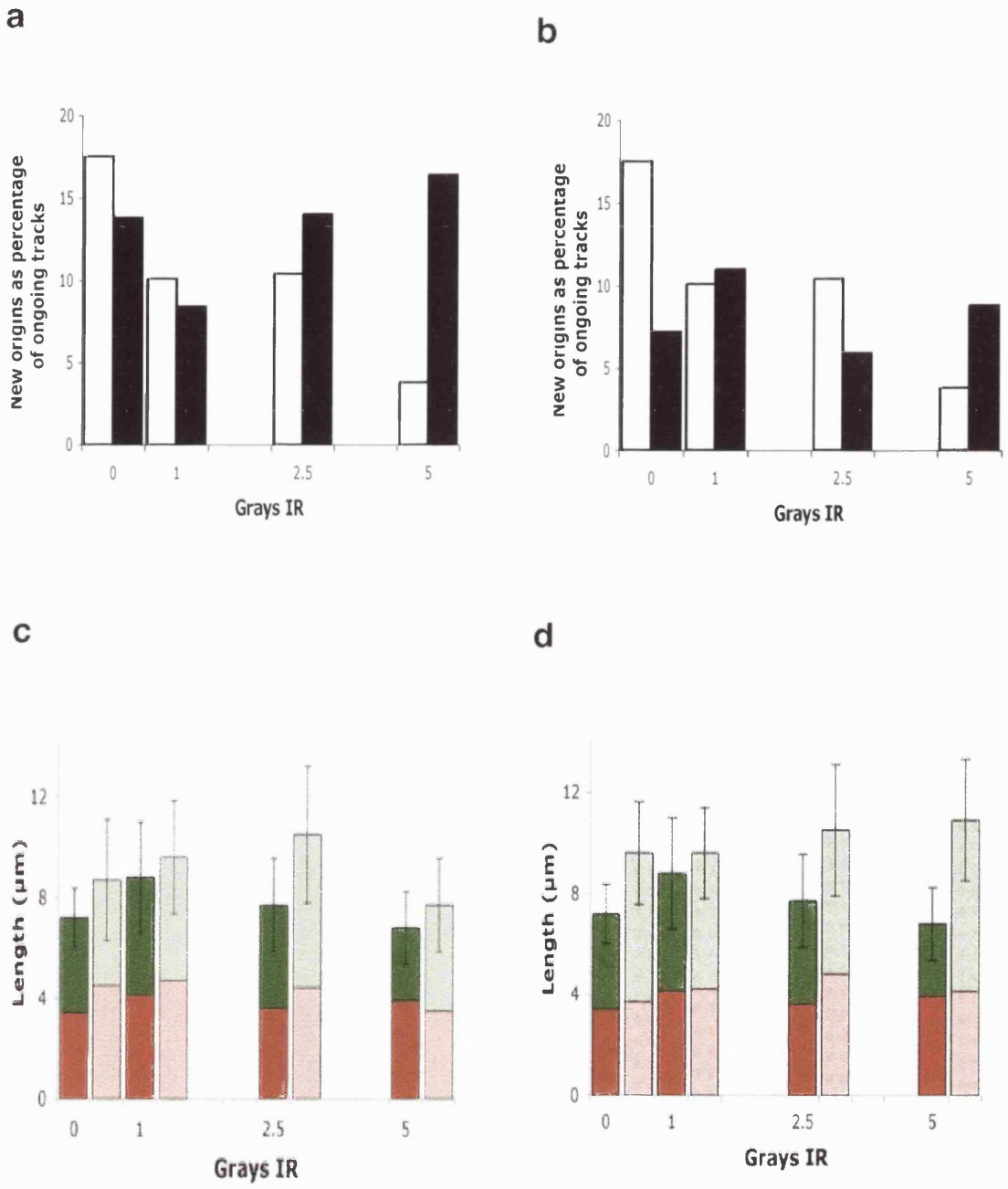
White bars = control IMR-90 cells, black bars = AT (GM03487) cells.

b) As in a), black bars = origin firing in NBS (NBS-ILB-I) cells.

c) The lengths of at least 50 unidirectional (red-then-green) tracks were measured from the experiment in 12a. Mean lengths are plotted with the green portion of each bar representing the CldU-labelled length (replicated after IR exposure).

Solid bars = control IMR-90 cells, textured bars = AT (GM03487) cells.

d) As in c, textured bars = NBS (NBS-ILB-I) cells.


a
b
New origins as percentage of ongoing tracks
20
15
10
5
0
0
1
2.5
5
Grays IR
c
d
Length (µm)
12
8
4
0
0
1
2.5
5
Grays IR

Turning to the CHK2-deficient cells, these are derived from an established cancer cell line, so identical CHK2-positive cells can be used as a matched control. HCT116 is an epithelial cell line which can be synchronised by mitotic shakeoff, so the experiments could be carried out - like the earlier experiments in HeLa cells - using fairly homogenous early S phase populations. However, HCT116 cells appear to label relatively inefficiently with halogenated nucleotides, so the protocol outlined in Fig.8a was carried out using twice the normal amounts of IdU and CldU, and extending the second pulse-label from 20 to 30mins. These cells also appeared to be relatively insensitive to IR, so doses of 2.5-10Gy instead of 1-5Gy were applied. (The insensitivity may be due to the mismatch repair deficiency of HCT116 cells, since MMR has been implicated in CHK2 activation by ATM (Brown et al., 2003). Alternatively, it may simply be a cell line-specific difference between HeLa and HCT116 cells.)

There was no significant failure to block origin firing after IR damage in the $CHK2^{-/-}$ cells (Fig.13a), nor did IR damage affect fork progression (Fig.13b), as was previously shown in HeLa cells. (The replication tracks in undamaged HCT116 cells appeared unusually short in this experiment, but this was not maintained in any of the IR-treated cells and may simply be an anomaly from counting tracks on a single slide). The effective suppression of origin firing in $CHK2^{-/-}$ cells was unexpected, since the acute inhibition of CHK2 function by over-expression of dominant negative CHK2 has previously been reported to cause RDS in U2-0S cells (Falck et al., 2001), and this RDS has been attributed to a failure to block origin firing (Falck et al., 2002). It was possible, however, that the established CHK2 knockout cell line used here had modified levels or increased activation of CHK1 – a second kinase which is also capable of

enforcing the intra-S phase checkpoint after IR damage (Sorensen et al., 2003). (The CHK2 knockout cells had previously been shown to have an intact G2 checkpoint, presumably due to CHK1, although their intra-S phase checkpoint was not tested (Jallepalli et al., 2003).) Western blotting showed that the levels of CHK1 were in fact not dramatically different in $CHK2^{-/-}$ and parental cells, although they may be very slightly elevated in the knockout cells (Fig.13c). CHK1, did, however, become more strongly phosphorylated after IR exposure in the $CHK2^{-/-}$ cells, whereas the parental cells had relatively little phosphorylated CHK1 despite showing large amounts of gel-shifted (activated) CHK2. By contrast, CHK1 was phosphorylated to a similar extent in both cell lines in response to MMS – a form of damage which is generally thought to activate ATR, not ATM, and which yields multiple bands of phosphorylated CHK1. Therefore, it seems that ATM (and/or ATR) activates CHK1 to greater extent after IR damage if CHK2 is not present. This may account for the checkpoint-proficiency of $CHK2^{-/-}$ cells, and it suggests that CHK1 and CHK2 are interchangeable in origin firing suppression. Since it is not clear whether this extra activation of CHK1 is unique to these $CHK2^{-/-}$ cells or whether it occurs in all cell lines after IR damage in the absence of CHK2, further experiments were carried out in HeLa cells using acute, siRNA-mediated knockdown of the Chk kinases.

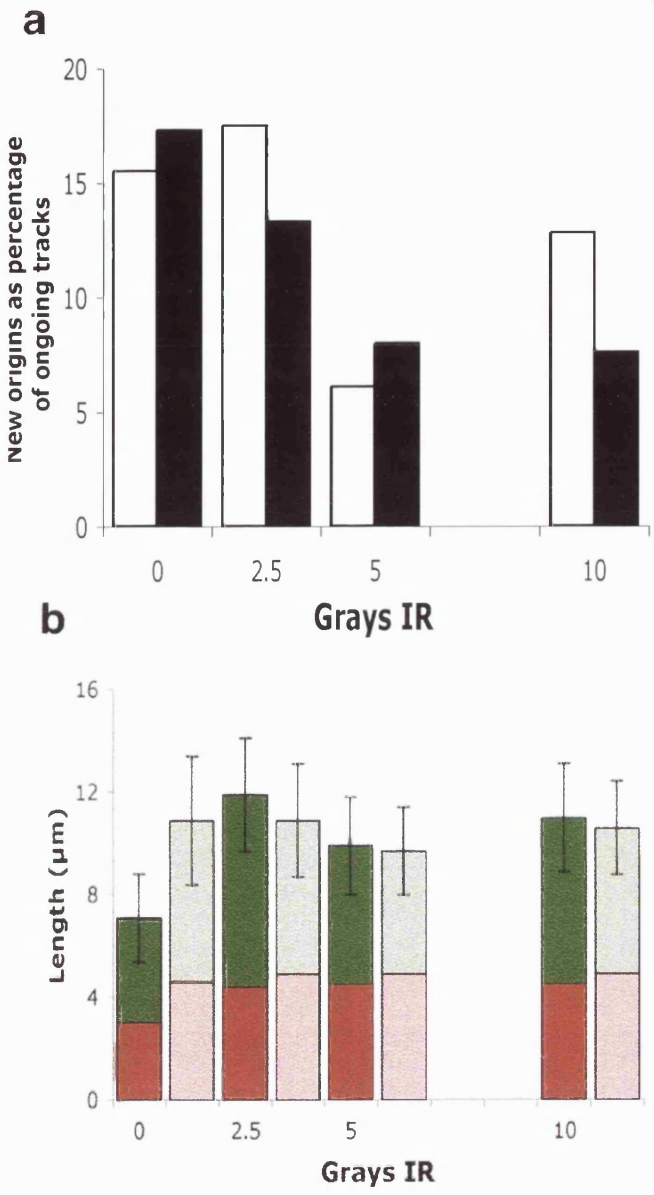
Figure 13: Response to IR in CHK2$^{-/-}$ HCT116 cells

a) The protocol in Fig.8a was used to quantify origin firing after 2.5-10Gy IR, labelling cells with 20μM IdU for 10mins and 200μM CldU for 30mins. The graph shows data from a single experiment: at least 100 replication tracks were counted for each dose of IR.

   White bars = control HCT116 cells, black bars = CHK2$^{-/-}$ HCT116 cells.

b) The lengths of at least 50 unidirectional (red-then-green) tracks were measured from the experiment in 13a. Mean lengths are plotted with the green portion of each bar representing the CldU-labelled length (replicated after IR exposure).

c) Extracts were made 30mins after 5Gy IR, 30mins after 0.02% MMS, or 90mins after 1mM HU treatment. Western blotting was carried out for P-Ser317-CHK1, total CHK1, total CHK2 and PCNA (loading control).


a
New origins as percentage of ongoing tracks
20
15
10
5
0
0
2.5
5
10
Grays IR
b
Length (µm)
16
12
8
4
0
0
2.5
5
10
Grays IR
c
Parental
Chk2-/-
- IR MMS HU - IR MMS HU
P-Chk1
Chk1
Chk2
PCNA

5c) Replication in an ATR conditional-knockout cell line

In order to investigate the role of ATR in the intra-S phase checkpoint, conditional knockout cells are required because ATR is essential for cell viability. Such a cell line has been derived, in the HCT116 background, with the first allele of the ATR gene disrupted and exon 2 of the second allele able to be excised by Cre recombinase (Cortez et al., 2001). The heterozygous $ATR^{flox/-}$ cells express about 20% of the normal level of ATR, possibly because the lox sites inserted into the remaining allele lead to reduced transcription. However, the cells are viable and are reported to have normal checkpoint responses (Cortez et al., 2001). Following Cre recombinase expression in these cells, ATR protein levels fall within 3-4 days and the cells then die with multiple chromosome aberrations by days 5-6. Therefore, the intra-S phase checkpoint in cells lacking ATR could theoretically be examined in the ~48hr window before apoptosis occurs.

The Cre recombinase is expressed from an adenoviral vector and since excessive infection with adenovirus can in itself be cytotoxic, the virus was first titrated to find a level which gave maximal excision with minimal associated cytotoxicity. A GFP-expressing adenovirus was tested in parallel, since this offered an easy visual read-out of successful infection. Both Ad-Cre and Ad-GFP were grown up in 293 cells, purified using caesium chloride gradients and titrated by the $TCID_{50}$ method. The stock of Ad-Cre obtained by this method had a concentration of 10^9 PFU/ml and the Ad-GFP, $10^{11.1}$ PFU/ml. $ATR^{flox/-}$ cells were then infected with both viruses at a theoretical multiplicity of infection (MOI) of 12, 25, 50 or 75. Infection is unlikely to be 100% efficient but any Ad-GFP infection above 25 MOI resulted in >95% of cells expressing GFP within 24hrs.

(The fluorescence levels varied significantly, however, suggesting that levels of infection were not uniform.) At all multiplicities of infection with Ad-Cre, excision of the floxable exon could be observed in genomic DNA prepared 48hrs after infection, although the PCR test is not linear and a product representing the unfloxed allele always appeared as well (Fig. 14a). Within 72hrs of even the lowest MOI, levels of ATR protein were reduced by ~90% compared to the levels in $ATR^{flox/-}$ cells (Fig.14b). At the same timepoint, HU-induced phosphorylation of CHK1 (P-CHK1) was similarly reduced, suggesting that ATR is functionally abrogated. (ATR has been reported to be principally responsible for phosphorylating CHK1 in response to HU in this cell system (Cortez, 2003).) It was important to verify, however, that ATR activity was actually absent in ~90% of individual cells, and furthermore, that these cells were still able to enter S phase rather than simply being arrested in G1 or G2. None of the ATR antibodies tested gave sufficiently specific signals in immunofluorescence, so instead, the phosphorylation of CHK1 in response to HU was used as an ATR indicator. Individual cells were stained for P-CHK1 together with the S phase marker, PCNA. Specific nuclear staining for P-CHK1 appeared in >90% of unfloxed S phase cells when they were treated with HU (Fig.14c). 72hrs after 25 MOI Ad-Cre, P-CHK1 staining was completely reduced to background levels in 60% of S phase cells, and was significantly reduced in a further 26% of cells (Fig.14d). This suggested that at least 86% of S phase cells should be significantly impaired in their ATR response. Infection at lower levels resulted in many more cells retaining a significant P-CHK1 signal while a higher MOI caused cells to die after 3-4 days instead of 5-6 days, suggesting that virus-associated death now preceded apoptosis through lack of ATR.

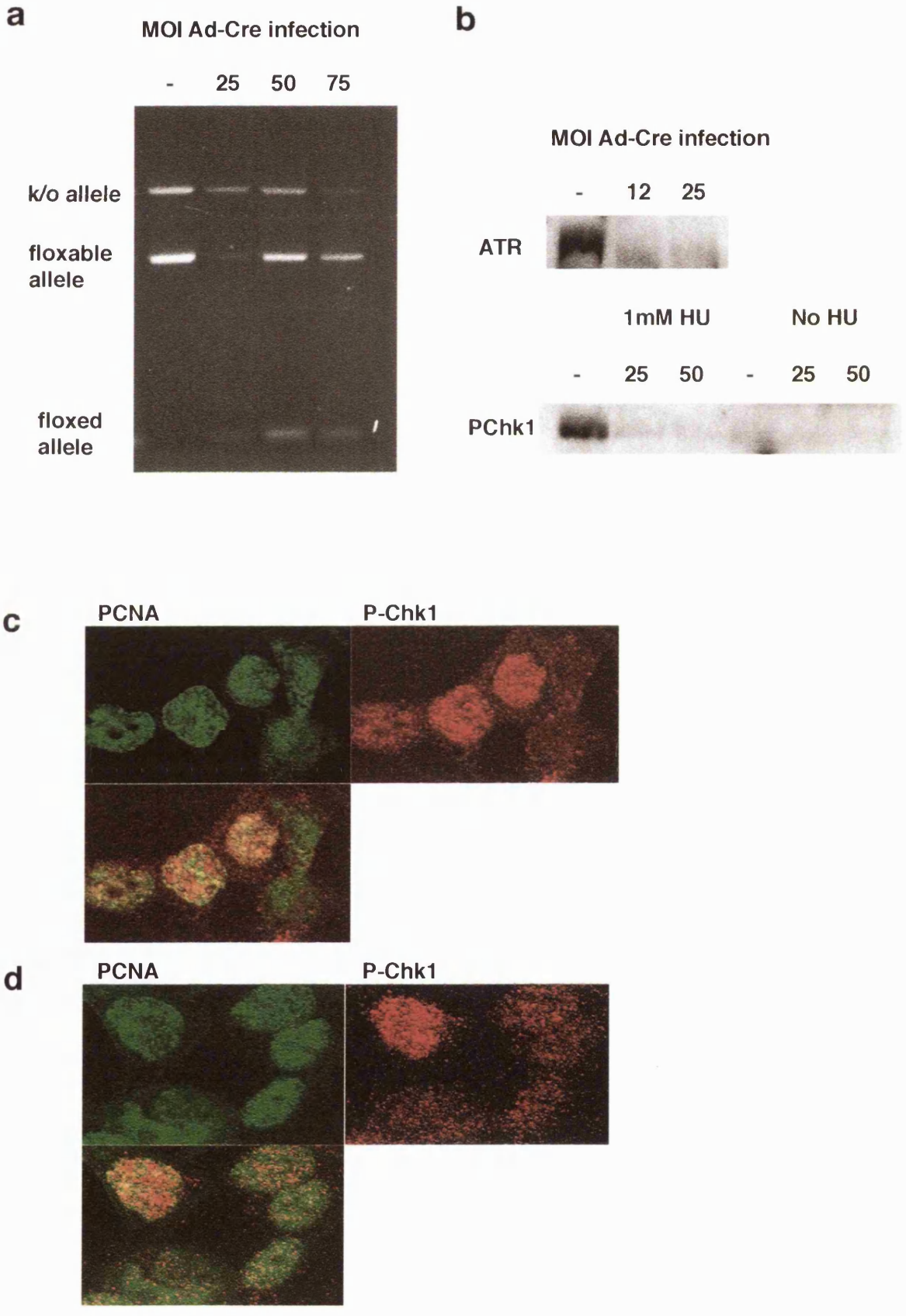
Figure 14: Reduction in ATR levels by Cre-mediated excision in $ATR^{flox/-}$ cells

a) PCR for ATR alleles was carried out on genomic DNA prepared 48hrs after infection with Ad-Cre at 0-75 MOI.

b) Extracts were made 72hrs after infection with Ad-Cre at 0-25 MOI, and blotted for ATR. Extracts were also made after treating the same cells with 1mM HU for 4hrs, and blotted for Ser317-phosphorylated CHK1 (P-CHK1).

c) Uninfected $ATR^{flox/-}$ cells were treated with 1mM HU for 2hrs, then fixed and stained for PCNA and P-CHK1. The three cells on the left show PCNA foci indicative of S phase and all three also show strong P-CHK1 signals.

d) Cells were fixed and stained as in (c) 72hrs after infection at 25 MOI. The three right-hand cells are in S phase but have very little P-CHK1, whereas the left-hand cell retains its P-CHK1 signal.


a
MOI Ad-Cre infection
- 25 50 75
k/o allele
floxable allele
floxed allele
b
MOI Ad-Cre infection
- 12 25
ATR
1mM HU
No HU
- 25 50 - 25 50
PChk1
c
PCNA
P-Chk1
d
PCNA
P-Chk1

All further experiments were therefore carried out after infection with Ad-Cre at 25 MOI. 72hrs after infection, significant growth inhibition was apparent but only a few cells showed fragmented nuclei and many cells were still entering S phase. The limited time window and progressive cell death, however, meant that it was not possible to synchronise floxed populations by mitotic shakeoff, so experiments were performed in asynchronous cells.

To assess the role of ATR in suppressing origin firing and in controlling fork movement after DNA damage, the double-labelling protocol outlined in Fig.8a was carried out on floxed and $ATR^{flox/-}$ cells treated with 0-0.02% MMS. The CldU (post-damage) label was extended from 20 to 30mins to allow for the relatively short replication tracks and high sensitivity to fork slowing in these cells.

Fig.15a shows that MMS caused a dose-dependent reduction in fork rate, in both $ATR^{flox/-}$ and in floxed cells, starting at only 0.005% MMS. The floxed cells also had consistently lower fork rates regardless of DNA damage, and showed a higher basal percentage of IdU-only tracks (stalled forks), although fork stalling was further increased with MMS damage (Fig.15b). Origin firing was reduced in a dose-dependent manner in the $ATR^{flox/-}$ cells (Fig.15c), but the threshold for this was significantly higher than in HeLa cells (Fig.8). In floxed cells, the threshold for an MMS-induced reduction in origin firing was higher still, with a response occurring only at the highest level of MMS. Finally, the activation status of both Chk kinases was assessed after MMS damage. Fig.15d shows that CHK1 was multiply phosphorylated in $ATR^{flox/-}$ cells and that this was reduced but not absent in the floxed cells, consistent with the subset of 'floxed'

cells that probably weren't infected and still showed P-CHK1 immuno-staining. In neither population was CHK2 activated, suggesting that there is no cross-talk on CHK2 within 30mins of MMS damage in these cell lines.

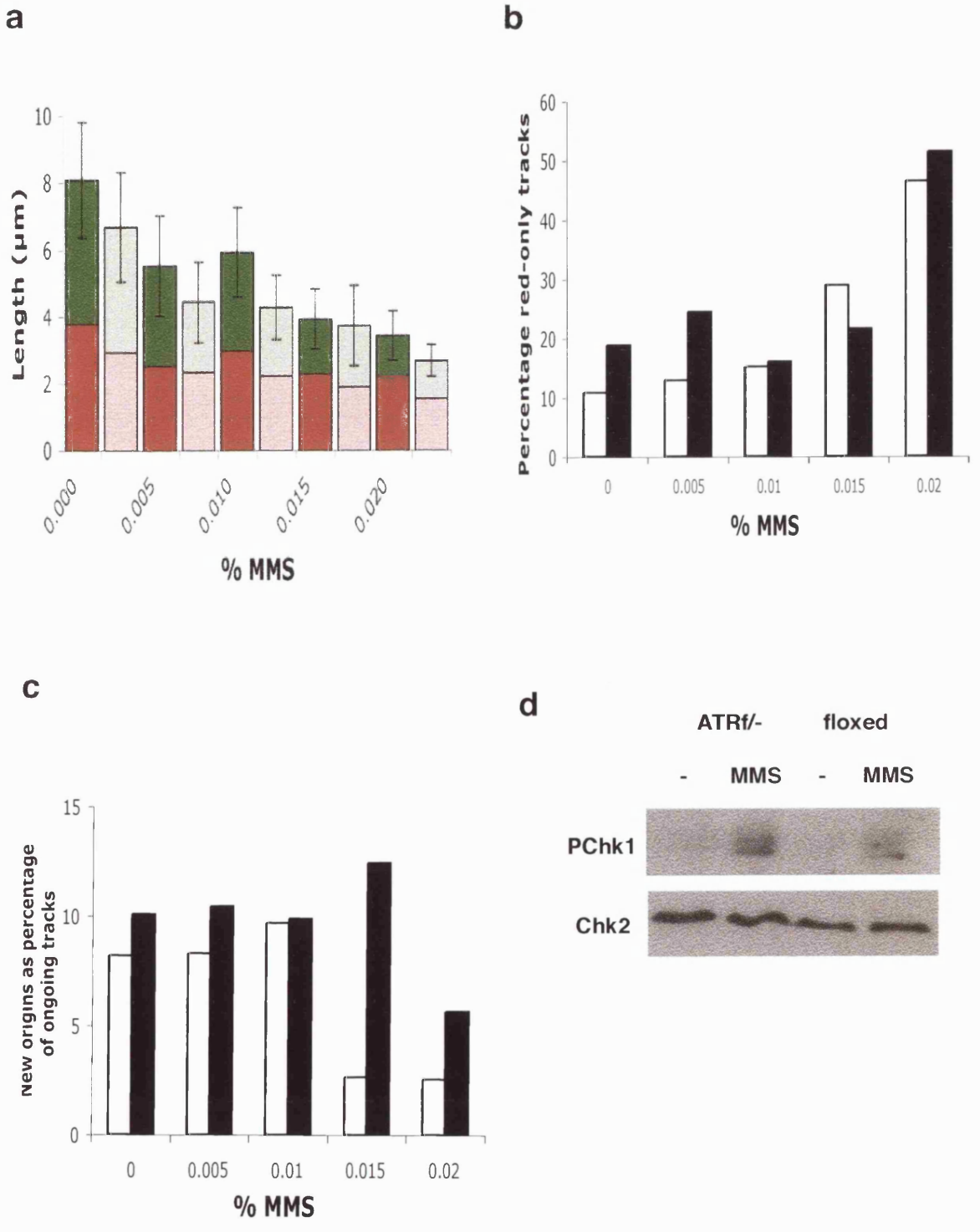
Figure 15: Response of $ATR^{flox/-}$ and floxed cells to MMS

a) Cells were labelled for 10mins with 10μM IdU, then 30mins with 100μM CldU in the presence of 0-0.02% MMS. The lengths of at least 50 unidirectional (red-then-green) tracks were measured and the mean lengths were plotted with the green portion of each bar representing the CldU-labelled length. At least 100 replication tracks were counted for each dose of MMS and the graph shows the average of two independent experiments.
Solid bars = $ATR^{flox/-}$ cells, textured bars = floxed cells.

b) From the experiments in (a), red-only tracks were counted as a percentage of ongoing tracks.
White bars = $ATR^{flox/-}$ cells, black bars = floxed cells.

c) From the experiments in (a), green-only tracks were counted as a percentage of ongoing tracks.
White bars = $ATR^{flox/-}$ cells, black bars = floxed cells.

d) Extracts were made after treating $ATR^{flox/-}$ and floxed cells with 0.02% MMS for 30mins, and blotted for P-CHK1 and CHK2.


a
Length (µm)
10
8
6
4
2
0
0.000
0.005
0.010
0.015
0.020
% MMS
b
Percentage red-only tracks
60
50
40
30
20
10
0
0
0.005
0.01
0.015
0.02
% MMS
c
New origins as percentage of ongoing tracks
15
10
5
0
0
0.005
0.01
0.015
0.02
% MMS
d
ATRf/-
floxed
-
MMS
-
MMS
PChk1
Chk2

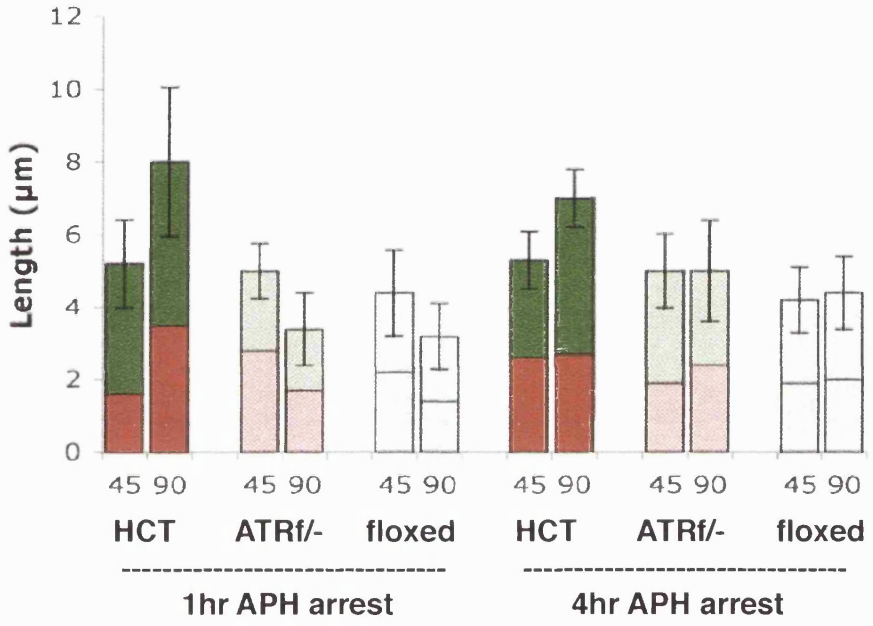
In a second set of experiments, the proposed role of ATR in stabilising stalled replication forks was directly tested. Floxed cells, $ATR^{flox/-}$ cells and also the HCT116 cells from which the $ATR^{flox/-}$ line is derived were tested for the ability to restart stalled forks after an APH arrest. Cells were briefly labelled with IdU, arrested for 1-4hrs with a high concentration of APH, then released into CldU for 45 or 90mins. Fig.16a shows that the restart of stalled forks was rather slow in all cases, compared with the expected rate for uninterrupted replication. This is consistent with fibre analysis from the *Xenopus* extract system, which has also showed that replication upon release from APH is initially very slow (Marheineke and Hyrien, 2004). More importantly, however, the restart was significantly worse in floxed and heterozygous cells than it was in parental cells. Replication tracks in floxed and $ATR^{flox/-}$ cells achieved no more than 2μm (~5kb) of replication in 45mins after APH release, and forks appeared to be permanently stalled by this point because replication proceeded no further in the following 45mins - indeed, tracks actually became fractionally shorter, suggesting that nascent DNA might even be degraded at terminally stalled forks. By contrast, HCT116 cells replicated ~4μm within 45mins of APH release and continued to replicate in the following 45mins such that most replicons had joined up and only the shortest subpopulation of tracks could actually still be counted. The efficiency of restart in the HCT116 cells did diminish with the length of the APH arrest, but restart remained relatively efficient even after a 4hr arrest. Blots for the Chk kinases showed that APH, like MMS, activated CHK1 efficiently in $ATR^{flox/-}$ cells and very much less efficiently in floxed cells. CHK2 was not activated regardless of the length of APH arrest (Fig.16b).

Figure 16: Restart of stalled forks in HCT116, $ATR^{flox/-}$ and floxed cells.

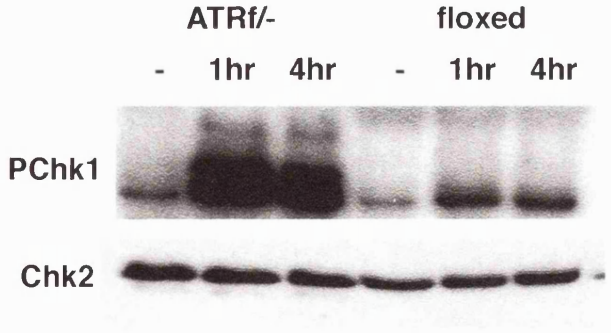
a) Cells were labelled for 10mins with 20μM IdU, then the IdU was replaced with 200μM CldU and 10μg/ml APH was added for 1hr or 4hrs. APH was washed out and CldU labelling was allowed for 45 or 90mins. The lengths of at least 50 unidirectional (red-then-green) tracks were measured and the mean lengths were plotted with the green portions of each bar representing the CldU-labelled length.

b) After the APH arrests detailed above, extracts were made and blotted for P-CHK1 and CHK2.

a


Length (µm)
12
10
8
6
4
2
0
45 90
HCT
ATRf/-
floxed
1hr APH arrest
4hr APH arrest

b


ATRf/-
floxed
-
1hr
4hr
PChk1
Chk2

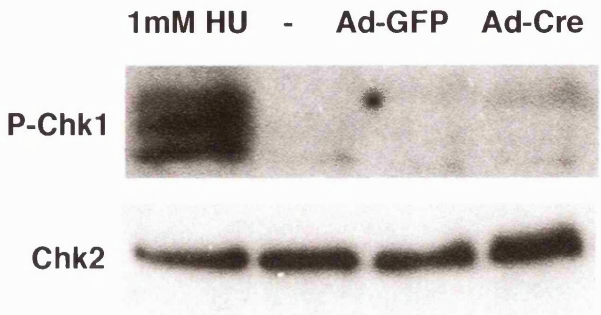
Finally, it was important to check that the dose-dependent cell death induced by Ad-Cre infection was not directly affecting the results of these experiments. Infection of $ATR^{flox/-}$ cells with high doses of Ad-GFP caused much less apoptosis than Ad-Cre, but it was difficult to separate death through loss of ATR from death from adenoviral infection. However, Ad-Cre also caused more apoptosis than Ad-GFP in non-floxable HCT116 parental cells, suggesting that not only adenoviral infection but Cre recombinase itself is somewhat cytotoxic. It was possible that the recombinase caused non-specific breaks in the genome and might therefore activate checkpoint proteins in infected cells. To test this possibility, HCT116 cells were infected with 25 MOI Ad-Cre or Ad-GFP and the activation of CHK1 and CHK2 was assessed after 72hrs. No shift in CHK2 was detected after any viral infection but Ad-Cre did cause a small amount of CHK1 to become highly phosphorylated (Fig.17a). Immuno-staining of Ad-Cre infected cells had previously shown that, in addition to any HU-induced P-CHK1 detected in S phase cells, a small number of non-S phase cells with rounded morphology and abnormal nuclei showed large clumps of very bright P-CHK1 staining (Fig.17b). This probably accounts for the highly phosphorylated CHK1 observed by Western blotting. Their abnormal morphology showed that these cells were probably captured in the process of apoptosis so the CHK1 phosphorylation is probably a result of the apoptotic process, during which DNA is extensively degraded. It is not clear why Ad-Cre induces apoptosis, but cells are unlikely to be actually replicating their DNA by this time and would therefore make no contribution to DNA fibre-labelling data.

Figure 17: Apoptosis and CHK1 activation in non-floxable Ad-Cre infected cells

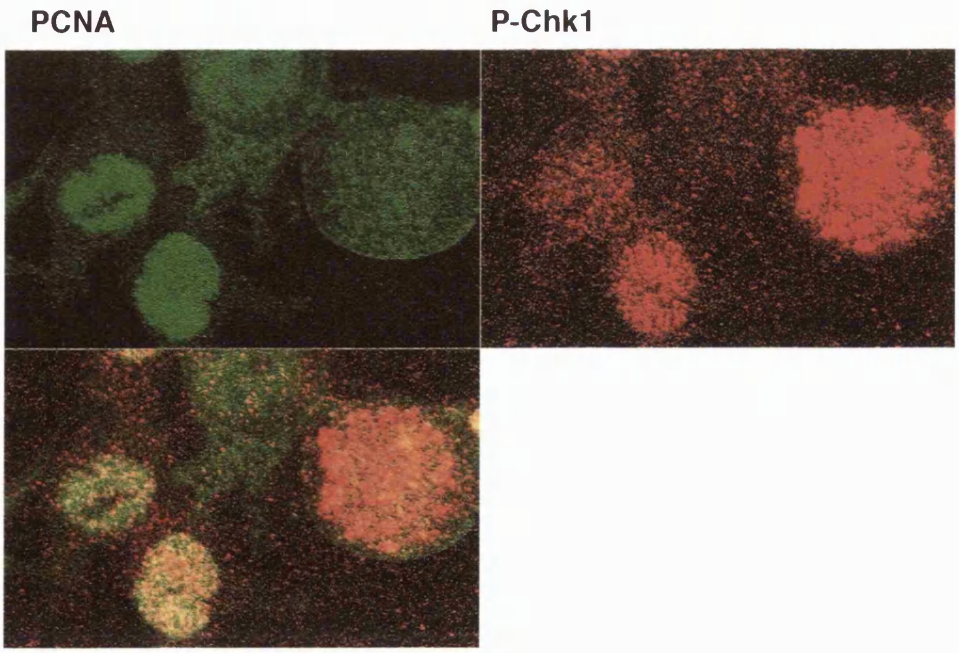
a) HCT116 cells were infected with 25 MOI Ad-Cre, Ad-GFP or no virus and extracts were made after 72hrs. The Chk kinases in uninfected cells treated with 1mM HU are shown for comparison.

b) Floxed cells treated with 1mM HU and stained for PCNA and P-CHK1. The right-hand cell has no PCNA foci but shows very strong P-CHK1 staining. It also has abnormal rounded morphology.

a



b



5d) Conditional knockdown of checkpoint proteins using siRNA

In order to investigate a complete range of both essential and non-essential checkpoint proteins in a uniform, well-characterised cell background, siRNA-mediated knockdowns of ATR, ATM, CHK1, CHK2 and NBS1 were all carried out in HeLa cells. SiRNA transfection does not in itself apparently affect replication kinetics, since a control RNA directed against pRb caused no change in either fork rate or origin firing when DNA fibres were prepared 48hrs after transfection and compared with fibres from mock-transfected cells. In all future experiments, control cells were therefore simply mock-transfected in parallel with the siRNA transfection. The disadvantage of this technique is that the target proteins are not completely knocked out and the residual levels may still be sufficient for certain checkpoint functions. Also, non-specific effects of particular siRNAs, although unlikely (Chi et al., 2003; Semizarov et al., 2003), cannot be excluded.

All five of the proteins targeted could be knocked down by at least 75% (Fig.18a) and no significant cross-effects on the other checkpoint proteins were detected (see for example siChk1, Fig.18a). All knockdowns were achieved within 40-48hrs, so cells could be examined in the first or second cell cycle after loss of the target protein. No apoptosis was observed within this time period and none of the cell cycle profiles were dramatically altered, suggesting that none of the siRNAs had toxic side-effects or caused serious cell cycle arrests (Fig.18b). The knockdown of the two essential proteins ATR and CHK1 did, however, seem to cause a slight reduction in the S phase population, as might be expected if ATR and CHK1 are required for normal cell proliferation. The absence of either protein has been reported to cause aberrant replication, and the DNA damage

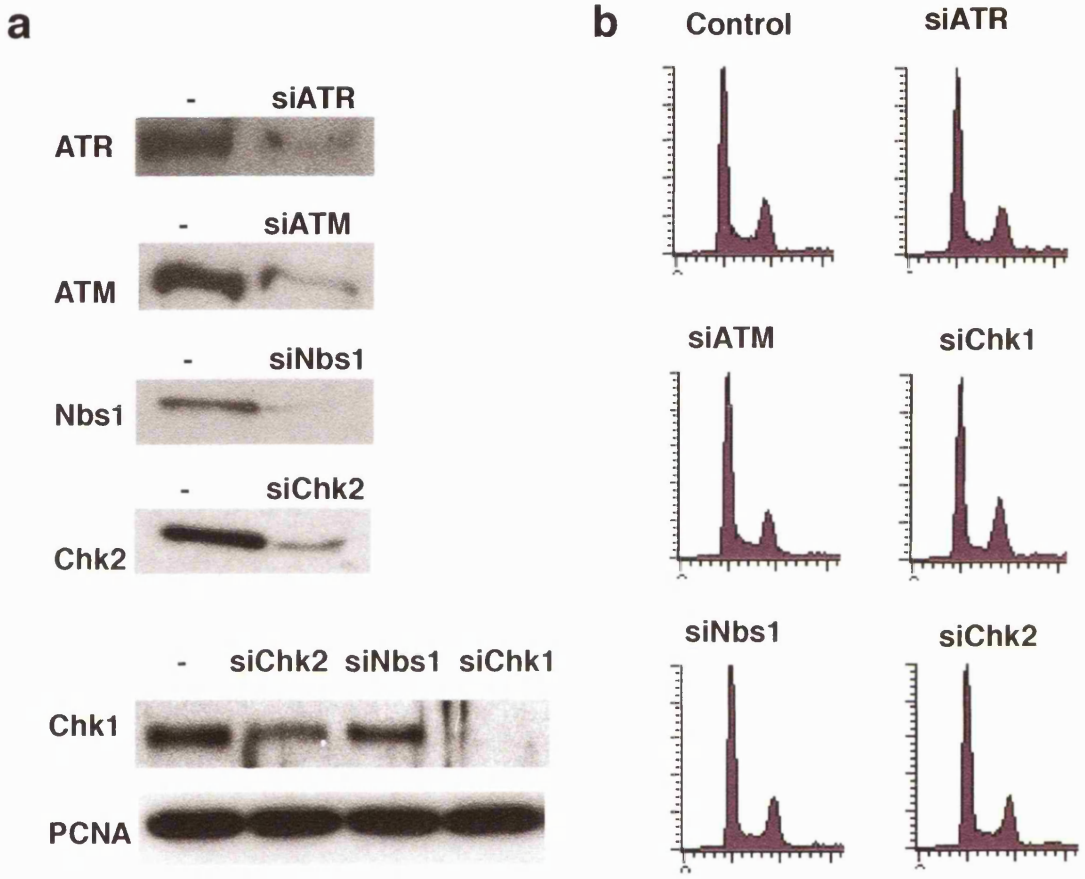
generated as a result forms a secondary checkpoint stimulus for arrest in G1 or G2 (Cortez et al., 2001; Lam et al., 2004). The cell cycle effect of siATR was examined in more detail by synchronising siATR-treated cells and following the subsequent cell cycle by flow cytometry. This confirmed that a significant percentage of cells 48hrs after siATR transfection failed to enter S phase with normal kinetics when released from nocodazole, appearing instead to be arrested in G1 (Fig.18c). By contrast, knockdown of the non-essential PIKK, ATM, caused no such defect. Therefore, when investigating siATR-treated cells (and possibly also siChk1), it was very important to use synchronised S phase populations for DNA fibre labelling: progressively reduced S phase entry in the transfected populations might otherwise skew any measurements of origin firing rates. Due to the difficulties of synchronising sufficient transfected cells, however, the experiments with ATM, CHK2 and NBS1 – non-essential proteins which do not affect the cell cycle - were all carried out in unsynchronised cells. This means that the G1/S checkpoint (the prevention of S phase entry by a PIKK/CDK-dependent suppression of the onset of origin firing) cannot be separated from the strictly 'intra-S phase' checkpoint. However, long-term checkpoint maintainance and recovery experiments were not carried out in these cells, and when measuring acute responses, the number of cells which could enter S phase within 20mins of DNA damage is far fewer than the number of cells already in S phase, so the response of cells within S phase should outweigh the response of cells at the G1/S border.

Figure 18: siRNA-mediated knockdown of ATR, ATM, CHK1, CHK2 and NBS1

a) Extracts were made 40-48hrs after transfection with the indicated siRNAs.

b) Cells were fixed for flow cytometry 45hrs after transfection.

c) Cells were transfected with siATR or mock-transfected, synchronised by mitotic shakeoff and fixed for flow cytometry over the following 27hrs. This corresponds to 32-59hrs post-transfection and mock-transfected cells entered S phase at ~48hrs.


a
-
siATR
ATR
-
siATM
ATM
-
siNbs1
Nbs1
-
siChk2
Chk2
-
siChk2
siNbs1
siChk1
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PCNA
b
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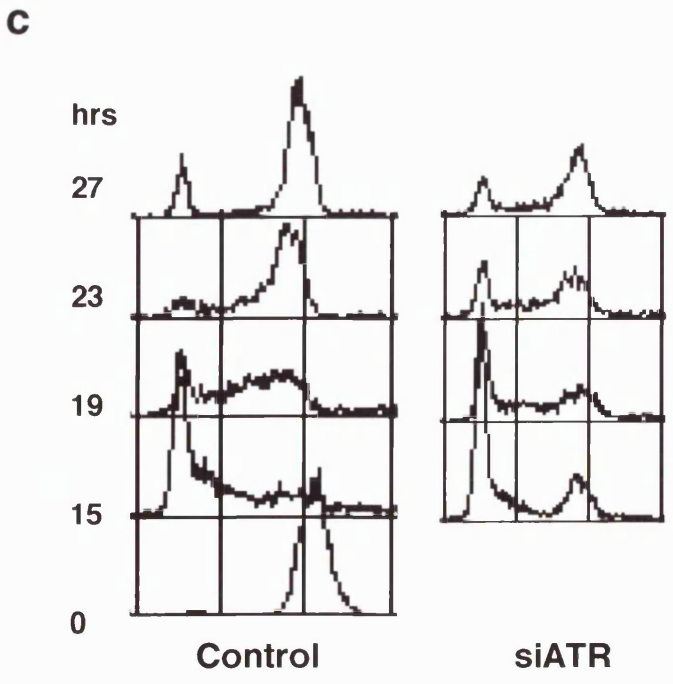
siATM
siChk1
siNbs1
siChk2


c
hrs
27
23
19
15
0
Control
siATR

The ATM pathway: response to IR

Experiments using the AT disease cell line had already suggested that in the absence of ATM, cells were unable to block origin firing in response to IR (Fig.12). However, no clear evidence was obtained to support the idea that either CHK2 or NBS1 have partial roles in transducing the ATM signal (Falck et al., 2002), nor was it clear whether fork progression was affected by any of these proteins. The response to IR was therefore systematically tested in HeLa cells knocked down for each of these proteins, using the protocol outlined in Fig.8a.

Cells lacking ATM did not block origin firing, as was previously shown in AT cells (Fig.19a). The lack of ATM did not have any effect on fork movement in undamaged cells but tracks replicated after the higher doses of IR were very slightly shorter in the ATM-knockdown cells than in control cells (Fig 19b). This slowing of replication was, however, much less significant than that caused by MMS or UV damage.

Cells treated with siChk2 showed a near-normal checkpoint response: they failed to suppress origin firing after the lowest dose of IR but after 5Gy or more, origin firing was reduced almost to control levels (Fig.19c). There was also no consistent change in fork rates (although once again, track lengths were very slightly shorter in the siChk2-treated cells after the highest dose of IR (Fig.19d)). To determine whether it is actually CHK1 that takes the major role in suppressing origin firing downstream of ATM, the same experiment was performed in siChk1-treated cells. Here, there was a more significant failure to prevent origin firing and only the highest dose of IR elicited a response (Fig.19e). Again, little change occurred in fork rates (Fig.19f). Thus, the IR-induced checkpoint operates principally through reduced origin firing, since no experiment showed a major

change in the rate of ongoing forks, and ATM appears to be completely responsible for suppressing origin firing. The two Chk kinases apparently share signal transduction downstream of ATM with CHK1 having the more important role. Consistent with this, both CHK1 and CHK2 became phosphorylated within 25mins in IR-damaged cells (Fig. 20c). When either Chk kinase was knocked down, there was no change in the phosphorylation profile of the other kinase, so neither is hyperactivated in the absence of the other. (This is in contrast to the situation in $Chk2^{-/-}$ HCT116 cells (Fig.13), which do seems to phosphorylate CHK1 somewhat more readily in the absence of CHK2.) Therefore both kinases are probably required for a maximally efficient checkpoint response to low-dose IR. After a high enough dose of IR, the single remaining kinase may get sufficiently activated to enforce the checkpoint alone, or a second, independent method of transducing the origin-blocking signal may become active.

To test the possibility that NBS1 is such a second transducer, as suggested by Falck et al (Falck et al., 2002) the same experiments were carried out in NBS1-knockdown cells. These experiments gave rather variable results but pooling the data from three independent experiments showed that on average, cells knocked down for NBS1 had a significantly higher basal rate of origin firing than control cells (Fig.19g). This *was* dose-dependently reduced after irradiation, but it remained higher than the rate of origin firing in control cells after all doses of IR. Again, siNbs1 did not have any notable effect on the rate of fork movement (Fig.19h).

Figure 19: Response to IR in siATM, siChk2, siChk1 and siNbs1-treated cells

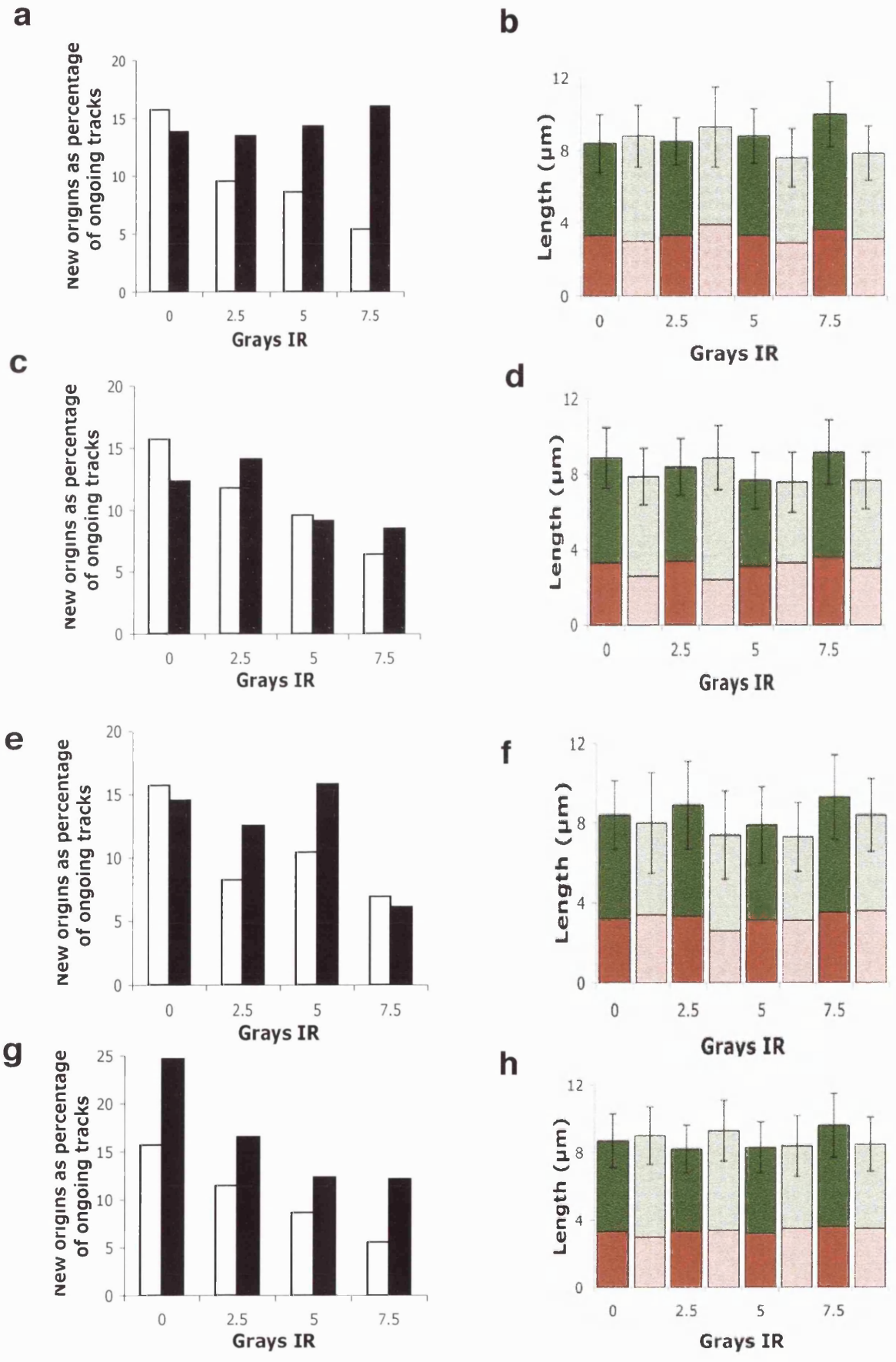
The protocol in Fig.8a was used to quantify origin firing and mean track lengths after 2.5-7.5Gy IR in cells treated with siATM (a,b), siChk2 (c,d), siChk1 (e,f) and siNbs1 (g,h). All graphs show the average of two independent experiments: at least 100 replication tracks being counted for each dose of IR.

Origin firing:

White bars = mock-transfected cells, black bars = siRNA-treated cells.

Track lengths:

Solid bars = mock-transfected cells, textured bars = siRNA-treated cells.


a
New origins as percentage of ongoing tracks
0
5
10
15
20
0
2.5
5
7.5
Grays IR
b
Length (µm)
0
4
8
12
0
2.5
5
7.5
Grays IR
c
New origins as percentage of ongoing tracks
0
5
10
15
20
0
2.5
5
7.5
Grays IR
d
Length (µm)
0
4
8
12
0
2.5
5
7.5
Grays IR
e
New origins as percentage of ongoing tracks
0
5
10
15
20
0
2.5
5
7.5
Grays IR
f
Length (µm)
0
4
8
12
0
2.5
5
7.5
Grays IR
g
New origins as percentage of ongoing tracks
0
5
10
15
20
25
0
2.5
5
7.5
Grays IR
h
Length (µm)
0
4
8
12
0
2.5
5
7.5
Grays IR

ATR pathway: response to UV

Previous reports have suggested that while ATM responds exclusively to dsbs and not to replication-blocking damage, ATR responds to a wider range of DNA damaging agents. As well as being activated by replication-blocking lesions from UV or MMS, (Tibbetts et al., 2000a), it also forms IR-responsive foci (Barr et al., 2003) and contributes to an IR-induced G2 arrest (Brown and Baltimore, 2003; Xu et al., 2002a). However, DNA fibre analysis in siATR-treated cells showed that ATR is not apparently required for the acute suppression of origin firing within 25mins of IR damage (Fig.20a). This is consistent with Fig.19a, which shows that the knockdown of ATM alone permits origin firing at control levels. Therefore, any activation of ATR by IR-associated damage must either occur relatively late, or only become relevant at later stages, perhaps when ATM ceases to be active.

Turning to replication-blocking damage, ATR *is* responsible for the acute suppression of origin firing after UV damage (Fig.20b). (UV was used as the replication-blocking stimulus in these experiments because the activity of ATR is better characterised with respect to UV than to MMS, because there is less evidence that UV causes dsbs which would cross-activate ATM, and because UV should cause DNA damage more specifically than MMS, which probably non-specifically alkylates proteins and other cellular components.) ATR appears to be solely responsible for the UV-induced block to origin firing, because origins were fired at control levels in the ATR-knockdown cells. Downstream of ATR, CHK1 is reported to be the principal transducer of the intra-S phase checkpoint (Heffernan et al., 2002), but CHK2 is also activated in certain cell systems and the relative importance of these two kinases with respect to UV damage is not entirely

clear (Feijoo et al., 2001; Zachos et al., 2003). In checkpoint-competent HeLa cells, only CHK1 is detectably activated within 25mins of UV damage but if CHK1 is knocked down, CHK2 *does* become weakly phosphorylated (Fig.20c). Furthermore, the loss of CHK2 seemed to cause a slight decrease in CHK1 activation. This is in contrast to the response to IR, where the loss of either Chk kinase did not significantly promote the activation of the remaining kinase.

In DNA fibre analysis, CHK1 was found to be only partially responsible for transducing the origin-blocking signal, since CHK1-knockdown cells still showed a partial reduction in origin firing (Fig.20d). This may be partly due to the ectopic activation of CHK2 in siChk1-treated cells, but it remains possible that CHK2 does have a minor role in the response to UV damage despite not being visibly phosphorylated. When the same experiment was carried out in siChk2-treated cells, a slight defect in origin firing suppression was observed after the lower UV doses, but the checkpoint could still be activated effectively after more severe doses (Fig.20e). Thus it appears, as in the case of IR damage, that both Chk kinases can contribute to the intra-S phase checkpoint in response to UV damage, but that CHK1 again has the more important role.

Figure 20: Origin-firing response of siATR, siChk1 and siChk2-treated cells to IR and UV

a) The protocol in Fig.8a was used to quantify origin firing after 2.5-5Gy IR in cells treated with siATR. The graph shows the average of two independent experiments: at least 100 replication tracks being counted for each dose of IR. .

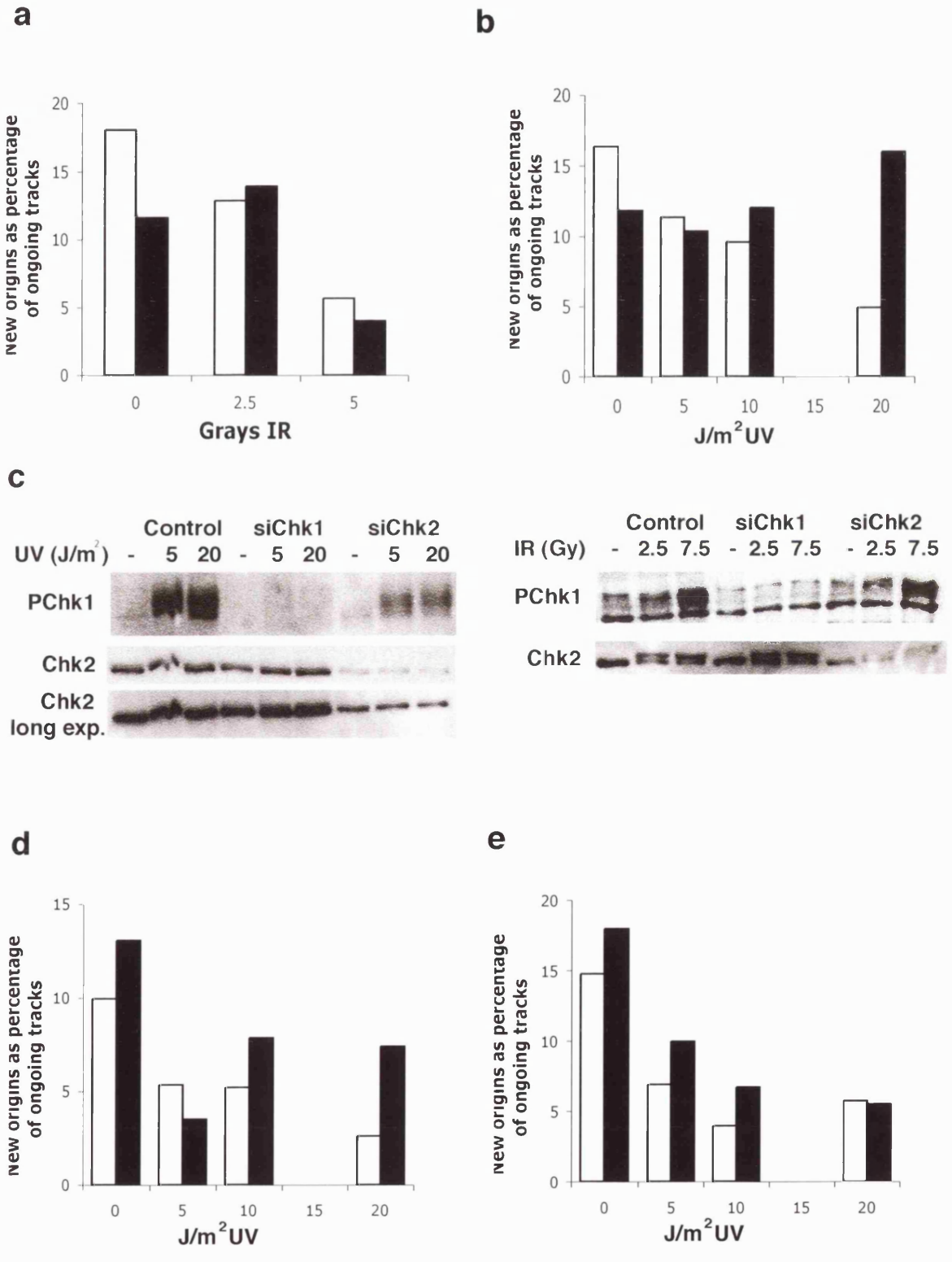
   White bars = mock-transfected cells, black bars = siATR-treated cells.

b) As in (a), using 5-20J/m^2 UV-C instead of IR.

c) Extracts were made from control, siChk1- and siChk2-treated cells 25mins after UV treatment (5J/m^2 or 20J/m^2) or IR treatment (2.5 or 7.5Gy). Western blotting was carried out for P-Ser317-CHK1 and CHK2.

d) As in (b), siChk1-treated cells.

e) As in (b), siChk2-treated cells.


a
New origins as percentage of ongoing tracks
20
15
10
5
0
0
2.5
5
Grays IR
b
New origins as percentage of ongoing tracks
20
15
10
5
0
0
5
10
15
20
J/m2UV
c
Control
siChk1
siChk2
UV (J/m2)
- 5 20 - 5 20 - 5 20
PChk1
Chk2
Chk2 long exp.
Control
siChk1
siChk2
IR (Gy)
- 2.5 7.5 - 2.5 7.5 - 2.5 7.5
PChk1
Chk2
d
New origins as percentage of ongoing tracks
15
10
5
0
0
5
10
15
20
J/m2UV
e
New origins as percentage of ongoing tracks
20
15
10
5
0
0
5
10
15
20
J/m2UV

With regard to fork movement, UV damage, like MMS, reduces mean fork progression in a dose-dependent manner. (Unlike MMS, no fork-slowing threshold was observed for UV damage in the range tested, suggesting that even $5J/m^2$ is sufficient to saturate NER and result in blocked replication forks.) As has been shown in *S,cerevisiae* (Tercero and Diffley, 2001), the slowing of replication forks is not apparently checkpoint-dependent because both siATR and siChk1-treated cells showed similarly reduced track lengths after UV damage (Fig.21a,b). This correlated with an overall S phase progression rate, as seen by flow cytometry, which was slowed comparably in siATR and control cells - supporting the idea that reduced fork movement is the major determinant of the severe S phase delay caused by replication-blocking damage (Fig.21c).

siATR-treated cells do, however, show a subtle alteration in the pattern of fork movement after UV damage. The double-labelled tracks, although progressively shortened, remained slightly longer in siATR-treated cells after all doses of UV, which might suggest that they stall somewhat less readily at UV lesions (see in particular Fig.21a, $20J/m^2$ UV). Furthermore, the control cells showed the expected dose-dependent increase in the number of IdU-only tracks, representing forks that are stalled without detectable CldU incorporation, but the siATR-treated cells did not show these tracks (Fig.21d). Downstream of ATR, CHK1 does not seem to be principally responsible for this phenomenon, since siChk1-treated cells showed near-control levels of fork stalling (Fig.21e) and much less of a difference in the lengths of double-labelled tracks (Fig.21b).

Preliminary experiments suggest that if a longer period of CldU labelling is allowed after UV damage, the difference in the number of stalled forks simply diminishes as those in control cells eventually restart or join up with new

replicons. Consistent with this, the lengths of ongoing tracks in control cells eventually meet and even exceed the lengths in siATR-treated cells (data not shown). It is not yet clear whether the extra fork progression seen in UV-treated ATR-deficient cells has any longterm consequences for fork movement or for S phase completion. Further investigation would be required to establish the viability of these cells, and also to clarify the exact kinetics of fork stalling and restart in siATR-treated cells, and the interplay between the checkpoint and DNA repair or lesion bypass in these cells compared to control cells.

Figure 21: Response of siATR and siChk1-treated cells to IR and UV: Fork movement

a) From the experiments in Fig.20, the lengths of at least 50 unidirectional (red-then-green) tracks were measured and the mean lengths were plotted with the green portion of each bar representing the CldU-labelled length. The graphs show the average of at least two independent experiments with at least 100 replication tracks counted in each case.

   Solid bars = mock-transfected cells, textured bars = siATR-treated cells.

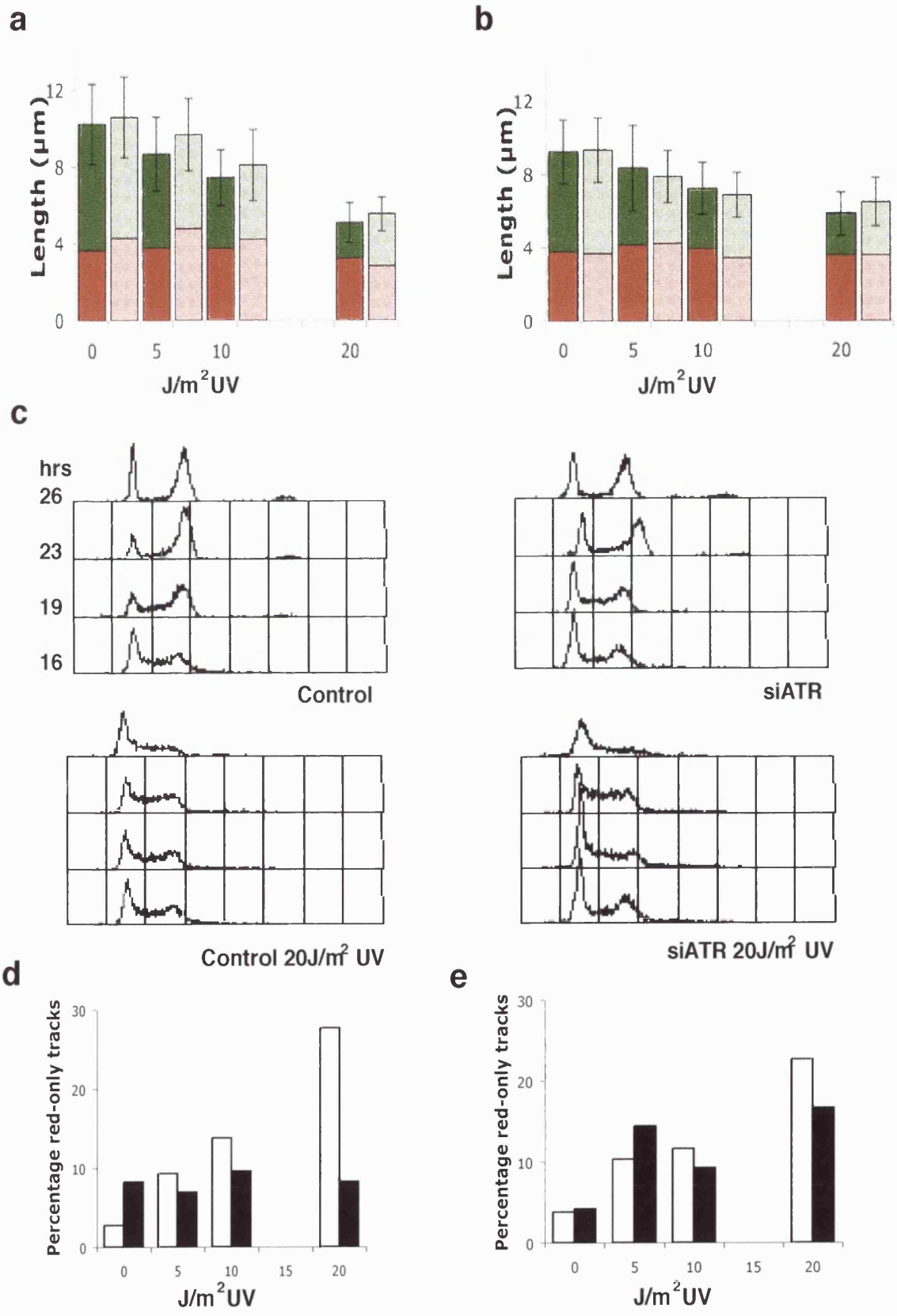
b) As in (a), siChk1-treated cells.

c) Synchronised siATR-treated cells were exposed to $20J/m^2$ UV in early S phase (16hrs after nocodazole release), then analysed by flow cytometry over the next 10hrs. This corresponds to 40-50hrs post-transfection.

d) From the experiments in Fig.20, IdU-only tracks were counted as a percentage of the total number of tracks.

   White bars = mock-transfected cells, black bars = siATR-treated cells.

e) As in (d), siChk1-treated cells.


a
b
Length (µm)
12
8
4
0
0
5
10
20
J/m²UV
c
hrs
26
23
19
16
Control
siATR
Control 20J/m² UV
siATR 20J/m² UV
d
e
Percentage red-only tracks
30
20
10
0
15
J/m²UV

ATR pathway: role in fork stabilisation

To examine the role of ATR in stabilising and restarting stalled forks without the added complication of DNA repair removing the fork-stalling lesions, the response of siATR-treated cells to HU and APH was examined. siATR-treated cells were arrested in early S phase with HU or APH, then the progression of S phase was followed by flow cytometry after release. Fig.22a shows that there was very little difference in bulk S phase progression between siATR-treated and control cells. Both populations released more efficiently from a short (30min) HU arrest than from a longer (3hr) arrest, as was seen before in terms of origin firing on DNA fibres (Fig.9). Only after 3hrs did a small delay in S phase progression appear in the siATR-treated cells compared to the control cells. The release from HU was also more efficient than the release from APH, regardless of the presence of ATR. Interestingly, cells treated with 5mM caffeine prior to an APH arrest *did* have a more severely retarded S phase than control cells, suggesting that caffeine treatment is *not* simply equivalent to the siRNA-mediated knockdown of ATR.

Western blotting to establish the pattern of Chk kinase activation during HU and APH arrest showed that during an HU arrest, CHK1 was phosphorylated in both control and siATR cells, but quantitatively less phosphorylated CHK1 appeared in the siATR cells and a super-shifted form of phospho-CHK1 which appeared in control cells after several hours remained specifically absent in siATR-treated cells. (The functional relevance of this form of CHK1 is not known). The gel-shifted form of CHK2 did not appear after any replication-stalling treatment, suggesting that CHK2 is never significantly activated in response to HU or APH (Fig.22b).

Despite the lack of any obvious defect in overall S phase recovery from an HU or APH arrest in the absence of ATR, fibre analysis was still carried out to detect any subtle defects in replication restart. In contrast to the severely impaired restart that was seen in floxed ATR-deficient HCT116 cells (Fig.17), stalled forks restarted quite efficiently in siATR-treated HeLa cells. Even after 9hrs in APH, stalled forks restarted almost as well in siATR-treated cells as in control cells (Fig.22c). Release from HU *was* consistently slightly slower in siATR-treated cells, but only by ~1μm (2.5kb) within 25mins (Fig.22d). Consistent with this, the number of IdU-only tracks representing forks which did not restart at all was not greatly increased after either HU or APH arrest (data not shown).

Although ongoing forks restarted unexpectedly well in these cells, ATR-knockdown did impair the firing of new origins within 25mins of HU release (Fig.22f), possibly accounting for the slight delay in bulk S phase progression shown in Fig.22a. This difference was not observed in the case of APH because almost no new origins fired within 25mins of release from APH even in the control cells (at least until the cells had been held in APH for at least 9hrs) (Fig.22e). This is consistent with the relatively slow release from APH which is seen by flow cytometry.

Figure 22: Response of siATR-treated cells to fork stalling by HU and APH

a) Synchronised siATR-treated, mock-transfected or caffeine-treated cells were arrested with 1mM HU or 10μg/ml APH in early S phase, then released 30mins or 3hrs later and analysed by flow cytometry over the next 9hrs.

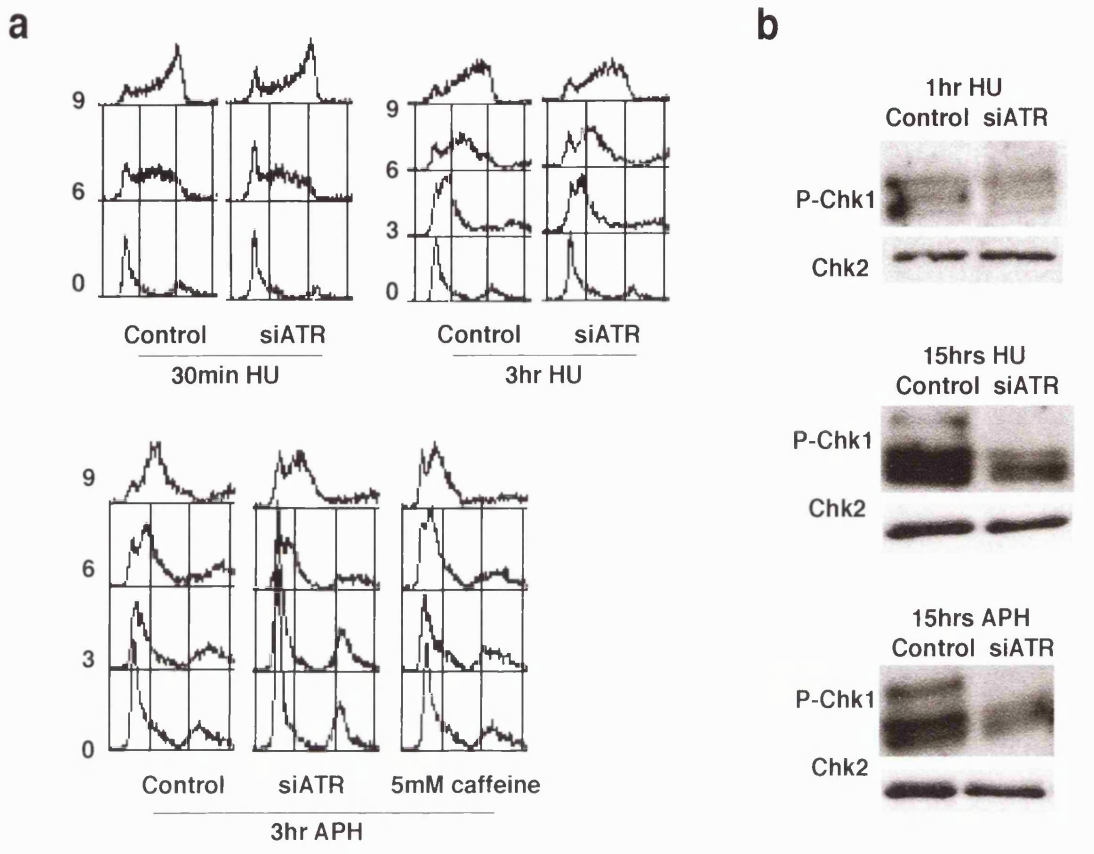
b) Extracts were made from siATR-treated or mock-transfected cells after 1hr or 15hrs in 1mM HU or 10μg/ml APH.

c) Cells were labelled for 15mins with 20μM IdU, then arrested for 0.5-9hrs with APH as above before releasing into 200μM CldU for 25mins. The lengths of at least 50 unidirectional tracks were measured and mean lengths calculated.

d) As (c), HU instead of APH.

e) The percentage of new origins (CldU-only tracks) was calculated from the APH-arrest/release experiment in (c).

f) The percentage of new origins (CldU-only tracks) was calculated from the HU-arrest/release experiment in (d).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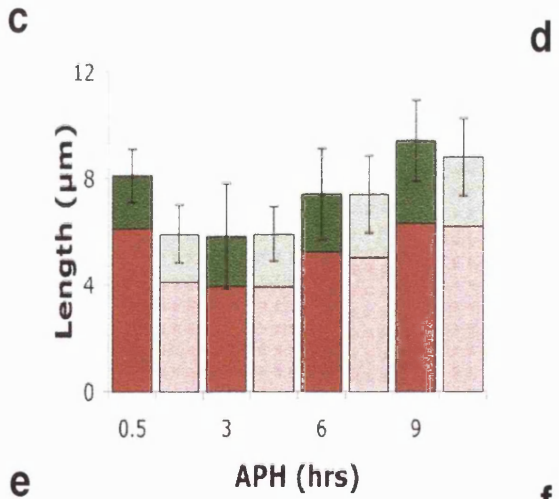
9
6
0
Control
siATR
30min HU
9
6
3
0
Control
siATR
3hr HU
9
6
3
0
Control
siATR
5mM caffeine
3hr APH
b
1hr HU
Control siATR
P-Chk1
Chk2
15hrs HU
Control siATR
P-Chk1
Chk2
15hrs APH
Control siATR
P-Chk1
Chk2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Length (µm)
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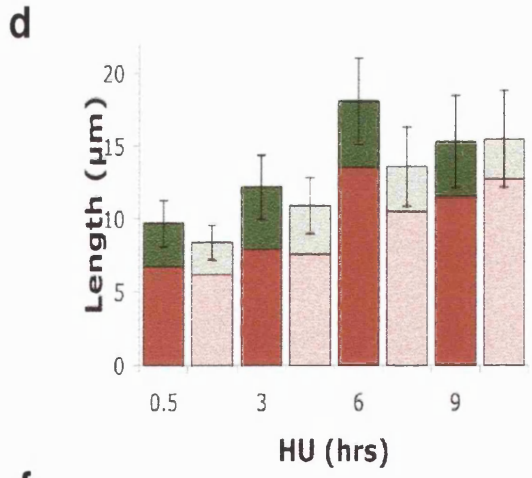
8
4
0
0.5
3
6
9
APH (hrs)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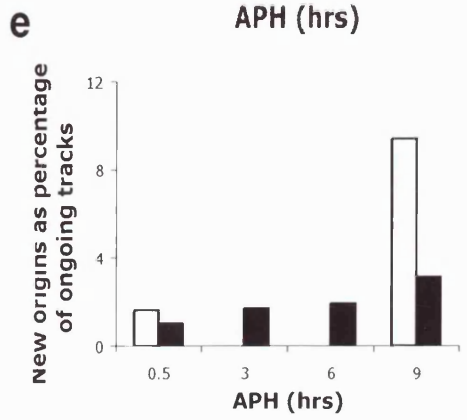
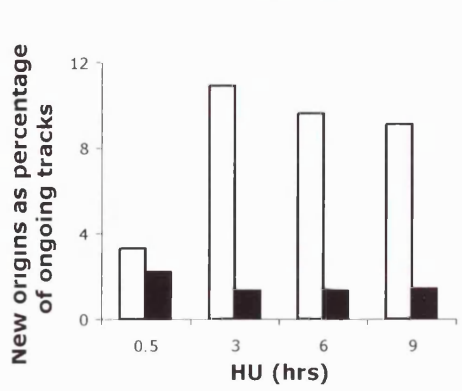
0.5
3
6
9
HU (hrs)


e
New origins as percentage of ongoing tracks
12
8
4
0
0.5
3
6
9
APH (hrs)


f
New origins as percentage of ongoing tracks
12
8
4
0
0.5
3
6
9
HU (hrs)

Chapter 5: Discussion

In order to investigate the roles of ATM and ATR in modulating replication during the S phase checkpoint, several different methods have been used to inhibit these kinases, including drug treatment and the use of various stable or conditional knockout/knockdown systems. First of all, the use of caffeine to inhibit ATM and ATR yielded somewhat confusing results. It is important to note that caffeine is a very poorly-specific inhibitor, so any interpretation of these data in terms of the ATR/ATM checkpoint system must be qualified with the possibility of off-target effects. For example, caffeine inhibits another PIKK, mTOR, with an IC_{50} of only 0.4mM: well below the level used in these experiments. mTOR has no known role in DNA damage checkpoints but it does control the translation of growth and proliferation factors in response to nutrient conditions (reviewed (Gingras et al., 2001)). This probably explains why caffeine interferes with cell cycle re-entry after serum starvation. mTOR also has a wide range of other roles: it regulates amino acid transport, is involved in the transcriptional activation of metabolic enzymes, and since it is a nucleocytoplasmic shuttling protein, it is thought to have further uncharacterised roles in the nucleus as well (Kim and Chen, 2000). It is not clear whether mTOR could affect the G1-S as well as the G0-G1 transition, so the reason for the caffeine-induced G1 arrest remains unknown. The same phenomenon has, however, recently been reported in primary fibroblasts, independent of both ATM and p53, so it is clearly not unique to HeLa cells (Kaufmann et al., 2003). Besides the non-specific inhibition of all PIKKs, caffeine inhibits various unrelated enzymes such as alkaline phosphatase (Wharton and Goz, 1979) and

p(ADPR) synthetase (Rowley et al., 1988), an enzyme which cleaves nicotinamide from NAD and is involved in many cellular processes including proliferation and DNA repair (Ueda and Hayaishi, 1985). Finally, even the well-established *in vitro* inhibition of ATR and ATM by caffeine has recently been called into question in certain *in vivo* situations (Cortez, 2003; Kaufmann et al., 2003). Despite all this, the effects of caffeine on S phase kinetics have been at least partially correlated with the effects of ATR-neutralising antibodies, or of up-regulating ATR/ATM targets in *Xenopus* extracts (Marheineke and Hyrien, 2004; Shechter et al., 2004), so ATM and ATR probably are at least some of the relevant targets of caffeine with respect to replication.

The role of ATM in the intra-S phase checkpoint

To avoid any unwanted effects of drug treatment, and to separate ATR from ATM, each PIKK was specifically targeted by two further methods. With regard to ATM, siRNA-mediated knockdown of ATM and an AT disease cell line gave fairly consistent results: a complete failure of the acute IR-induced suppression of origin firing but no major effect on the rate of ongoing forks. Therefore, the principal response to IR damage appears to be blocked origin firing and this is entirely dependent on ATM: no role for ATR was detected, at least within 25mins of IR exposure. Moreover, an efficient checkpoint response requires quite high levels of ATM, since the residual protein in siRNA-treated cells is apparently not sufficient. By contrast, the dramatic slowing of forks induced by caffeine certainly does not seem to be due to ATM inhibition. Undamaged ATM-deficient cells replicated at normal rates, and average tracks lengths were only very slightly shortened in siATM-treated cells after the higher

IR doses. This may be because forks have to pass through a certain number of IR-induced ssbs and damaged bases and, although a moderate dose of IR does not affect the rate of ongoing forks in checkpoint-competent cells, ATM may actually have a minor role in promoting efficient replication through such lesions. This might be mediated via ATM's phosphorylation of RAD9, since RAD9 in *S.pombe* promotes lesion bypass by translesion polmerases (Kai and Wang, 2003).

The role of ATR in the intra-S phase checkpoint

Turning to ATR, Cre-mediated excision, siRNA-mediated knockdown and inhibition by caffeine all gave slightly different results. This may be due to the different cell lines used (HCT116 and HeLa cells) together with the various flaws in each of the knockdown methods. The Cre/lox system carries the risk of non-specific effects on DNA replication associated with overexpression of Cre recombinase and/or adenoviral infection. Most of the infected cells die soon after the experiments are carried out, so the multiple changes associated with apoptosis may already be beginning by the time the cells are labelled for DNA fibre analysis. In addition, the heterozygous $ATR^{flox/-}$ cells have only ~20% of normal ATR levels and although this was reported to be sufficient for normal checkpoint responses (Cortez et al., 2001), Seckel syndrome cells which have similarly reduced ATR were recently reported to show dose-dependent deficiencies in ATR activity on several target proteins (O'Driscoll et al., 2003). Therefore, the assessment of a 'normal checkpoint response' may depend very much on the level of DNA damage applied and the endpoint measured. More importantly, $ATR^{flox/-}$ cells may have accumulated secondary mutations or adaptations allowing them tolerate reduced ATR levels and therefore to respond better to DNA damage. The

second knockdown method using siRNA was carried out in standard HeLa cells: it is non-toxic and cells do not die before they recover from the transient transfection, so any interference from the knockdown method itself or from incipient cell death is unlikely. However, the siRNA method is not 100% efficient so some ATR probably remains in all the transfected cells. By contrast, the Ad-Cre infected cells irreversibly lose their ATR gene, so there is probably a small time-window in which they represent a more complete knockdown. This issue of residual ATR may be very important – for example, in the case of CHK1, knockdown with siRNA can yield viable cells (Chen et al., 2003b) whereas an *in vivo* Cre-mediated knockout of CHK1 in the mouse mammary gland causes widespread apoptosis (Lam et al., 2004). Therefore, the exact amount of ATR in cells must be considered when attempting to reconcile the results from the two knockdown systems, together with the results from caffeine-treated cells. Furthermore, in either the Ad-Cre or the siRNA system, a subpopulation of uninfected or untransfected cells will dilute the data, making it very important to count a large number of tracks from many different areas of each fibre spread, and to average the results of several independent experiments.

ATR in normal S phase progression

Firstly, caffeine did not appear to affect the overall progression of S phase either with or without MMS damage, yet it halved the average rate of replication fork movement. These results are apparently the opposite of those obtained from similar experiments in CHO cells (Dimitrova and Gilbert, 2000a): a caffeine-treated S phase took twice as long as an untreated S phase when assessed by counting early and late replication patterns, yet *in vitro* run-on assays measured

the same amount of replication in nuclei with or without caffeine – a result interpreted as a normal rate of fork elongation. In more recent experiments, this time using *Xenopus* extracts, caffeine apparently increased the rate of origin firing by a factor dependent on the concentration of nuclei in the extract (Marheineke and Hyrien, 2004; Shechter et al., 2004). Concomitantly, it reduced fork movement by 25-50% so that there was no net change in the length of S phase. These studies led to the idea that ATR (and also ATM) plays a physiological role in regulating the rate of origin firing: PIKK inhibition by caffeine therefore abrogates the temporal programme of S phase and allows origins to fire rapidly and synchronously.

The model proposed from the *Xenopus* experiments could explain the results in Fig.11, since S phase in caffeine-treated cells could be completed in a normal timeframe if twice as many origins fired but the resultant forks moved at half their normal speed. The same could theoretically be true of the CHO cells studied by Dimitrova et al. (2000) since twice as many slow-moving forks in the caffeine-treated nuclei would actually generate the same amount of *in vitro* replication. It is not clear how the extra origins would be distributed in terms of replication foci, but the focal patterns in caffeine-treated cells may not be comparable to normal patterns: the vast majority of origins could fire early in 'type 1' patterns, while the delayed appearance of 'type 4/5' patterns might actually account for very little bulk replication as seen by flow cytometry. Thus, the model that caffeine induces extra origin firing but a slower rate of fork progression is consistent with results from mammalian cells.

In *Xenopus* extracts, ATR appears to be the most relevant target of caffeine, since when ATR is inhibited using ATR-neutralising antibodies, bulk

replication can be increased to the same extent as in caffeine-treated extracts (~50%) (Shechter et al., 2004). (ATM antibodies also elicit a smaller increase in replication (~20%), so a minor role for ATM has been proposed as well.) However, in a second study examining individual origins on DNA fibres, ATR-neutralising antibodies were much less effective than caffeine in inducing extra origin firing, suggesting that the exact level of ATR inhibition is very important and that low residual levels of ATR may be enough to regulate origin firing, at least in the absence of DNA damage (Marheineke and Hyrien, 2004). It is not yet clear whether this constitutive role for ATR is conserved as far as *S.cerevisiae*, since any change to the timing of specific late origins in the absence of Mec1/Rad53 remains the subject of conflicting reports (Santocanale and Diffley, 1998a; Shirahige et al., 1998). As for mammalian cells, the experiments described here provide no direct evidence that either ATR or ATM actually regulates origin firing in undamaged cells. Unlike the *Xenopus in vitro* system, specific *in vivo* knockdowns using siRNA are not instantaneous, so the number of new origins cannot be counted directly following PIKK inhibition. Once a knockdown is in effect, the entire steady state of origin firing, termination and ongoing forks may be shifted, but the percentage of new origins compared to ongoing forks would not necessarily increase. Indeed, the numbers of new origins in siATR-treated cells did *not* increase, although this may be due to insufficient ATR knockdown, since by contrast, floxed cells did show slightly higher rates of origin firing than $ATR^{flox/-}$ cells. In both knockdown systems, there was also a higher percentage of IdU-only tracks, which represent both termination events and stalled forks. More origin firing might logically lead to more terminations within a particular time window, but it might also lead to suboptimal replication rates

(the fork rate in floxed cells was indeed decreased compared to $ATR^{flox/-}$ cells) and thus to increased fork stalling. Therefore, it is not possible to definitively assign these IdU-only tracks as evidence of increased origin firing. In the siRNA-treated cells, forks moved at normal rates but there were still more IdU-only tracks, which could be taken as indirect evidence that termination, rather than fork stalling, is indeed increased. However, ATR may alternatively have an additional positive role in preventing the appearance of stalled forks, as discussed below. In conclusion, the results presented here do not definitively show that the lack of ATR allows increased origin firing in mammalian cells, although such a phenomenon may be still occurring.

The mechanism by which PIKKs suppress origin firing – both constitutively and in response to exogenous DNA damage - is relatively well-established in the *Xenopus* system: S phase promoting kinases (SPKs) are inhibited, leading to the prevention of CDC45 loading onto chromatin (Costanzo et al., 2000; Costanzo et al., 2003). It remains less clear, however, how inhibiting PIKKs with caffeine could simultaneously reduce the rate of ongoing forks (Fig.11b, (Marheineke and Hyrien, 2004)). If CDC45 is needed for the elongation as well as initiation of replication, then extra CDC45 loading in the absence of PIKKs might be expected to increase rather than decrease replication rates at ongoing forks. Therefore, it is possible that the PIKKs only control CDC45 loading at initiation and not at ongoing forks, or that extra CDC45 is simply unable to accelerate ongoing replication. Instead, ATR and/or ATM may have a second independent role in actively promoting fork progression and this may be abrogated by caffeine. For ATM, this seems unlikely as ATM is not chromatin-

associated or activated in the absence of dsbs. ATR, however, is associated with normally replicating chromatin (Dart et al., 2004) and might conceivably promote fork movement via claspin, which is probably present at all ongoing forks (Katou et al., 2003; Lee et al., 2003). Claspin has already been proposed to somehow 'promote' replication (Lin et al., 2004a).

In a simpler model, the change in fork rate caused by caffeine may be an indirect effect of increased origin firing rather than a direct effect of the lack of ATR. Either CDC45 or some other replisome component, or the level of dNTPs in the cell, could become rate-limiting for ongoing replication when too many origins fire all at once. In support of this idea, simply increasing the number of nuclei – and therefore the number of active origins - in a *Xenopus* extract can cause a decrease in fork rate (Marheineke and Hyrien, 2004). Furthermore, the degree of fork slowing seen in HeLa cells in the experiments described here generally increased after longer caffeine pre-treatments (data not shown), so this rate-limiting factor might develop over time in mammalian cells as well as in *Xenopus* extracts. No reduction in fork movement was seen in siATR-treated HeLa cells, but ATR may be insufficiently reduced in these cells. In floxed cells, fork rates *were* slightly reduced and the cells also had slightly higher origin firing rates.

Finally, it remains possible that the reduction in fork rates is simply a non-specific effect of caffeine and has nothing to do with ATR. The drug is known to intercalate into DNA (Tornaletti et al., 1989) and this could somehow impede fork movement, exacerbating if not actually causing the severe fork-slowing seen in caffeine-treated DNA. In conclusion, although the results described here are broadly consistent with ATR controlling fork movement in mammalian cells as

well as in *Xenopus* extracts, the model has yet to be directly proved and it seems that only low levels of ATR might be sufficient for this function.

ATR in the response to DNA damage

When cells are challenged with exogenous DNA damage, ATR is proposed to become hyper-activated compared with its normal S phase-regulating equilibrium state and this accordingly leads to a much more severe suppression of replication. Indeed, ATR does appear to severely suppress origin firing in response to UV or MMS just as ATM suppresses it in response to IR (Fig.15,20). Moreover, an efficient checkpoint response requires quite a lot of ATR: more than remains in siATR-treated cells. In the case of UV damage, the suppression of origin firing appears to be dependent solely on ATR even after $20J/m^2$ of UV. In the case of MMS, origin firing *was* still suppressed in floxed cells after the highest level of MMS damage, but this could be because the small amounts of ATR remaining in floxed cells trigger a relatively weak dose-response to MMS. This is consistent with the ability of these cells to weakly activate CHK1 (Fig.15d). Origin firing was suppressed after intermediate levels of MMS damage in $ATR^{flox/-}$ cells, further supporting the concept of an increasingly insensitive dose-response mediated by increasingly-reduced levels of ATR. A model in which the level of active ATR monitors the overall level of DNA damage (perhaps via ssDNA) is supported by several studies in yeast (Shimada et al., 2002; Tercero et al., 2003). (An alternative explanation for the MMS response seen in floxed cells is that there is some relatively insensitive crosstalk from ATM, perhaps activated by replication-induced dsbs. However, no such crosstalk was observed after an

equally replication-blocking level of UV damage, and CHK2 was never detectably activated so ATM would have to be acting exclusively through CHK1.)

In contrast to γ-irradiation, the types of DNA damage which activate ATR affect replication in a second way, by causing a dose-dependent slowing of replication forks. This appears to be checkpoint-independent, since neither siATR nor siChk1 prevented the slowing of fork movement. (In fact, as was discussed for ATM, ATR could actually have a minor role in minimising fork slowing by maximising lesion bypass via RAD9 phosphorylation.). The observed slowing of forks is consistent with the MMS response seen in checkpoint deficient *S.cerevisiae*, but in mammalian cells it seems to be the major reason for the very severe delay in S phase seen by flow cytometry, whereas in *S.cerevisiae,* most of the delay must be due to Mec1-dependent blocked origin firing because bulk DNA synthesis in an MMS-treated mec1 mutant proceeds as fast as an undamaged S phase (Paulovich and Hartwell, 1995; Tercero and Diffley, 2001). This probably indicates a difference in the way that the intra-S phase checkpoint is observed in *S.cerevisiae* compared to mammalian cells, rather than a fundamental difference in the checkpoint mechanism. A high density of stalled forks *was* detected in the yeast mec1 mutants, but they nevertheless finish bulk synthesis in a normal timeframe, so they must either be able to resolve stalled forks relatively efficiently, or be able to replicate most of their genome despite the occasional stalled fork. The mec1 mutants studied by Tercero et al. (01) do have elevated dNTP levels due to the Sml1 deletion, so perhaps their lesion bypass system is more efficient than it is in mammalian cells. Nevertheless, at least on a population level, stalled replication still persisted for long periods in the mec1

mutants, so lesion bypass is unlikely to be the whole explanation. Instead, differences in genome size and the length of S phase may mean that the extra origins which are fired in checkpoint-mutant yeast cells are sufficient to replicate most of the genome despite the stalled forks, whereas the extra origins fired in mammalian cells may not be enough to contribute significantly to bulk replication. This would suggest that mammalian cells, which fire origins throughout ~8hrs in a normal S phase, cannot simply fire all of these origins when the checkpoint is inhibited. Possibly other constraints, such as chromatin structure or insufficient replication factors, mean that only a subset of unfired origins can fire. In any case, whether the amount of unreplicated DNA left by terminally stalled replication forks is large or very small, it is likely to be lethal to the cell, making stalled forks a potent cytotoxic consequence of DNA damage in checkpoint-deficient cells.

Since slowed or stalled replication forks are clearly a significant issue after ATR-activating DNA damage, the last phenomenon examined in ATR-deficient cells was the stabilisation and restart of stalled forks. Several studies have implicated Mec1 in fork stabilisation in yeast, whether in MMS-damaged DNA (Tercero and Diffley, 2001), HU-arrested replication (Desany et al., 1998; Lopes et al., 2001b; Sogo et al., 2002) or at RSZs during normal replication (Cha and Kleckner, 2002). In mammalian cells, there is circumstantial evidence that ATR plays a similar role in somehow promoting stable replication when forks are especially prone to stalling, for example, in fragile sites (Casper et al., 2002). However, no direct visualisation of stalled forks by 2D-gel or electron microscopy has been achieved in ATR-deficient mammalian cells. DNA fibre labelling

certainly supported such a role for ATR, both in normal replication and in damaged DNA, but conflicting results were obtained from the two different ATR-knockdown systems.

In the $ATR^{flox/-}$ system, ATR was clearly required to allow forks to restart after an APH arrest, and this seemed to require quite high levels of ATR, since forks were not stabilised in either floxed or $ATR^{flox/-}$ cells. ATR activity might therefore be needed at each individual stalled fork, as has been proposed in yeast, to prevent replisome breakdown and/or to recruit recombination proteins to stalled forks (Katou et al., 2003; Lucca et al., 2004). However, in HeLa cells when ATR was acutely knocked down, no serious defect in fork restart was observed, either in the short term, by DNA fibre analysis, or on a wider scale by flow cytometry. It is possible that the siRNA simply did not reduce ATR levels enough, but it is also possible that HeLa cells are inherently resistant to the breakdown of stalled forks in the absence of ATR, having been selected for many other growth-promoting mutations. Perhaps, for example, they have constitutively increased levels of recombination and repair proteins which help to restart stalled forks. By contrast, ATR is still needed for checkpoint signalling to suppress origin firing in these cells, suggesting that the *in cis* and *in trans* roles of ATR can be separated, as has been demonstrated for Mec1 in *S.cerevisiae* (Tercero et al., 2003). It is notable that the $ATR^{flox/-}$ cells which did not restart stalled forks nonetheless showed strong phosphorylation of CHK1 (Fig.16b) and CHK1-depleted Hela cells showed near-control levels of fork stalling after UV damage whereas ATR-depleted cells did not. This might suggest that CHK1 is needed for *in-trans* checkpoint signalling from ATR, but not for the *in cis* activity at replication forks. Consistent with this, Mec1 but not Rad53 is required to stabilise pol α and pol ε at

stalled forks in yeast (Cobb et al., 2003), although a subsequent study did detect a more subtle defect in Rad53 mutants as well. The Rad53 mutants were defective in the long-term maintainance of polymerases at stalled forks (Lucca et al., 2004) and they also show aberrant fork structures in 2D gels and in EM (Lopes et al., 2001b; Sogo et al., 2002) but it is not clear whether this is an indirect consequence of the failure to suppress further origin firing – perhaps leading to the removal of replication factors and/or dNTPs from earlier forks – or whether Rad53 actually does have a *direct* role in fork stabilisation.

Returning to mammalian cells, the experiments described here show that although the restart of ongoing forks was not defective in siATR-treated cells, the firing of new origins *was* specifically defective after HU release. It is possible that a small number of the stalled forks which are not properly stabilised degenerate into dsbs and activate ATM, but no CHK2 activation was ever observed during an HU arrest, so significant DNA breakage and ATM activation seems unlikely. An alternative explanation is that new origins may fail to fire because dNTP levels are not restored fast enough in siATR-treated cells. This would also explain the slightly delayed appearance of CldU labelling on the ongoing tracks. Thus, although no mechanism has yet been reported, ATR – like Mec1 - might actually regulate dNTP levels in mammalian cells.

The data regarding fork stalling after UV or MMS damage is rather confusing due to the differences between MMS-damaged floxed HCT116 cells and UV-damaged siATR-treated HeLa cells. In the $ATR^{flox/-}$ system, fork stalling at MMS-induced lesions did not seem to be affected by the different levels of ATR in floxed and $ATR^{flox/-}$ cells: forks stalled readily in both systems and the sensitivity of these cells was even higher than that observed in HeLa cells.

Although HeLa and HCT116 cells are not directly comparable, this would be consistent with quite high levels of ATR being needed to keep fork stalling to a minimum. Paradoxically, however, in siATR-treated HeLa cells, the lack of ATR seemed to actually prevent forks from stalling at UV lesions: the lack of ATR apparently allowed forks to continue further than normal through UV-damaged DNA. Perhaps in unstabilised replisomes, lagging strand synthesis becomes uncoupled from leading strand synthesis and the extra CldU labelling represents aberrant replication of only one DNA strand (Sogo et al., 2002). This, however, might be expected to occur at alkylated bases in the $ATR^{flox/-}$ cells as well as at UV lesions in the HeLa cells, since either type of lesion can stall DNA polymerases *in vitro* (Larson et al., 1985; Moore and Strauss, 1979). To account for the difference, the repair proteins which are recruited to DNA lesions could contribute to fork slowing, and ATR could perhaps be required to recruit NER components to UV lesions but *not* to recruit BER components to alkylated bases. Thus, in the absence of ATR, translesion synthesis and replication run-on might occur instead of fork stalling and NER. This is currently entirely speculative and further experiments are required to clarify ATR's exact role in fork stalling, both after nucleotide depletion and after DNA damage by different agents.

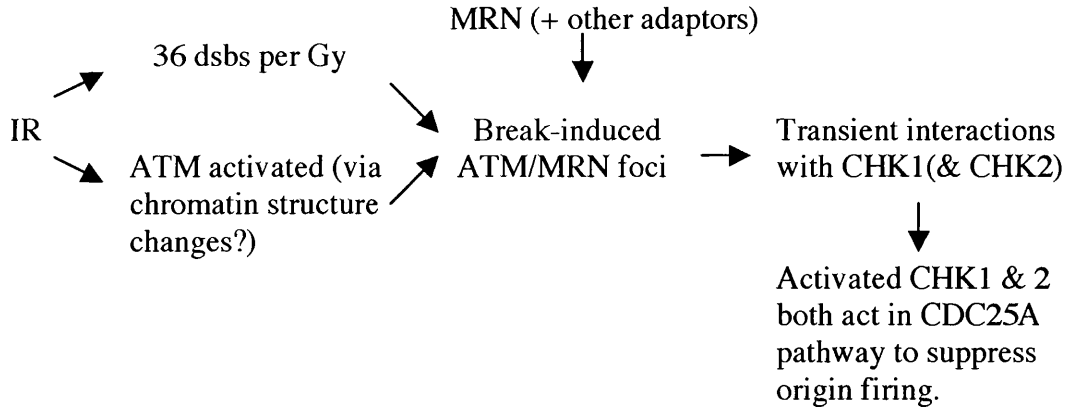
The roles of CHK1 and CHK2 downstream of ATM and ATR

To determine which of the Chk kinases is responsible for the S phase checkpoint effects of ATM and ATR, each Chk kinase was knocked down and the suppression of origin firing after UV or IR damage was measured. Downstream of ATM, CHK1 and CHK2 both appear to have roles in transducing the IR-induced checkpoint signal: after moderate doses of damage, cells lacking either

kinase were defective in their IR response. CHK1-deficient cells were more severely affected, suggesting that CHK1 has the more important role, although the relative contributions of the two kinases are hard to assess accurately because the two knockdowns were not identically effective. (CHK2 targeted-knockout cells are, however, entirely checkpoint proficient, further supporting a more central role for CHK1 than CHK2.) After high doses of IR, neither knockdown prevented the cells from suppressing origin firing, suggesting that either kinase can enforce the checkpoint alone after severe damage. It is also possible that a third transducer begins to act after severe damage: both Chk kinases were not successfully knocked down simultaneously so it is not clear whether together they account for the entire checkpoint response. No other signal-transducer has been mechanistically defined, but NBS1 has been reported to have a non-overlapping, partial effect in preventing the RDS phenotype of ATM cells (Falck et al., 2002). When NBS1 was knocked down with siRNA, cells still showed a dose-dependent suppression of origin firing but they seemed to have significantly higher basal levels of origin firing. Theoretically, this could account for the partial RDS phenotype of NBS cells: when more origins are firing, the normal amount of Chk kinase activation might only partially suppress these origins, so NBS cells would synthesise more DNA than normal cells despite the activation of the CHK1/CHK2 checkpoint. However, further work is required to establish whether NBS1-deficient cells are genuinely firing origins at a higher rate and if so, why. The most obvious explanation would be that NBS1 is required in the physiological control of origin firing – presumably acting as an adaptor for CHK1 activation at fork-associated ssDNA. MRN *is* necessary for the activation of CHK1 (as well as CHK2) after IR damage (Gatei et al., 2003) but it is not yet clear whether MRN is

also needed at ssDNA, where the adaptors are thought to be 9-1-1, RAD17 and claspin. Circumstantial evidence has recently linked MRN to ssDNA via the RPA foci which form at putative sites of HU-stalled replication (Robison et al., 2004), but further work is required to establish whether stalled forks and ssDNA are actually the relevant signals for MRN focus formation. Alternatively, it must be considered that the extra CldU-labelled tracks do not actually represent new origins at all, since in the DNA fibre labelling system, a steady state of elevated origin firing might logically lead to an equally elevated number of ongoing forks. Perhaps the lack of NBS1 instead allows a high level of some sort of repair synthesis (although this would have to be very rapid and extensive in order to appear as stretches of several kb within 25mins).

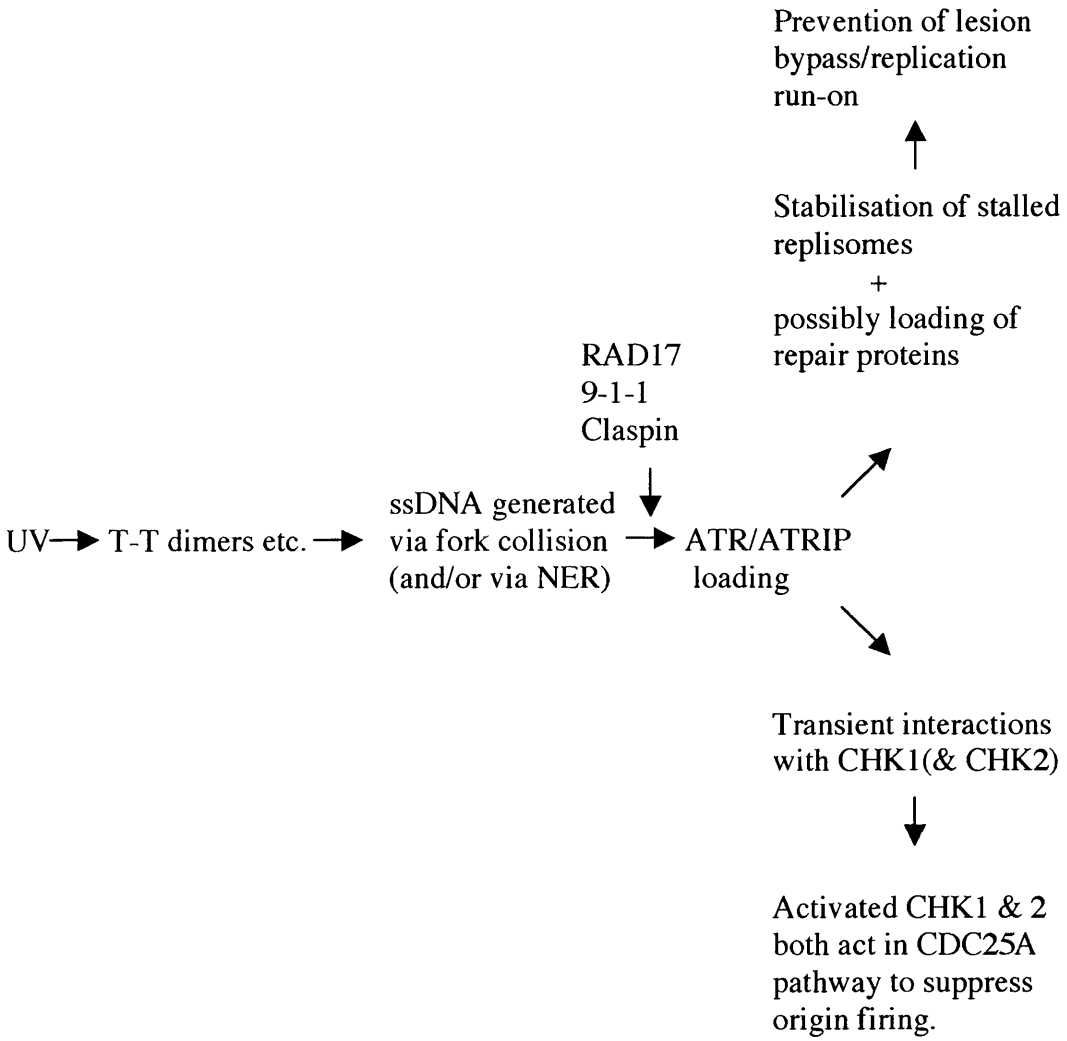
Diagram 1: The S phase response to IR



Turning to ATR, CHK1 appears to be the principle transducer of ATR-activating damage since this kinase is strongly phosphorylated after UV exposure whereas CHK2 is not detectably phosphorylated and CHK2-knockdown cells are relatively proficient in suppressing origin firing in response to UV. However, CHK1-knockdown cells are not completely deficient in the origin firing response and this could be because CHK2 becomes hyper-activated when cells lacking CHK1 experience UV damage, a phenomenon which has also been observed in CHK1-knockout DT-40 cells (Zachos et al., 2003). In the absence of CHK1, UV damage may be processed into dsbs which activate ATM – and thence CHK2 – and/or ATR may act directly on CHK2 at ssDNA.

By contrast to the origin-firing response, it seems possible that neither Chk kinase is involved in ATR's second role of stabilising stalled forks. CHK2 was never activated by HU or APH and CHK1, although it was strongly activated in APH-arrested cells, did not prevent the irreversible stalling of arrested forks. It remains to be established whether ATR carries out its fork-stabilising role directly, or whether other transducers – for example, claspin – might be needed instead.

Diagram 2: The S phase response to UV



Summary

- IR causes a reduction in DNA synthesis during S phase via a dose-dependent suppression of origin firing.

- This is principally ATM-dependent. CHK1 and CHK2 collaborate downstream of ATM to enforce the block to origin firing, with CHK1 taking the major role.

- UV and MMS cause reduced fork movement, probably via stochastic fork stalling and restart, as well as causing suppressed origin firing.

- The origin-firing response to UV is entirely dependent on ATR with downstream roles for CHK1 and CHK2, as above.

- Reduced fork movement, by contrast, is not primarily dependent on ATR or CHK1.

- ATR does have a role in efficient fork stalling at UV lesions, but not at MMS lesions, and also in stabilising the stalled forks caused by polymerase inhibition and probably also by UV lesions so that replication is able to restart.

Future Work

A number of the questions raised here about the mechanics of the intra-S phase checkpoint merit further investigation. Firstly, the issue of dNTP pools in mammalian cells should be clarified by testing p53-positive cells for changes in dNTP levels after DNA damage, both in S phase and in G1. When yeast experience DNA damage, increased dNTP levels could clearly offer survival advantages for a single-celled organism, but the balance of advantages between mutagenesis and cell survival may be different in metazoan organisms.

Secondly, it remains unclear whether ATR (and/or ATM) actually directly regulate origin firing in discrete mammalian cells as well as in the *Xenopus* extract system: the whole balance between checkpoint proteins and replication factors in the two systems may be entirely different. If origin firing *is* regulated by ATR or ATM, are PIKKs wholly responsible for the temporal program of origin firing, or can they only modulate it within the constraints of chromatin structure or particular DNA sequence elements? And does ATR directly regulate fork movement as well as origin firing, or is this an entirely non-specific effect of caffeine? If ATR *does* regulate fork movement, is this a direct effect, or an indirect effect of the failure to suppress origin firing so that replication factors become limiting? In order to definitively separate possible roles for ATR in fork-slowing and in origin firing, an *in vitro* system would be needed in which replication occurred from a set number of origins, supplied with an excess of all replication factors, in the presence or absence of a checkpoint signal. If the checkpoint signal still caused reduced fork movement, this would suggest that ATR does affect ongoing forks as well as unfired origins. Such a system would, however, be difficult to arrange if the checkpoint signal actually consists of ATR

monitoring active replication forks. In an alternative approach to investigating ATR's role in fork progression, claspin currently seems to be the best candidate for transducing such an activity, so claspin could simply be knocked down to look for an effect on fork progression rates.

The longterm effects of aberrant origin firing during a PIKK-deficient S phase have not yet been directly investigated, either in *Xenopus* or in mammalian cells, although the fact that chromosome breakage occurs, especially at fragile sites, in ATR-deficient cells suggests that some forks stall irreversibly and that replication cannot therefore be completed. It would be interesting to know if this is a direct result of too many active origins leading to suboptimal replication rates and hence to fork stalling: if so, it would also be interesting to know which factor actually becomes limiting for replication. To address this question, PIKKs would have to be inhibited to levels that caused measurable increases in origin firing, and evidence of DNA damage, such as chromosome rearrangements or dsbs in a comet assay, would then have to be correlated with the rate of origin firing. With regard to the limiting replication factor, it should then be possible to suppress the DNA damage by overexpression of the relevant factor, or by adding an excess of dNTPs if the limiting factor turned out to be dNTPs. (This latter would probably be the easiest to achieve, and the fact that chromosome breakage at RSZs in yeast can be suppressed by the Sml1 deletion (Cha and Kleckner, 2002) suggests that dNTPs are indeed the limiting factor, at least in *S.cerevisiae*.)

Several more focused questions have also been raised by the knockdown of particular checkpoint proteins. CHK1 and CHK2 still remain to be eliminated simultaneously in order to establish whether together, they are entirely responsible

for controlling origin firing. This could potentially be done by double-siRNA or by using siChk1 in $CHK2^{-/-}$ cells. The extra CldU-labelled tracks which appeared in NBS1-knockdown cells also need to be confirmed as either new replicons or repair patches, and the effect of either of these on the RDS phenotype needs to be clarified.

With regard to ATR, the reason for the conflicting results regarding ATR-dependent fork stalling and restart in HCT116 cells and HeLa cells remains to be established, and any role for CHK1 in fork stabilisation has not been directly tested. This would require CHK1 knockdown in the HCT cell system, since these were the cells that showed defective stabilisation in the absence of ATR, whereas HeLa cells did not. Furthermore, the differences between the two cell systems regarding fork stalling after different forms of DNA damage should be investigated in detail - initially by looking at MMS damage in siATR-treated HeLa cells and, conversely, UV damage in floxed HCT116 cells. This would determine whether ATR's role in preventing fork stalling is damage-specific – with replication run-on occurring exclusively at UV lesions but not at MMS lesions - or whether it is merely HeLa-cell-specific. If ATR is genuinely needed for some specific aspect of the UV response, this could be investigated by knocking down ATR in NER-deficient cells or XP-V cells. It might also be possible to use antibodies raised against UV lesions to actually detect the fork-stalling stimuli on DNA fibres.

Finally, the functions of other transducers which have as-yet-unknown roles in the RDS phenotype – for example, Smc1 and FANCD2 – could be investigated by DNA fibre analysis.

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Visualization of Altered Replication Dynamics after DNA Damage in Human Cells*

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Eukaryotic cells respond to DNA damage within the S phase by activating an intra-S checkpoint: a response that includes reducing the rate of DNA synthesis. In yeast cells this can occur via checkpoint-dependent inhibition of origin firing and stabilization of ongoing forks, together with a checkpoint-independent slowing of fork movement. In higher eukaryotes, however, the mechanism by which DNA synthesis is reduced is less clear. We have developed strategies based on DNA fiber labeling that allow the quantitative assessment of rates of replication fork movement, origin firing, and fork stalling throughout the genome by examining large numbers of individually labeled replication forks. We show that exposing S phase cells to ionizing radiation induces a transient block to origin firing but does not affect fork rate or fork stalling. Alkylation damage by methyl methane sulfonate causes a slowing of fork movement and a high rate of fork stalling, in addition to inducing a block to new origin firing. Nucleotide depletion by hydroxyurea also reduces replication fork rate and increases stalling; moreover, in contrast to a recent report, we show that hydroxyurea induces a strong block to new origin firing. The DNA fiber labeling strategy provides a powerful new approach to analyze the dynamics of DNA replication in a perturbed S phase.

Many types of DNA damage can cause mutations in the genome of a cell, not only by direct mutagenesis but also by generating lesions that are processed into mutations when DNA is replicated during S phase. Mechanisms that guard against this include multiple DNA repair systems and also cell cycle checkpoints that coordinate cell cycle progression with the DNA damage response (1). One such checkpoint acts within the S phase to reduce the rate of DNA synthesis, presumably minimizing the risk of damage being fixed into potentially dangerous mutations before it can be repaired.

The reduction in rates of DNA synthesis in the intra-S checkpoint may be due to any of a combination of parameters: the overall number of active origins, the temporal program of origin firing, the rates of movement of all active forks, and the occurrence of "fork stalling" events. Any or all of these parameters may be affected by DNA damage, either as a direct physical result of DNA lesions or via the action of checkpoint proteins. This issue has been addressed in some detail in the budding yeast *Saccharomyces cerevisiae*, in which replication from specific origins has been examined after treatment with methyl methane sulfonate (MMS)[1] and hydroxyurea (HU) using a combination of Southern blot, two-dimensional gel, and density transfer analyses of replication intermediates. These techniques can separate effects on origin firing from effects on fork rate, at least on a population level, and they have shown that origin firing is blocked in response to MMS or HU (2, 3) and that rates of fork movement are also reduced after MMS damage (2).

The block to origin firing in yeast depends on the checkpoint kinases Mec1 and Rad53, whereas the reduction in fork rate appears to be independent of these kinases. Mec1 and Rad53 (homologues of human ATM/ATR and Chk2, respectively) are also central to several other aspects of the S phase checkpoint: the induction of a transcriptional program of damage response genes (4, 5), the prevention of irreversible fork stalling after MMS damage (2, 6, 7), and the increase of dNTP levels in the cell after damage (8). It is not clear, however, whether these additional checkpoint responses actually affect the rate of DNA synthesis.

S phase responses to DNA damage have also been examined extensively in human cells. However, by contrast to the techniques described above, the standard assay for an S phase checkpoint response in mammalian cells, the radioresistant DNA synthesis assay, simply measures rates of overall DNA synthesis by pulse labeling a population of cells with tritiated thymidine after DNA damage. Because it only measures bulk synthesis, this assay cannot distinguish effects on origin firing from those on either fork movement or fork stalling. Moreover, it is affected not only by intra-S phase changes to DNA synthesis but also by inhibition of the G_1-to-S transition. In addition, to correlate the incorporation of tritiated thymidine with DNA synthesis, it is necessary to assume that the specific activity of the endogenous dNTP pools remains constant. These pools may, however, be affected by changes in the rates of *de novo* nucleotide synthesis and/or nucleotide salvage after damage. Therefore, in the absence of a good range of efficient, sequence-defined early and late origins in mammalian genomes (which might facilitate the use of the same techniques employed to study *S. cerevisiae*), various alternative assays have been used to further investigate specific aspects of the mammalian S phase checkpoint.

Size separation of ^{3}H-labeled DNA on an alkaline sucrose gradient after treating cells with ionizing radiation (IR) led to the inference that origin firing is blocked because the proportion of small DNA fragments, assumed to represent recently fired origins, is reduced after IR damage (9, 10). Longer frag-

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[1] The abbreviations used are: MMS, methyl methane sulfonate; HU, hydroxyurea; IR, ionizing radiation; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline; Gy, gray(s).

ments of labeled DNA, assumed to represent ongoing forks, were also found to be reduced but only after much higher doses of IR. A similar block to origin firing was observed after MMS and UV damage, with fork movement again being affected to a lesser extent and only after longer time periods (11, 12). The response to both IR and UV was found to be deficient in ATM cells. It is important to note, however, that alternative interpretations of much of these data could be made because it is not possible to tell how the large and small DNA fragments actually originated, and like the radioresistant DNA synthesis method, this assay could be skewed by changes to dNTP levels as well as cell cycle effects outside the S phase. Indeed, a subsequent investigation of ^{3}H labeling of DNA in asynchronous *versus* synchronized cell populations showed that at least 50% of the reduction in ^{3}H labeling that follows exposure to IR in an asynchronous population was due to the complete prevention of S phase entry via a G_1/S checkpoint, as opposed to any intra-S phase change in replication dynamics (13). Nevertheless, the existence of a block to origin firing that is genuinely intra-S phase and ATM-dependent has been corroborated by a second method: two-dimensional gel analysis of replication in rDNA (one of the few areas in the mammalian genome showing sequence-defined "early" and "late" replication). This showed, at least qualitatively, that unfired origins could be blocked following IR damage *within* S phase, whereas fork movement appeared to be minimally affected, at least after moderate IR doses (14).

Replication dynamics and their dependence on checkpoint proteins were not tested by the two-dimensional gel method after other forms of DNA damage such as alkylation by MMS. However, an alternative approach has been used to examine origin firing after aphidicolin or HU treatment, drugs that stall replication. This technique, involving the fluorescent labeling of characteristic patterns of "early S" and "late S" foci in Chinese hamster ovary cells, revealed an ATR/Chk1-dependent block to the appearance of late replication patterns when the cells are treated with aphidicolin (15, 16). This was interpreted as a checkpoint-dependent block to origin firing; however, the method does not yield quantitative data on the numbers or proportions of affected origins in the labeled foci, nor can it address other parameters such as fork rate or fork collapse.

Finally, all of the techniques described above, including those used to examine replication in yeast, rely on examining replication intermediates in populations of cells. Thus, all such approaches may miss important information that can be obtained by examining individual replication forks.

To integrate all these different pieces of information using a single experimental system, a DNA fiber-labeling strategy has been developed in which all the various parameters determining DNA synthesis during the S phase can be assessed individually, on the level of single replication forks as opposed to whole cell populations. This method measures DNA synthesis across the entire genome, independently of sequence or structure; it is quantitative, and the results can be subjected to statistical analysis. The technique has been used in a systematic investigation of both the immediate and longer term changes to replication dynamics, which occur after a variety of DNA-damaging and replication-stalling stimuli.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—HeLa cells were grown as monolayers in Dulbecco's modified Eagle's medium +10% fetal calf serum. Synchronization was carried out by adding 0.17 μM nocodazole (from stock solution 3.4 mM in Me_2SO). After 4–5 h, rounded mitotic cells were shaken off into prewarmed PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EDTA, 2 mM $MgCl_2$, pH 6.9), collected with minimal centrifugation (~130 × *g* for 5 min) and replated in fresh medium. DNA damaging treatments were applied 15–16 h after replating, when the majority of cells were in the early S phase. The experiment in Fig. 4*e* was carried out in unsynchronized IMR90 cells, also grown in Dulbecco's modified Eagle's medium + 10% fetal calf serum.

Flow Cytometry—Cell samples were prepared by trypsinizing, washing in cold PBS, and fixing for at least 2 h in 70% ethanol at 4 °C. The cells were then washed in complete PBS and incubated for 30 min in 0.5 ml of complete PBS containing 40 μg/ml propidium iodide and 0.5 mg/ml RNase A. Flow cytometry was carried out using a BD Biosciences FACScan.

DNA Damaging Treatments—MMS (100% solution; Sigma) was added directly to the culture medium at final concentrations of 0.005–0.03% (0.59–3.54 mM). After 20-min treatments the MMS was removed, and cells were washed twice with MMS-free medium before incubating in further fresh medium.

IR exposures were carried out at between 1 and 10 Gy (~25–250 s; control cells were removed from the incubator for the same time period). HU (Sigma) was dissolved in water and added to the culture medium at final concentrations of 20 μM to 2 mM.

Replication Labeling and DNA Fiber Spreads—The cells were single-labeled with 50 μM IdU for 10–60 min, or, for double-labeling, 10 μM or 20 μM IdU for 10 min and then 100 μM CldU for 20 min. In the experiments in Fig. 5, the cells were pulsed with 20 μM IdU for 10 min directly before DNA damage, then incubated with 50 μM thymidine for 15 min to wash out the IdU, and then kept in fresh medium before double-labeling 1.5–4.5 h later.

DNA spreads were made as described by Jackson and Pombo (18), with certain modifications. Briefly, the cells were trypsinized and resuspended in ice-cold PBS at 2.5 × 10^5 cells/ml. The labeled cells were diluted 1:8 in unlabeled cells, and 2.5 μl of cells were mixed with 7.5 μl of spreading buffer (0.5% SDS in 200 mM Tris-HCl, pH 7.4, 50 mM EDTA) on a glass slide. After ~8 min the slides were tilted at ~15°, and the resulting DNA spreads were air-dried, fixed in 3:1 methanol/acetic acid, and refrigerated overnight.

Immunolabeling—The slides were treated with 2.5 M HCl for 1 h, washed several times in PBS, and blocked in 1% bovine serum albumin, 0.1% Tween 20. The slides were then incubated at room temperature with the following antibodies, rinsed three times in PBS, and then washed three times for 20 min in blocking buffer between each incubation: 1) overnight in 1:2000 rat anti-bromodeoxyuridine (detects CldU) (OBT0030F Immunologicals Direct); 2) 2 h in 1:1000 Alexafluor 633-conjugated anti-rat (A-21094 Molecular Probes); 3) 2 h in 1:500 mouse anti-bromodeoxyuridine (detects IdU) (MD5100 Caltag); and 4) 2 h in 1:1000 Cy3-conjugated anti-mouse (C-2181 Sigma). The slides were then counterstained for 20 min with 1:20 000 YOYO-1 in PBS (Molecular Probes) before rinsing three times in PBS and mounting in PBS/glycerol. Microscopy was carried out using a Zeiss LSM Meta 510 confocal microscope.

RESULTS

S Phase Progression is Slowed by IR, MMS, and HU—Many techniques used to synchronize cells in S phase, such as aphidicolin, mimosine, or double thymidine blocks, interfere with replication forks and are likely to activate DNA damage responses. Therefore, in this study, HeLa cells were synchronized by nocodazole arrest, mitotic shake-off, and release for 16 h, at which point most cells are in the early S phase. Initially, we used such synchronized cells to examine the effects of various treatments on overall S phase progression. First, the cells were treated with 20-min pulses of 0.001–0.03% MMS, the MMS was removed, and S phase progression was followed by flow cytometry over the next 12 h. Fig. 1*a* shows that S phase was slowed in a dose-dependent manner, ranging from a mild effect after 0.005% MMS to nearly complete arrest over 12 h after the 0.03% treatment. Second, S phase progression was followed after exposure to 1 or 5 Gy of IR (exposures that should cause ~36 and 180 double-stranded breaks/cell, respectively (17)). 1 Gy did not cause a detectable slowing of S phase, but 5 Gy resulted in a moderate slowing of S phase progression (Fig. 1*b*). Third, 5–100 μM HU was added to the cells in the early S phase. Again, a dose-dependent slowing of the S phase was observed (Fig. 1*c*); 5 μM HU had little effect, 20 μM caused a significant slowing of S phase, and 100 μM lead to arrest with a nearly 2C DNA content.

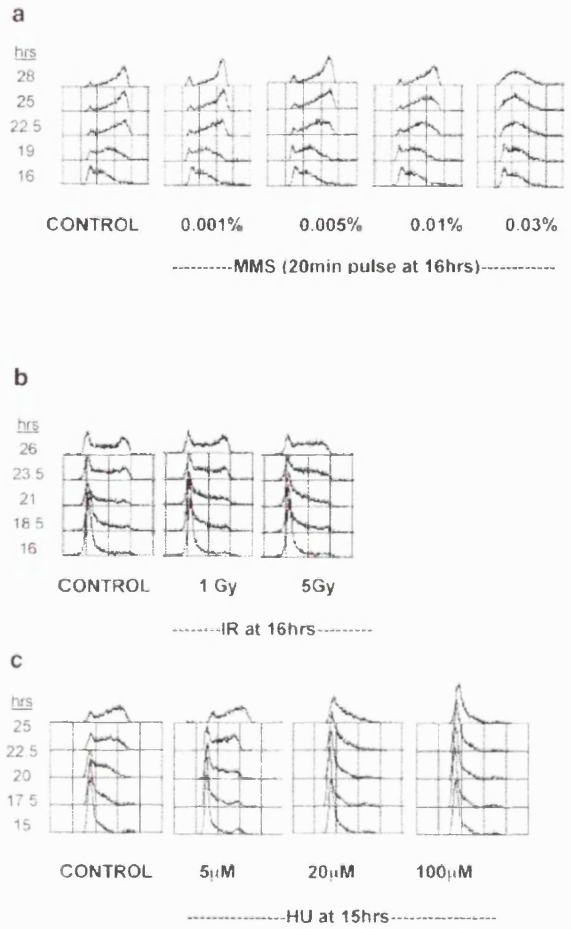

FIG. 1. **S phase progression is slowed by IR, MMS, and HU.** *a*, cells were synchronized by mitotic shake-off and treated in early S phase with 0.005–0.03% MMS for 20 min. Cell cycle progression was followed over the next 12 h by flow cytometry. *b*, cells as in *a*, exposed to 1–5 Gy IR in the early S phase. *c*, cells as in *a*, with 5–100 μM HU added to the medium in the early S phase.

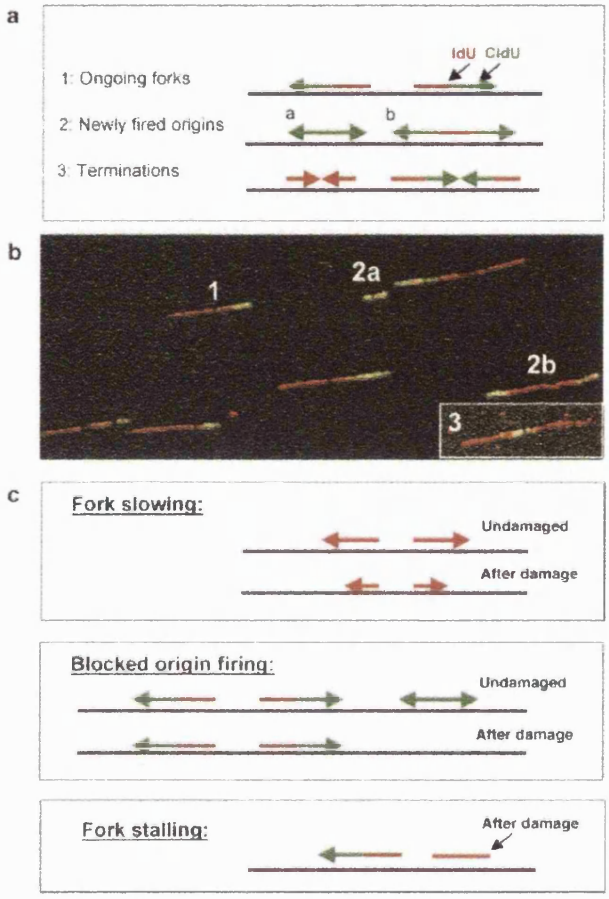

FIG. 2. **Replication track forms visualized by fiber labeling.** *a*, schematic of double-labeled replication tracks. *b*, example of labeled replication tracks. *c*, schematic showing alterations to replication tracks as a result of DNA damage.

Fork Movement Is Reduced by MMS and HU but Not by IR Damage—The DNA fiber labeling (DIRVISH) technique (18) has been adapted in this study such that two distinguishable modified nucleotides, IdU and CldU (19), could be used to label replication within a single S phase. In this technique (itself adapted from the classical DNA fiber autoradiography technique (20) in which newly replicated DNA is labeled with tritiated thymidine), the cells are pulse-labeled with halogenated nucleotides, then collected, and lysed on a glass slide. By tipping the slide, DNA from the cells is spread out in the form of single fibers. This DNA is subsequently fixed, denatured, and immunolabeled to detect the halogenated nucleotides. In these experiments, all of the DNA was then counterstained in a third color with YOYO-1 DNA dye, allowing the exclusion of any broken or tangled fibers. Consecutive pulse labeling of the S phase cells with IdU and then CldU yields double-fluorescently labeled tracks on the DNA that can be interpreted unambiguously as either ongoing forks, newly fired origins, terminations, or fork stalling events (Fig. 2). The length of any track after a given labeling period is proportional to its fork rate, whereas counting the relative numbers of different track forms can determine changes in the rates of origin firing or fork stalling after DNA damage.

DNA fiber assays were carried out after each of the three treatments examined in Fig. 1 to establish which parameter(s) of DNA synthesis contributed to the overall slowing of S phase seen by flow cytometry. To quantify any change in fork rates after DNA damage, the cells were exposed to 20-min pulses of MMS (0.005–0.03%) and then, after removal of the MMS, immediately labeled with IdU for 10–60 min before preparing DNA fiber spreads. The mean length of at least 50 IdU-labeled tracks was calculated for each time period. Fig. 3*a* shows that fork rates were reduced for at least 60 min after more severe MMS treatments. The severity of slowing was correlated with the MMS dose, but slowing was only observed above ~0.01% MMS.

Fig. 3*b* shows that reduction of cellular dNTP pools by treatment with hydroxyurea also slows replication forks in a dose-dependent manner. When cells were treated with sufficiently high levels of HU (above ~100 μM), the forks were essentially stalled, and very little progression occurred over several hours (data not shown). By contrast to MMS and HU, IR did not cause detectable fork-slowing, even at doses that do reduce overall S phase progression. Fig. 3*c* shows no significant change in the mean lengths of tracks labeled after IR exposures of up to 5 Gy.

Origin Firing Is Rapidly Inhibited after IR, MMS, or HU—DNA fiber labeling can be used to distinguish newly fired origins from ongoing forks using the experimental protocol outlined in Fig. 4*a*. Active replication forks prior to damage were labeled with IdU, and the cells were then treated with damage and the IdU was replaced by CldU. During the subse-

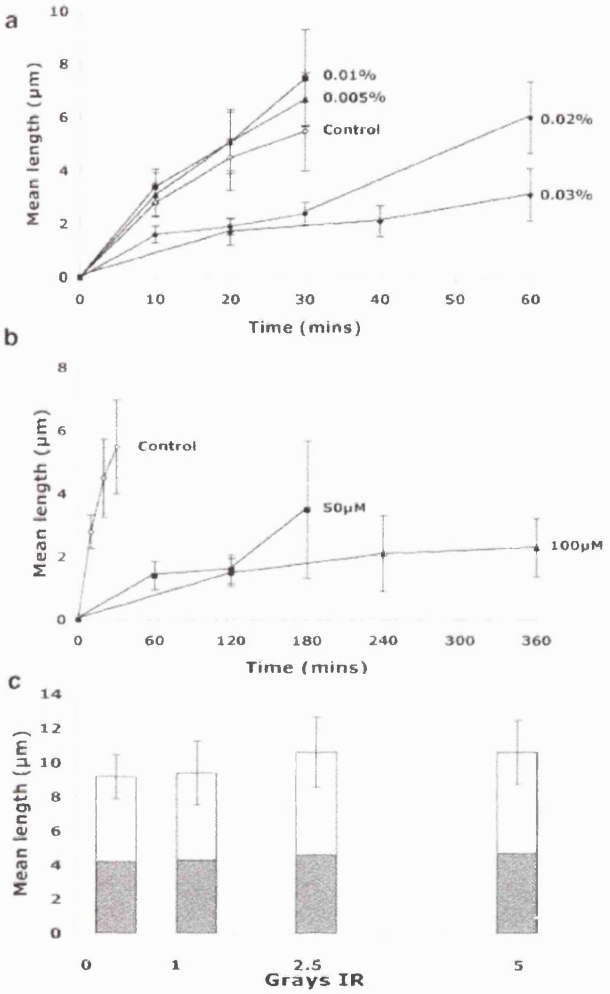

FIG. 3. **Reduced fork progression after DNA damage.** *a*, cells were treated with 0.005–0.03% MMS for 20min, and then MMS was removed, and the cells were labeled with 50 μM IdU for 10–60 min before preparing DNA fibers. The mean length of at least 50 replication tracks is plotted for each time point. *b*, synchronized cells in early S phase were labeled with 10 μM IdU for 10 min, then 50–100 μM HU was added, and the IdU was replaced with 100 μM CldU. The mean length of track extension in CldU over the subsequent 6 h is plotted for each HU concentration. *c*, synchronized cells in the early S phase were labeled with 10 μM IdU for 10 min, exposed to 1–5Gy IR, and then labeled with 100 μM CldU for 20 min. The mean total lengths of at least 50 unidirectional (*red* then *green*) tracks are plotted with the *white portions* of each bar representing the CldU-labeled length (replicated after IR exposure).

quent 20 min, any newly fired origins will generate tracks labeled along their entire length with CldU, and they can be counted against the number of double-labeled (ongoing) forks that were tagged with IdU prior to damage.

Fig. 4*b* shows that origin firing was inhibited in response to MMS and that the severity of inhibition was dose-dependent over the range tested (20-min pulses of MMS at 0.005–0.02%). Exposure to IR also inhibited origin firing, but unlike the response to MMS, this may show a threshold between 1 and 2.5 Gy (Fig. 4*c*). No further decrease in origin firing was then seen after IR exposures up to 10 Gy (data not shown). This damage-insensitive subset of initiation events, seen after the maximum doses of both IR and MMS damage, may represent the proportion of the total origins, which are already committed to fire within this 20-min labeling period at the time of damage.

The response of cells to HU was also tested in this origin blocking assay, because nucleotide depletion has been shown to inhibit origin firing via the S phase checkpoint in *S. cerevisiae* (2, 3, 21). In higher eukaryotes the S phase checkpoint response to HU has not been tested, but aphidicolin, which stalls replication by inhibiting DNA polymerases, does inhibit the appearance of late S phase foci in Chinese hamster ovary cells (16).

Replication forks were prelabeled for 10 min with IdU as before, and then the IdU was replaced with CldU together with 250 μM HU. The accumulation of new (CldU-labeled) origins was then counted against the IdU-tagged ongoing tracks over the subsequent 2–6 h. Fig. 4*d* shows that origin firing is greatly reduced, such that it takes 6 h to accumulate the same number of origin firing events that occur in control cells in less than 1 h. It is unlikely that many new origins *did* fire but were simply not labeled because of nucleotide depletion, because most existing forks were able to progress, incorporating CldU, for a further 1–2 μm over the 6 h of HU arrest. To confirm this, the experiment was repeated using only 50 μM HU, a concentration that allows existing forks to elongate more extensively, growing by 3–4 μm over 3 h. As before, new origin firing was severely inhibited (Fig. 4*d*). Because of a recent report indicating an increase in origin firing after treatment of a modified hamster fibroblast cell line with HU (22), this experiment was repeated using primary human fibroblasts instead of HeLa cells, and a similar inhibition of new origin firing was observed (Fig. 4*e*).

Origin Firing Recovers at Different Rates after IR, MMS, and HU—A modified version of the origin-firing assay described above was used to assess recovery in the rate of firing over longer periods after DNA damage (Fig. 5*a*). As in Fig. 4, active replication forks were tagged with a pulse of IdU prior to DNA damage (Fig. 5*a*, *tracks a*), and then the IdU was washed out before MMS or IR were applied. This generates exclusively IdU-labeled (*red*) tracks representing the number of active replication forks before DNA damage. At time points from 1.5 to 4.5 h later, the cells were then double-labeled with consecutive pulses of IdU (*red*) and CldU (*green*). This protocol distinguishes any new origins actually firing at each time point (exclusively *green* or *green* at both ends: labeled *c* in Fig. 5*a*) from ongoing replication forks (*red* then *green*: *b* in Fig. 5*a*). These new origins were counted against the exclusively red tracks that form an internal control because they had been tagged identically in all the cells before any DNA damage.

Fig. 5*b* shows that a 20-min pulse of 0.01% MMS (*gray bars*) elicited a sustained block to origin firing when compared with the levels occurring in undamaged cells (*white bars*); origin firing recovered to only a very limited extent during at least 4.5 h after the MMS treatment. In comparison, 5 Gy IR (Fig. 5*c*) caused a much more transient block to origin firing with significant recovery after only 1.5 h. By 3 h post-IR exposure, origin firing had returned to normal levels.

The efficiency of origin firing recovery was also assessed after release from an HU arrest. As before, replication forks were prelabeled with IdU and then completely arrested by adding a high level of HU for 1–4 h. Upon release from HU, the IdU was replaced with CldU, and new origins fired within 1 h were counted against the prelabeled tracks. By comparison with either IR or MMS damage, origin firing recovered relatively well after a brief (1 h) HU arrest, but recovery became progressively less efficient after longer periods (2–4 h) (Fig. 5*e*). This is unlikely to be an artifact because of under-detection of CldU-labeled tracks after HU release, because the nucleotide balance within the cells recovered sufficiently fast to allow the origins that did fire to elongate by ~3 μm within 30 min and 6 μm within 60 min (data not shown).

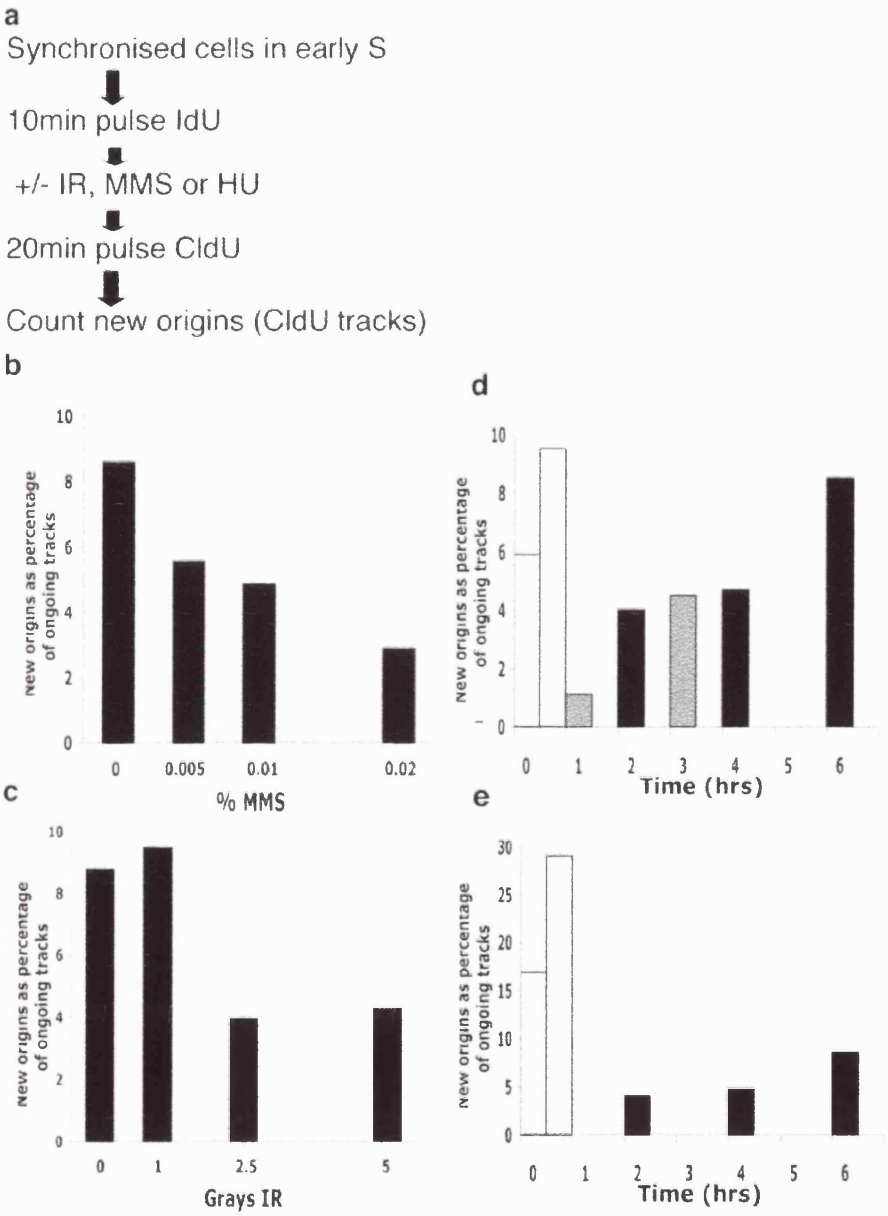

FIG. 4. **Inhibition of origin firing after DNA damage.** *a*, outline of protocol for measuring origin firing within 20 min of DNA damage. *b*, the protocol in *a* was used to quantify origin firing after 20-min pulses of MMS (0.005–0.02%). The graph shows pooled data from two independent experiments; at least 100 replication tracks were counted in each experiment for each MMS level. *c*, as in *b*, using 1–5 Gy IR instead of MMS. *d*, as in *b*, prelabeling with 20 μM not 10 μM IdU and adding 50 or 250 μM HU together with the CldU directly after this prelabel. Newly fired (CldU-labeled) origins were then allowed to accumulate for 30–50 min (control, *white bars*), 1–3 h (50 μM HU, *gray bars*), or 2–6 h (250 μM HU, *black bars*) and were quantified, as before, as a percentage of ongoing tracks. *e*, as in *D*, using unsynchronized IMR90 cells instead of HeLa cells.

Replication Forks Stall at an Elevated Rate after MMS and HU but Not after IR—The slowing of replication forks after MMS damage, which was documented in Fig. 3, could result from at least two distinct modes of altered fork progression. DNA damage may provoke a pan-nuclear change to a slower mode of replication, for example, by modification of all replication forks or a change to a different polymerase. Alternatively, there could simply be a series of transient stalling events at each fork in isolation as it encounters successive DNA lesions.

If such fork stalling does occur within the time frame of a double-labeling experiment (Fig. 4*a*), it should be detectable in the form of IdU-labeled tracks, which fail to incorporate the subsequent 20-min pulse of CldU because they are currently stalled. These events will therefore appear as an elevated number of red-only tracks (Fig. 2).

When the percentage of these red-only tracks was counted, a significant level of fork stalling was indeed found after higher MMS treatments (Fig. 6*a*), supporting the hypothesis that fork slowing occurs via stochastic stalling events. By contrast, IR did not cause significant fork stalling, consistent with the lack of overall fork slowing after IR damage (Fig. 6*b*).

In the case of HU treatment, all forks are essentially stalled by sufficiently high levels of HU. In lower levels of HU, however, replication does proceed at reduced speed (Fig. 3*b*), and in this situation there is elevated fork stalling,

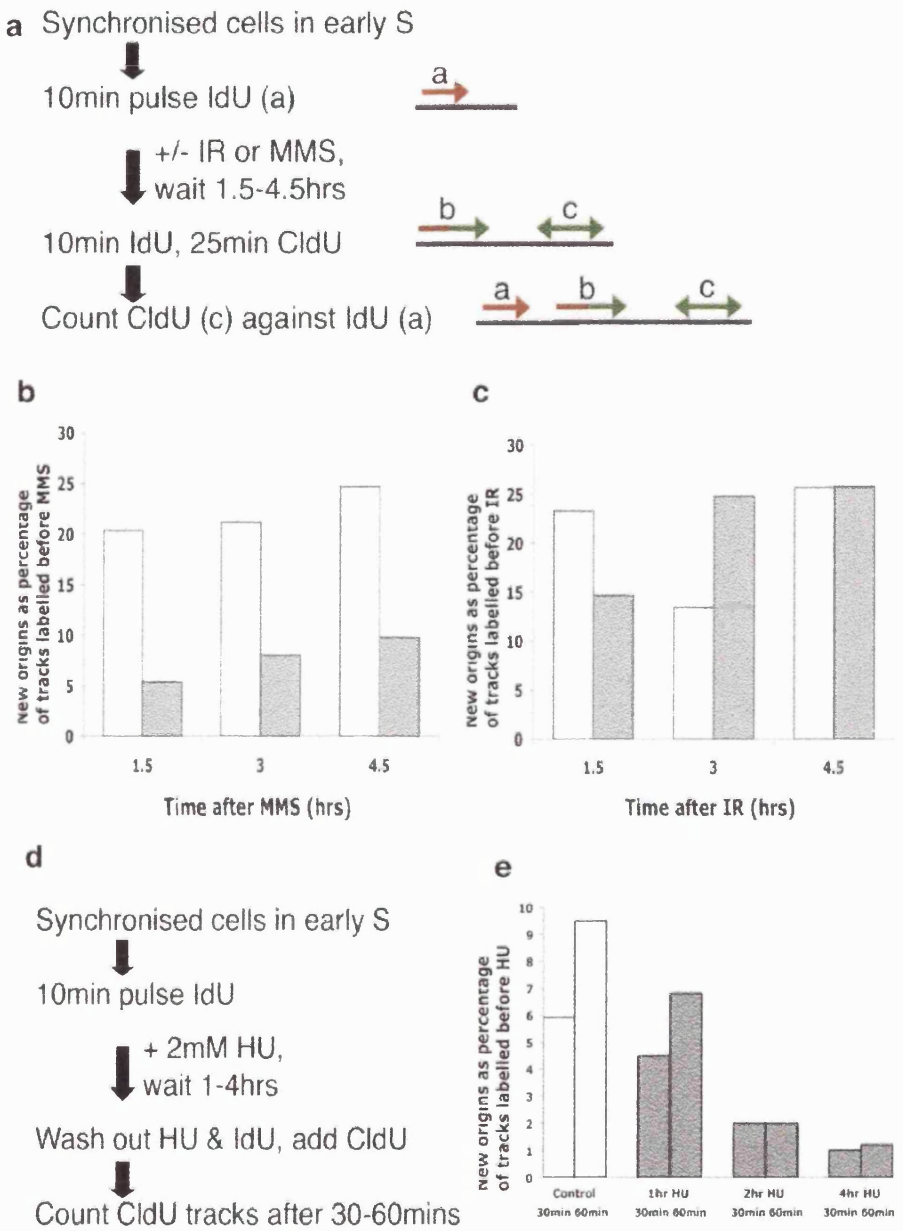

FIG. 5. **Recovery of origin firing after DNA damage.** *a*, outline of protocol to measure origin firing at time points up to 4.5 h after DNA damage. New origins are counted as percentages of tracks prelabeled with IdU before damage. *b*, the protocol in *a* was used to quantify origin firing 1.5–4.5 h after a 20-min pulse of 0.01% MMS (*gray bars*), and also in undamaged cells (*white bars*). The graph shows pooled data from two independent experiments; at least 100 replication tracks were counted in each experiment for each MMS level. *c*, as in *b*, using 5 Gy IR instead of MMS. *d*, outline of protocol to assess recovery of origin firing after 1–4 h of HU arrest. *e*, the protocol in *d* was used to quantify origin firing within 30 or 60 min of release from 2 mM HU.

detectable in as little as 5 μM HU and increasing in a dose-dependent fashion to very high levels when S phase cells are subjected to 20 or 50 μM HU (Fig. 6*c*).

DISCUSSION

This work comprises the first systematic investigation of all the various parameters that determine the rate of DNA synthesis in mammalian cells during S phase and the ways in which these parameters are affected by DNA damage. The fiber labeling technique developed here is an improvement on other methods that have been used to investigate S phase checkpoint responses because it unambiguously separates changes in the rate of origin firing from changes in the rates of fork movement and fork stalling. Using this technique, each of these parameters can be examined quantitatively and under comparable conditions, using the same experimental method throughout. (Labeling cells with short pulses of modified nucleotides does not in itself perturb the S phase (23) or activate the S phase checkpoint in yeast (24), so the technique should measure only changes in DNA synthesis that are induced by IR, MMS, or HU.) Fiber labeling also offers the advantage of revealing replication dynamics on the level of individual forks rather than as an average of an entire cell population. It does not allow any analysis of replicon clustering in relation to higher order chro-

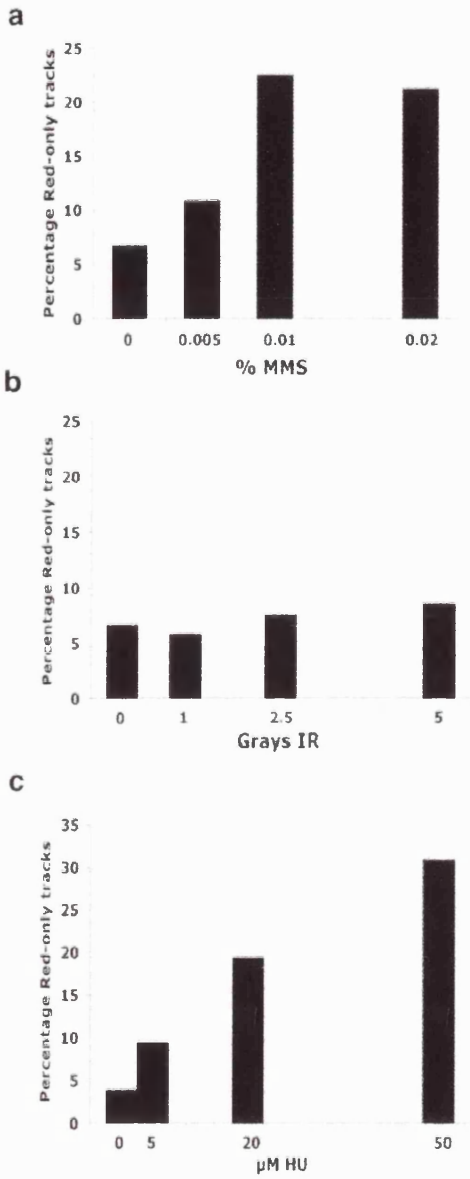

FIG. 6. **Fork stalling after DNA damage.** *a*, from the labeling protocol outlined in Fig. 4*A*, tracks labeled with IdU only were counted as percentages of the total number of tracks after MMS damage. (A proportion of these will represent terminations (see Fig. 1), but any significant increase over the control percentage is taken as evidence of fork stalling.) *b*, as in *a*, using 1–5 Gy IR instead of MMS. *C*, as in *a*, labeling cells after 3 h of replication in low levels of HU.

matin or nuclear structure, but it does allow subtle yet potentially important effects on a minority of individual forks to be detected and quantified.

Effects of IR, MMS, and HU on Replication Dynamics: Mammalian Cells Compared with S. cerevisiae—This study shows that different forms of DNA damage affect replication in different ways. Moderate levels of ionizing radiation, alkylation by MMS, or nucleotide depletion by HU can all slow down the overall progression of S phase. In the case of IR, this slowing appears to be entirely due to a rapid but fairly transient block to origin firing. Alkylation by MMS elicits a similar block to origin firing, but this persists for much longer after the removal of the drug than does the block to origin firing after IR. MMS also causes additional changes to replication: a general slowing of fork movement and the stalling of many forks for significant periods, phenomena that are not observed after levels of IR that block origin firing to a similar extent. Finally, nucleotide depletion by HU reduces fork movement (as might be expected because HU blocks RNR and therefore depletes the cell of dNTPs), and this is accompanied by elevated levels of fork stalling. HU treatment also blocks origin firing, but unlike the block elicited by MMS, this is relatively rapidly reversible; there is good recovery of initiation events within 1 h after a brief HU arrest, although the efficiency of recovery declines after longer arrests.

Most of the experiments described were carried out in HeLa cells, which lack functional p53. However, the intra-S phase checkpoint is believed to be p53-independent, and because all experiments were carried out in synchronized S phase cells, any p53-dependent G_1/S checkpoint defects should not be relevant. HeLa cells have been previously shown to down-regulate their DNA synthesis in response to both IR and MMS damage (as measured by reduced [^{3}H]thymidine incorporation), supporting the existence of a functional S phase checkpoint in these cells (11, 25).

The S phase responses to DNA damage observed here in HeLa cells are thus essentially similar to the responses observed in checkpoint-competent *S. cerevisiae*. Exposure of synchronized yeast cells to IR during S phase results in an extension of S phase, probably because of reduced origin firing (26). The response to MMS or HU involves reduced fork movement and fork stalling as well as blocked origin firing (2, 3, 7); all the same phenomena as are observed here in mammalian cells.

In *S. cerevisiae*, the relative checkpoint dependence of each of the phenomena described above has been established; blocked origin firing depends on the Mec1 and Rad53 checkpoint kinases (3, 21), and the same proteins are responsible for increasing dNTP levels and for maintaining stalled forks in a stable state (2, 6, 7). By contrast, the slowing of fork movement is independent of Mec1/Rad53 and has been proposed to be a direct physical result of replisomes encountering alkylated bases or their repair intermediates on DNA (2). It will be of interest to determine whether the fork slowing in yeast is a consequence of high rates of fork stalling as appears to be the case in human cells.

Because HeLa cells should be proficient in the intra-S phase checkpoint, they would therefore be expected to show a full range of checkpoint-dependent as well as checkpoint-independent DNA damage responses, and this work would suggest that this is indeed true. A degree of ambiguity remains, however, as to which replication phenomena are actually dependent on which, if any, of the mammalian checkpoint proteins. Regarding the two mammalian Mec1 homologues, ATM and ATR, radioresistant DNA synthesis is known to occur in ATM-deficient cells (9), and there are more recent suggestions that the different subpathways acting downstream of ATM play distinct roles in origin firing and fork elongation (27). By contrast, the direct study of the replication role of ATR has until recently been hindered by the fact that ATR is an essential protein. However, cells deficient in the ATR-pathway proteins Hus1 (28) and Chk1 (15) have been studied, and ATR has thus been implicated, albeit indirectly, in the inhibition of origin firing after UV and aphidicolin treatment. Future work will use the fiber labeling technique described here, together with recent advances in recombinational knockout and/or small interfering RNA technology, to make a direct comparison of each replication phenomenon separately in checkpoint-competent *versus* specifically checkpoint-compromised mammalian cells.

Further Insights into Replication Dynamics Obtained from DNA Fiber Labeling—The fiber labeling technique used in this study offers a quantitative assessment of replication dynamics with detailed time resolution; it therefore lends itself to the analysis of both dose-dependent and time-dependent effects. This has revealed several aspects of the S phase response to DNA damage that were not apparent from radioresistant DNA synthesis experiments nor from the population level studies previously carried out in *S. cerevisiae*.

The slowing of replication forks after MMS treatment, for example, has a nonlinear dose dependence. It is possible that this is due to a thresholded checkpoint response that acts *in trans* to slow down all ongoing forks once a critical level of DNA damage is detected; however, a threshold at comparable levels of MMS was not detected in the origin blocking response (suggesting that if a checkpoint *is* responsible for both origin blocking and fork slowing, the two must at least be differently thresholded). Because in *S. cerevisiae* the slowing of forks is entirely independent of Mec1/Rad53 (2), it is likely that fork slowing is similarly checkpoint-independent in mammalian cells, being instead a direct result of replication forks encountering DNA lesions. After lower levels of MMS damage, these lesions may be cleared by methods such as base excision repair sufficiently fast that they are not detected by the subsequent fiber labeling assay, although Fig. 4 would suggest that they do generate a sufficient checkpoint signal to inhibit origin firing. At higher levels of MMS, however, repair may become saturated, and alkylated lesions and/or repair intermediates may therefore accumulate on the DNA. This would be consistent with the slight recovery in fork rates seen at later times after intermediate MMS treatments, because the accumulated fork-blocking lesions would presumably be progressively removed over time.

Ionizing radiation differs from MMS in that no fork-slowing was detected after IR doses of up to 5 Gy. If ~35 double-stranded breaks are induced per Gy (17), then these would be far too infrequent to be detected as fork-blocking lesions, and broken DNA strands are in any case excluded when the data are collected. However, IR is also thought to cause many single-stranded breaks and other more minor DNA lesions. In striking contrast to the persistent lesions caused by MMS, it would appear that any single-stranded lesions induced by IR are either also too sparse to be detected, that they do not impede fork movement for any significant length of time, or that they are repaired extremely rapidly. It will be interesting to investigate whether this is still the case, and whether fork slowing after MMS damage is also altered, in cells lacking specific checkpoint pathways. For example, do checkpoint proteins have secondary roles in promoting specific pathways of damage repair, as well as simply slowing down the cell cycle?

Regarding origin firing, the results presented here differ markedly from recent findings regarding the response to HU in Chinese hamster lung fibroblast cells (22). In these cells, origin firing was not simply inhibited by HU; the firing of a particular "dominant" origin became less efficient, but this was accompanied by the activation of normally dormant origins and an increase in the overall density of origin firing. By contrast, in both of the cell types tested here, HeLa cells and primary fibroblasts, origin firing throughout the genome was severely inhibited, even by 20-fold lower HU than the amount used by Anglana *et al.* (22) (50 μM compared with 1 mM). This difference may be explained by the fact that the Chinese hamster cells had been selected for resistance to coformycin, an inhibitor of adenylate deaminase 2, and this may have selected for cells with a mutated checkpoint response to nucleotide depletion and/or adaptations to reduced nucleotide concentration allowing them to tolerate HU. Consistent with this, fork slowing exhibited by the Chinese hamster cells in response to HU was also different; in HeLa cells or IMR90 cells, 1 mM HU is more than enough to completely arrest cells in early S phase with little or no fork progression. The Chinese hamster cells, however, were reported to show significant S phase progression and great heterogeneity in replication track lengths. This again implies that these cells may have adapted to tolerate disturbed nucleotide metabolism; for example, they may possess a mutated form of ribonucleotide reductase that has some resistance to HU. It is also possible that the origins examined by Anglana *et al.* (22) are somehow regulated differently from the majority of genomic replication origins.

It is notable that although initiation events are blocked after both IR and MMS damage, the response to IR appears to be thresholded between 1 and 2.5 Gy, whereas the response to MMS increases linearly. This may be due to the fact that IR damage is transduced via ATM and MMS damage via ATR (29). It has recently been proposed that ATM is activated via a very rapid and sensitive signaling cascade involving intermolecular autophosphorylation and dissociation of ATM dimers, perhaps after the protein senses a global change in chromatin structure induced by double-stranded breaks (30). The mechanism of ATR activation is presently unknown, but if the lesions sensed by ATR do not induce any global changes to chromatin, and/or if they require processing by a replication fork or damage repair pathway before detection, then a comparable all-or-nothing checkpoint response involving ATR might not be expected. In budding yeast and *Xenopus*, checkpoint activation by MMS requires active replication forks (31–33). Experiments in yeast indicate that the activation of Rad53 in response to HU and MMS requires some threshold number of forks (34). However, above this threshold there is the capacity for differential activation of Rad53 depending on the number of stalled replication forks (32).

The allocation of different types of DNA damage to ATM and ATR may also explain the different rates of recovery from IR, MMS, or HU. Origin firing appears to recover relatively well after a brief HU arrest but less efficiently after longer periods of arrest. It also recovers much faster after IR than after MMS. There are at least two possible explanations for these observations. Firstly, recovery from a short HU arrest may be rapid because the transient depletion of nucleotides causes little actual DNA damage, and the stimulus for checkpoint signaling would therefore be removed as soon as dNTPs were restored and stalled forks were able to restart. After progressively more time in HU, it may take longer to restore dNTP levels to normal, and/or stalled forks may begin to lose their integrity or be processed via recombinational repair. It has been proposed that recombination is directly responsible for the slowing of fork progression that is elicited by UV or cisplatin, treatments that also cause potentially fork-stalling lesions on DNA (35). If forks stalled by HU are channeled into the same pathway, then they may not restart so efficiently after HU release and may themselves be sensed as DNA damage, resulting in a more persistent checkpoint signal in the form of stalled forks and/or damage repair structures.

At the opposite end of the spectrum, MMS may cause the greatest number of persistent lesions, generating an ongoing checkpoint signal for longer than do the lesions caused by IR. However, it is not clear that IR-induced lesions *are* any less persistent than MMS lesions; in fact, recent studies of the persistence of H2AX foci suggest that at least a subset double-stranded breaks can remain unrepaired for many hours after irradiation (36), and exposure of HeLa cells to 5 Gy in early S phase results in a G_2 arrest in most cells up to 40 h later (data

not shown). A second, and not mutually exclusive, hypothesis is therefore that the checkpoint signal during S phase is "turned off" at different rates after IR and MMS damage, perhaps because ATM ceases to respond to persistent double-stranded breaks faster than ATR becomes insensitive to any persistent MMS-induced lesions.

Relevance of the S Phase Checkpoint to Cancer Therapy—The study of the S phase checkpoint is particularly important in view of its potential role in determining the efficacy and specificity of cancer therapy. If the same checkpoint deficiencies that sensitize tumor cells to chemotherapy or radiotherapy also allow them to continue replicating regardless of damage and simultaneously reduce their efficiency of DNA repair, then any DNA-damaging therapy could carry a double risk of allowing further dangerous mutations to accumulate in any tumor cells that survive the treatment.

Therefore it is important to consider the actual S phase responses of particular tumors to particular forms of damage. For example, IR-induced double-stranded breaks may not themselves prevent the DNA on either side of a break from being correctly replicated, and if any other IR-induced lesions, such as single-stranded breaks, are rapidly repaired and/or non-fork-blocking, IR may actually be relatively benign during S phase and lethal only if the cell reaches G_2/M with unrepaired chromosome breakage.

By contrast, alkylation by MMS does appear to cause multiple fork-blocking lesions on DNA, giving alkylating drugs a much greater impact on cells within the S phase. If the fork stalling caused by such drugs is irreversible in checkpoint-deficient tumor cells (as in checkpoint-mutant yeast strains) (2, 6, 7), then all of the stretches of DNA between collapsed forks would remain unreplicated at the end of the S phase. Catastrophic chromosome breakage and rearrangement would result in any cells that survived mitosis from such a state.

An understanding of all the replication phenomena that are induced by DNA damage and of the proteins that control these phenomena may thus lead to the development of better chemotherapeutic drugs, perhaps causing different spectrums of DNA lesions or activating specific aspects of the checkpoint response. In this regard, future work will include the study of replication responses to clinically relevant chemotherapeutic drugs including alkylating agents and topoisomerase inhibitors such as etoposide, whose mode of action on a molecular level remains debatable (37). In the longer term, the information gained from these studies may also contribute to the development of targeted cancer therapies designed on the basis of known checkpoint deficiencies in individual tumors.

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