Oncogene (2010), 1–12

www.nature.com/onc

ORIGINAL ARTICLE

Reducing MCM levels in human primary T cells during the $G_0 \rightarrow G_1$ transition causes genomic instability during the first cell cycle

SJ Orr[1], T Gaymes[1], D Ladon[1], C Chronis[1], B Czepulkowski[1], R Wang[2], GJ Mufti[1], EM Marcotte[2] and NSB Thomas[1]

[1]*King's College London, Department of Haematological Medicine, Leukaemia Sciences Laboratories, Rayne Institute, London, UK and* [2]*Department of Chemistry and Biochemistry, University of Texas at Austin, Institute for Cellular and Molecular Biology, Austin, TX, USA*

DNA replication is tightly regulated, but paradoxically there is reported to be an excess of MCM DNA replication proteins over the number of replication origins. Here, we show that MCM levels in primary human T cells are induced during the $G_0 \rightarrow G_1$ transition and are not in excess in proliferating cells. The level of induction is critical as we show that a 50% reduction leads to increased centromere separation, premature chromatid separation (PCS) and gross chromosomal abnormalities typical of genomic instability syndromes. We investigated the mechanisms involved and show that a reduction in MCM levels causes dose-dependent DNA damage involving activation of ATR & ATM and Chk1 & Chk2. There is increased DNA mis-repair by non-homologous end joining (NHEJ) and both NHEJ and homologous recombination are necessary for Mcm7-depleted cells to progress to metaphase. Therefore, a simple reduction in MCM loading onto DNA, which occurs in cancers as a result of aberrant cell cycle control, is sufficient to cause PCS and gross genomic instability within one cell cycle.
Oncogene advance online publication, 3 May 2010; doi:10.1038/onc.2010.138

Keywords: cell cycle; MCM; chromosomal abnormalities; DNA damage; premature chromatid separation

Introduction

Normal DNA replication must be accurate and occur only once per cell cycle. Sites of DNA replication are 'licensed' (Chong and Blow, 1996) by binding the origin recognition complex that recruits MCM proteins (Mcm2–7), which are conserved from yeast to human beings (Bell and Dutta, 2002). In human cells undergoing a normal mitotic cell cycle, once a replication origin has fired, re-replication is prevented until the next cycle (Blow and Dutta, 2005; Mailand and Diffley, 2005). The consequences of triggering re-replication in the same cell cycle are a change in ploidy or potentially abnormal chromosomes.

Genome instability is a hallmark of cancer (Nakanishi *et al*., 2006). A cell is most vulnerable to genomic instability as DNA is replicated in S-phase and then as chromosomes segregate during mitosis. The expression of MCM proteins is abnormal in cancer cells, and anti-MCM antibodies can be used to identify proliferating cells in many cancers (Williams and Stoeber, 2007). Such abnormal expression is probably a consequence of abnormalities in oncogenes and/or tumour suppressor genes that abrogate cell cycle checkpoints and lead to unscheduled entry into the cell cycle. Expression of nuclear cyclin D1 leads to an increase in Cdt1 expression, increased MCM loading, re-replication and genomic instability (Aggarwal *et al*., 2007). In contrast, prevention of pre-replication complex formation in yeast by the induction of increased G_1-dependent cdk activity induces genomic instability (Tanaka and Diffley, 2002). In *Xenopus laevis* egg extracts, over-expression of cyclin E-cdk2 prevents DNA replication initiation by abrogating the binding of Mcm3 to chromatin (Hua *et al*., 1997). Cyclin E is also deregulated in many human cancers and the loading of MCM proteins onto chromatin is reduced in these cells (Ekholm-Reed *et al*., 2004). However, in *Xenopus* and human cancer cell lines (for example, HeLa), there is a >20-fold excess of MCM proteins over replication origins (Bell and Dutta, 2002; Hyrien *et al*., 2003; Blow and Dutta, 2005). Therefore, a reduction in MCM levels would not be expected to have a profound effect on DNA replication.

In the normal state, most T cells in human peripheral blood are quiescent (G_0) and they do not have many of the proteins necessary for cell cycle mechanisms and their control, including proteins required for DNA replication. These proteins are synthesized in response to mitogenic stimulation as cells progress from G_0 through G_1 for the first time (Lea *et al*., 2003). Quiescent cells in the body have low levels of MCM proteins compared with proliferating cells (Stoeber *et al*., 2001; Supplementary Figure S1), and recently, we identified Mcm7 in a mass spectrometry screen of proteins in human primary T cells that are induced and become bound to chromatin during the $G_0 \rightarrow G_1$ transition (Orr *et al*., in preparation). Thus, we investigated the

Correspondence: Dr NSB Thomas, King's College London, Department of Haematological Medicine, Leukaemia Sciences Laboratories, Rayne Institute, 123 Coldharbour Lane, London SE5 9NU, UK.
E-mail: nicholas.s.thomas@kcl.ac.uk
Received 29 June 2009; revised 7 January 2010; accepted 11 February 2010

importance of controlling the precise levels of MCM induction as primary human T cells enter the cell cycle for the first time.

Results

Mcm7 is up-regulated during the $G_0 \rightarrow G_1$ transition

Non-activated, quiescent human T cells from the peripheral blood of normal donors were stimulated with CD3/CD28 beads. Mcm7 is present at a very low level in quiescent T cells and is induced in mid/late G_1 (16 h). Mcm7 is induced with the same kinetics when the cells are induced with PMA/ionomycin, and gene expression array analyses show that mRNA encoding Mcm7 is induced fourfold by late G_1 (not shown). Analysis of T cells transiently stimulated before and after the $G_0 \rightarrow G_1$ commitment point that we described earlier (Lea *et al*., 2003) shows that Mcm7 is only induced in T cells that are committed to entering the cell cycle (Figure 1a). Mcm2–6 are present in G_0 and are also induced as cells progress through late G_1, albeit with different kinetics (Supplementary Figure S3A).

Transcription can only occur where there is an open chromatin conformation, which is maintained by epigenetic modifications of histones, such as histone H3 acetylation (H3Ac). H3Ac frequently occurs at the transcriptional start site of inducible genes in quiescent T cells before cell stimulation and gene induction (Smith *et al*., 2009), 'priming' gene promoters for transcription factor activation. This study showed that H3Ac priming occurs at the *MCM3* transcriptional start site. Given that Mcm7 is induced highly in G_1 from a low/undetectable level in G_0, we determined whether the same was true for the *MCM7* promoter. The minimal *MCM7* promoter contains E2F sites (Suzuki *et al*., 1998), and transfection of E2F-1 induces mRNA encoding Mcm7 (and Mcm5 and 6) (Ohtani *et al*., 1999; Bruemmer *et al*., 2003). ChIP analysis of H3Ac across the E2F sites in the *MCM7* promoter shows that this positive epigenetic mark is at a low level in G_0 and is induced significantly during the $G_0 \rightarrow G_1$ transition (Figure 1b). E2F-1 is not expressed in quiescent T cells, but is induced in mid-G_1 (Lea *et al*., 2003) and ChIP analysis shows that it then binds to the *MCM7* promoter (Figure 1c). Thus, the *MCM7* promoter is not 'primed' in G_0 and is epigenetically remodelled before E2F-1 binding and gene activation.

Primary T cells have normal cell cycle controls and DNA damage responses (Gaymes *et al*., 2002a; Lea *et al*., 2003) and respond to the DNA cross-linking agent

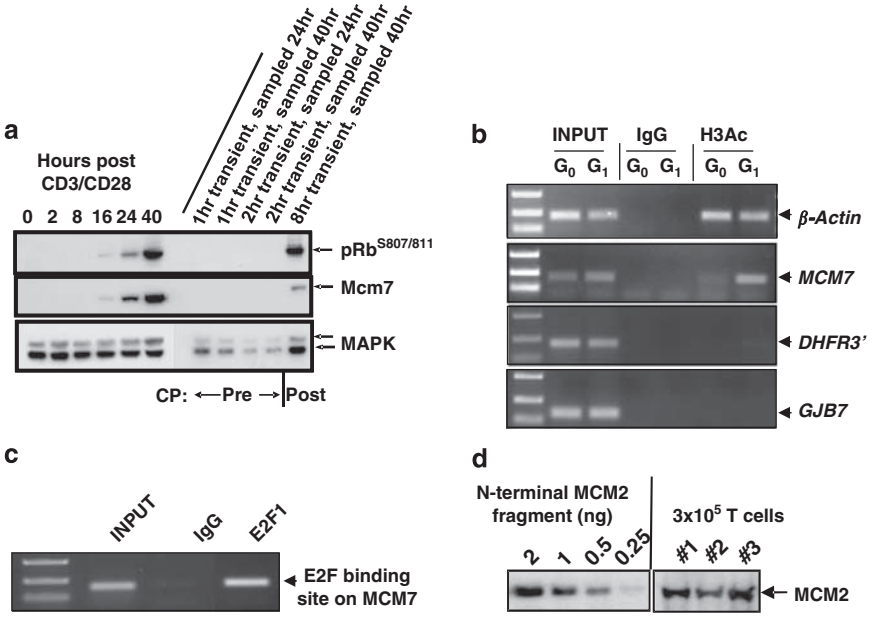

Figure 1 Mcm7 expression in T cells. (**a**) Mcm7 is induced in T cells after the $G_0 \rightarrow G_1$ commitment point (CP). Non-activated human primary quiescent T cells were isolated from peripheral blood by negative selection. The cells were stimulated continuously with anti-CD3/CD28 and samples were taken for western blotting during the transitions from $G_0 \rightarrow G_1 \rightarrow$ S-phase (0–40 h). Cells were also stimulated transiently with anti-CD3/CD28 before (1 or 2 h) and after (8 h) the $G_0 \rightarrow G_1$ CP (Lea *et al*., 2003) and samples were taken at 24 or 40 h. The expression of Mcm7 was determined by western blotting. Phosphorylation of pRb at $S^{807/811}$ and expression of MAPK were used as controls for cell cycle entry and as loading controls, respectively. Representative of $n = 6$ time-courses and $n = 3$ CP experiments. (**b**) H3Ac in *MCM7* in G_0 and G_1. Mono-nucleosomes were isolated from quiescent T cells and 72 h post-stimulation with PMA/ionomycin (G_1). The presence of nucleosomes containing H3Ac at the E2F sites in the *MCM7* promoter was determined by native ChIP-PCR. IgG: control ChIP. Input: PCR with total nucleosomal DNA. Representative of $n = 3$ individual T-cell isolates. (**c**) E2F-1 binding to the *MCM7* promoter. Binding of E2F-1 to the *MCM7* promoter was determined by cross-linked-ChIP-PCR for T cells in G_0 and G_1. Representative of $n = 3$ individual T-cell isolates. (**d**) Quantification of Mcm2 in primary human T cells. The amount of Mcm2 expressed in T cells stimulated for 72 h with PMA/ionomycin was quantified by comparing western blots of protein extracts from three independent isolates of primary human T cells with known amounts of an N-terminal Mcm2 recombinant protein. The N-terminal Mcm2 recombinant protein is smaller than endogenous Mcm2, but for clarity, they are shown alongside each other. Representative of $n = 3$ blots.

mitomycin C by S-phase arrest (Supplementary Figure S4). Thus, we used primary human T cells to investigate the consequences of reducing the levels of MCM proteins as these quiescent cells entered and progressed through the first cell cycle.

Quantifying the number of MCM proteins in T cells

We determined the number of molecules of MCM proteins expressed in primary T cells by comparing the expression of Mcm2 in T cells stimulated for 72 h with PMA/ionomycin against a titration of a recombinant N-terminal Mcm2 fragment (Figure 1d; Supplementary Figure S2). We found that T cells have about 3.5×10^{-19} mol of Mcm2 per cell, equivalent to 2×10^5 molecules per cell. PMA/ionomycin stimulates all cells, but at 72 h not all the T cells have entered S-phase (Lea *et al*., 2003). However, the same Mcm2 levels occur in T cells proliferating for 3 days in IL-2 (not shown). As the MCM helicase consists of one molecule each of Mcm2–7, the amount of Mcm2 can be used to estimate the amount of available MCM helicases. The availability of any one of the MCM proteins will constrain the number of active MCM helicases that can bind to chromatin. There are thought to be between 2×10^4 and 5×10^4 replication origins in the human genome (Françon and Mechali, 2006), each requiring two MCM helicases. Our results show that there are between two and five MCM helicases available per replication origin in human primary T cells. Therefore, in contrast to *Drosophila* or *Xenopus*, MCM proteins are not in great excess in human primary T cells.

Reducing MCM induction during $G_0 \rightarrow G_1$

We determined the consequences of reducing the induction of MCM expression as T cells enter the cell cycle. We transfected quiescent T cells with siRNA against Mcm7, before stimulation with PMA/iomomycin. A non-targeting siRNA pool was used as a control (Smartpool, Dharmacon, Thermo Fisher Scientific, Epsom, UK). Nearly 100% of the cells are transfected with siRNA, and Mcm7 levels were reduced to $<5\%$ of normal. This was judged by comparing the levels of Mcm7 in cells transfected with Mcm7 siRNA with a titration of control siRNA-transfected cell extract run on the same western blot (Figure 2a; see Materials and methods). A pool of target siRNA (Smartpool, Dharmacon) was used in these studies, but a similar reduction in Mcm7 was achieved with three of the four individual siRNA species in the pool (Supplementary Figure S3B). Therefore, the reduction in Mcm7 is unlikely to be due to off-target effects. Transfection with Mcm7 siRNA did not affect the overall levels of expression of Mcm2–6, but did reduce chromatin binding of all MCM proteins as cells become licensed in late G_1 (Figure 2b). This is consistent with the fact that MCM proteins bind DNA as a complex and reducing one MCM protein would be expected to reduce the binding of all the others. The Cdc6 protein also forms part of the DNA replication origin licensing complex, but binds DNA before MCM proteins (Bell and Dutta, 2002). Consistent with this, the level of chromatin-bound Cdc6 was not affected by reducing Mcm7 (Figure 2a).

Depletion of Mcm7 leads to a prolonged G_2 phase and induces DNA damage responses

An earlier study showed that *X. laevis* cells still enter S-phase with 5–10% normal levels of MCM proteins (Blow and Dutta, 2005). However, *X. laevis* have a >20-fold excess of MCM proteins over replication origins. In contrast, reducing Mcm7 to $<5\%$ of normal levels in primary T cells, in which we have shown that MCM proteins are not in excess, would be expected to severely inhibit proliferation. We reduced Mcm7 to $<5\%$ of the normal levels in primary T cells with siRNA, and rather than being inhibited in G_1, these cells progressed from $G_0 \rightarrow G_1 \rightarrow$S-phase when stimulated with PMA/ionomycin. The proportion of cells in S-phase was reduced, but there was a significant increase in the numbers of cells in G_2/M as compared with control siRNA-transfected cells (Figure 2c; S-phase: $P<0.001$; G_2/M: $P<0.02$; paired *t*-test). In agreement with the flow cytometry data shown in Figure 2c, BrdU incorporation in control-siRNA-transfected cells was $55 \pm 2.5\%$ greater than in cells transfected with Mcm7 siRNA (ELISA assays on replicate experiments with $n=3$ T-cell isolates; $P<0.0001$).

Next, we determined whether the G_2/M block was in G_2 or M by analysing the presence of phospho-histone H3-S^{10}, which occurs in mitosis (Hans and Dimitrov, 2001). Mcm7 depletion did not increase the proportion of cells containing phospho-histone H3-S^{10} as compared with cells transfected with control siRNA (Figure 2d). Therefore, the cell cycle block is not in mitosis, rather these data indicate an increase in the number of cells in G_2.

An increase in cells in G_2 is probably the result of a prolongation of G_2, consistent with a DNA damage response caused by DNA replication fork stalling. Indeed, reducing Mcm7 to 2–5% of normal levels resulted in cells with foci of phospho-γH2AX, indicative of DNA double-strand breaks, and foci of phospho-ATM, -ATR, -Chk1 and -Chk2 (Figure 3a), indicating DNA repair responses (Supplementary Figure S6, control western blots for phospho-ATM, -ATR, -Chk1 and -Chk2 antibodies). The same responses were observed in T cells treated with etoposide as a control. There was a significant increase in the number of cells with >5 foci per cell of all these markers when Mcm7 was depleted (Figure 3b). Cells also contained Rad51 foci (Figures 3a and b), consistent with engaging the homologous recombination (HR) pathway (Niida and Nakanishi, 2006). Similar data were obtained with each of the three individual Mcm7 siRNA that caused a reduction in Mcm7 (not shown). Furthermore, there was a dose-dependent increase in DNA repair responses with Mcm7 reduction, as titrating siRNA increased the number of cells with phospho-Chk1, -Chk2 and Rad51 foci (Figure 3c).

Mcm7 interacts with ATRIP (Cortez *et al*., 2004) and so reduction in Mcm7 might trigger DNA damage responses directly. Therefore, we carried out similar assays after reducing another MCM protein, Mcm4,

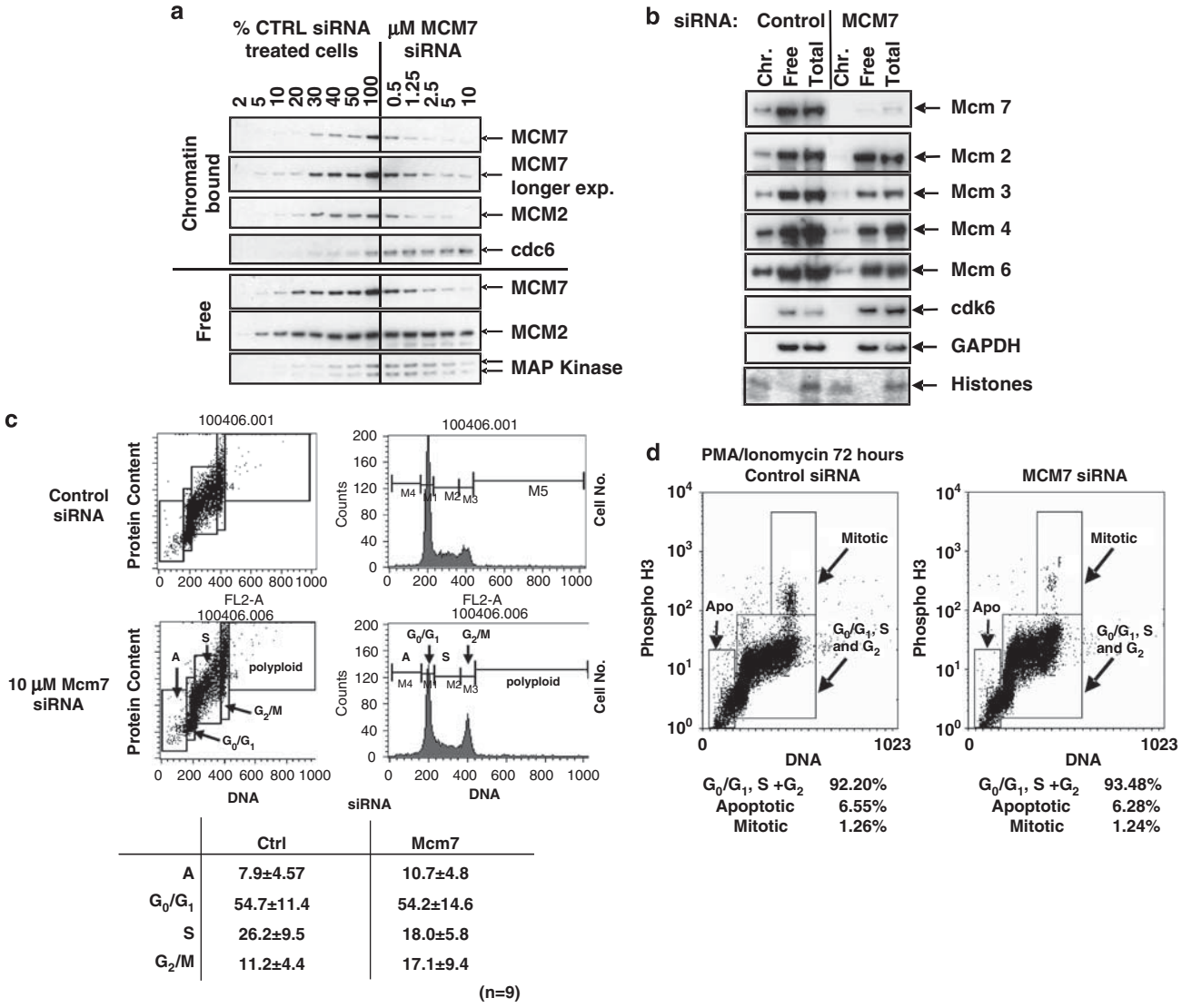

Figure 2 Inhibiting the induction of Mcm7 with siRNA. (**a**) Reducing Mcm7 with siRNA. Quiescent T cells were transfected with siRNA to Mcm7 (0.5–10 μM final; Smartpool, Dharmacon) or scrambled control (10 μM) and stimulated with PMA/ionomycin for 72 h. Chromatin-bound and free proteins were isolated by extracting cells with CSK buffer and the expression of Mcm7 as well as Mcm2, Cdc6 and MAPK were assayed by western blotting. The level of Mcm7 expression in cells transfected with Mcm7 siRNA was determined as a percentage of that in cells transfected with control siRNA by titration/western blotting (see Materials and methods). Representative of $n=3$ experiments. (**b**) Effect of reducing Mcm7 on expression and chromatin binding of Mcm2–6. Quiescent T cells were transfected with 10 μM Mcm7 or control siRNA and chromatin-bound and free fractions were prepared by extraction with CSK buffer after 72 h stimulation with PMA/ionomycin. Western blotting was carried out for Mcm2–7. As a control for loading chromatin-bound and total cell protein extracts, the bottom of the gel was cut before blotting and stained with Coomassie blue (<25 kDa; predominantly histones). Representative of $n=2$ experiments for all MCMs and $n>10$ for Mcm7 and Mcm 2. (**c**) Effect of reduced Mcm7 on the cell cycle. Quiescent T cells were transfected with 10 μM Mcm7 or control siRNA and stimulated as for (**a**), fixed and the percentage of cells in each cell cycle phase was determined by PI (DNA content) and FITC (protein content) staining and flow cytometric analysis. The percentage of cells in each cell cycle phase is tabulated (mean ± s.e.m.; $n=9$ experiments). (**d**) Effect of reduced Mcm7 on mitosis. The proportion of mitotic cells was determined by staining with Alexa Fluor 488-labelled phospho-Histone H3-S^{10} (Cell Signalling) and analysing by flow cytometry. Cells were also labelled with propidium iodide to determine DNA content. Cells that had 4n DNA content and were positive for phospho-Histone H3-S^{10} staining were counted as mitotic.

with siRNA. Reducing Mcm4 to 2–5% of normal levels also caused DNA damage responses (Supplementary Figure S5A) and the number of cells with >5 foci per cell was significantly increased in Mcm4-depleted cells (Supplementary Figure S5B). Thus, the induction of DNA damage is likely to be due to a decrease in binding the MCM complex to DNA rather than because of the reduction of a particular MCM protein.

Depletion of Mcm7 leads to gross chromosomal abnormalities

In spite of causing DNA damage, reducing Mcm7 did not cause significant apoptosis, as judged by sub-G_1 staining and PARP cleavage (Figure 2c; Supplementary Figure S5C). We used standard cytogenetic analyses to investigate whether this led to chromosomal changes. Our data show that Mcm7 depletion causes

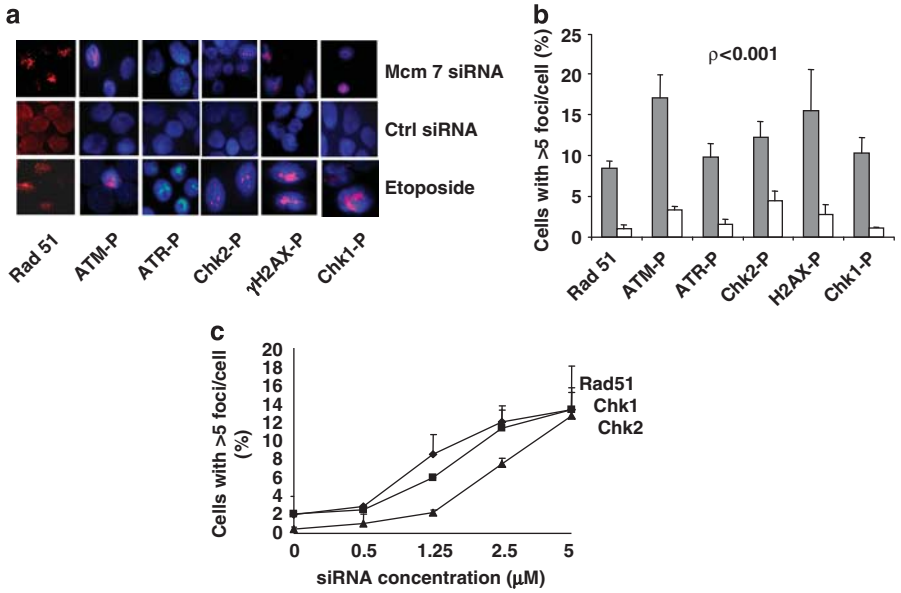

Figure 3 Induction of DNA damage foci in cells depleted of Mcm7. (**a**, **b**) DNA damage proteins in Mcm7-depleted cells. Quiescent T cells were transfected with 10 μM Mcm7 (grey bars) or control siRNA (white bars), stimulated with PMA/ionomycin for 72 h and the formation of foci in individual nuclei was determined by staining cytospins for the proteins shown. As a control, cells were also cultured with the topoisomerase inhibitor etoposide (25 μM) to induce DNA damage. The percentage of cells with >5 foci/cell were determined by scoring 300 cells/slide of $n=3$ experiments. (**c**) Quantification of DNA damage markers. Quiescent T cells were transfected with 0.5–5 μM Mcm7 or control siRNA and the percentage of cells with >5 foci per cell of phospho-Chk1, phospho-Chk2 and Rad51 was determined.

chromosomal abnormalities of varying severity. Metaphase spreads showed individual cells in the population that had few abnormalities, such as chromosome fragments and additions (for example add(9)), whereas others were grossly abnormal and contained tri-radial figures, terminal fragments, chromatid breaks and asymmetric interchanges (Figure 4Af). Each metaphase observed comes from a single cell and there were no multiple metaphases in any of the samples analysed that had exactly the same pattern of abnormalities. The proportion of abnormal cells increased as Mcm7 levels were decreased (Figures 4Ab–e) and abnormalities were detectable even when the level of chromosome-bound Mcm7 was reduced by only 50% (Figure 4Ab). Transfection with the four individual siRNAs also led to gross chromosomal abnormalities (Supplementary Table S3), indicating that this is not an off-target effect. The percentage of cells with chromosomal abnormalities also increased when Mcm4 was reduced (control siRNA 5.25%, Mcm4 siRNA 19.75%; mean of $n=2$ experiments), indicating that such gross chromosomal abnormalities are not specifically due to reduction of Mcm7. Similar chromosomal abnormalities also occur when cells from patients with genomic instability syndromes such as Fanconi anaemia or Bloom's syndrome are subjected to a mild replication stress (Howell and Taylor, 1992). However, reducing Mcm7 does not alter the level of chromatin bound or free FANCD2 or BLM proteins (Supplementary Figure S5D), indicating that the chromosomal abnormalities seen in Mcm7-depleted cells are unlikely to be due to aberrant recruitment of these pathways.

The cytogenetic analyses of chromosomal abnormalities caused by Mcm7 depletion do not allow detailed characterization of chromosomal rearrangements. Therefore, we carried out more detailed analyses of the chromosomal abnormalities caused by reduction in Mcm7 using M-FISH (Figure 4B). Depletion of Mcm7 causes chromosomal translocations, breaks, deletions, fragments as well as loss and gain of whole chromosomes (Figures 4Bb and c; quantified in Figure 4C). There were significantly more abnormalities per cell as compared with cells transfected with control siRNA (2.45 ± 0.05 vs 0.22 ± 0.07, mean ± s.e.m.; $P < 3.3 \times 10^{-12}$). Such abnormalities were also evident in slides stained with centromere probes (Figures 4Dd–f). Chromosomal translocations, losses of parts of chromosomes as well as loss and gain of whole chromosomes can occur in the same cell (Figure 4Bc). Thus, simply depleting Mcm7 is sufficient to account for translocations as well as changes in ploidy and these are not mutually exclusive events. We did not detect a gross poly-ploidy in the population of cells depleted of Mcm7, as judged by flow cytometric analyses of DNA content (Figure 2c) or multi-nucleated cells by microscopic examination of MGG-stained slides (not shown). However, we noted small numbers of individual DAPI-stained cells that were poly-ploid (<1%; Figure 4Df).

Depletion of Mcm7 causes premature chromatid separation

In addition to causing chromosomal abnormalities, depletion of Mcm7 also causes premature chromatid

separation (PCS) (Kajii *et al*., 1998), evidenced by partially separated chromosomes in some metaphase spreads (Figure 4A, compare panel a with e). To quantify PCS, we stained metaphase spreads with a centromere-specific probe and quantified the distances between centromeres of sister chromatids (Figure 4D). The distance between centromeres increased from 6.7 ± 0.3 (pixels) in control siRNA-transfected cells to

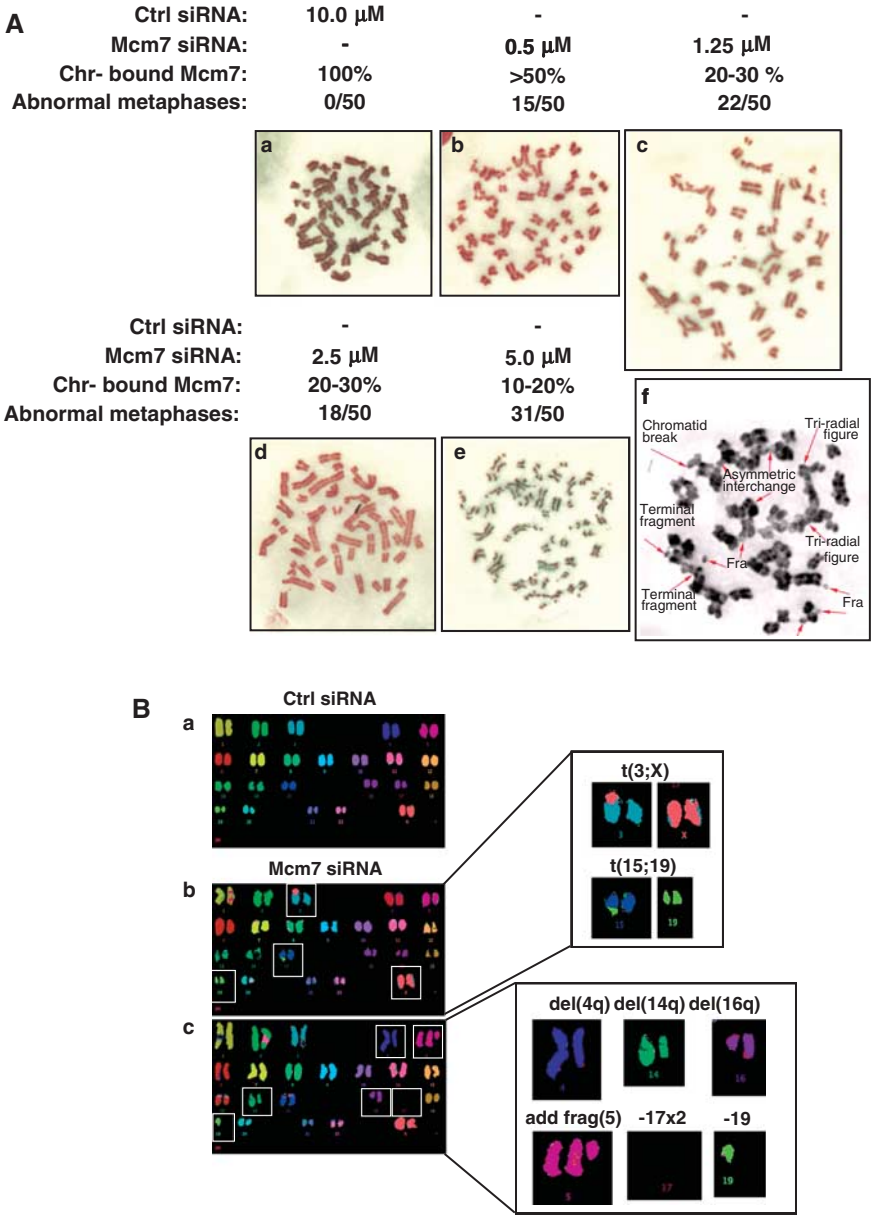

Figure 4 The karyotype of cells with reduced Mcm7. (**A**) Cytogenetic analyses. Quiescent T cells were transfected with (a) 10 μM control siRNA or (b–e) 0.5–10 μM Mcm7 siRNA and stimulated for 72 h with PMA/ionomycin. G-banded metaphase spreads were prepared and 50/sample were scored for chromosome abnormalities. The level of chromatin-bound Mcm7 in each sample was determined by titration/western blotting as described for Figure 2a and this is indicated above each panel. Panel f highlights a range of abnormalities that were detected. Representative of $n = 4$ experiments. (**B**) M-FISH analyses. T cells were transfected with control or Mcm7 siRNA as for (**A**) and chromosomal abnormalities were analysed and quantified by M-FISH. Examples are shown of chromosome abnormalities in Mcm7 siRNA-transfected cells: (b) chromosomal translocations (t(3;X) and t(15;19)) and no loss or gain of chromosomes and (c) del 4q, 14q and 16q, add fragment of chromosome 5, deletion of both copies of chromosome 17 and one missing chromosome 19. Panel a shows normal chromosomes that occurred in all control siRNA-transfected cells. (**C**) Frequency of chromosomal abnormalities detected by M-FISH. T cells were transfected with Mcm7 or control siRNA as for (**A**) and the chromosomal abnormalities detected by M-FISH in three separate experiments were quantified by analysing 20 metaphases/sample. Chromosomal deletions (del), breaks, fragments (frag), translocations (trans) as well as loss and gain of individual chromosomes were scored (mean ± s.e.m.). *P*-values for differences in Mcm7 vs control siRNA are $< 1.2 \times 10^{-3}$ for fragments and $< 2.1 \times 10^{-5}$ for all other categories. (**D**) FISH with locus-specific and centromeric probes. T cells transfected with control or Mcm7 siRNA were analysed by FISH with the centromeric probe CEP8 (8p11.1-q11.1-α satellite sequence) and with the LSI MYC (8q24) as a control for chromatid distance. Sections of panels a and b are enlarged for clarity and the 8q24 probe (red) is arrowed. Some metaphases (for example panel c) were also probed for the IgH locus on 14q32 as independent confirmation.

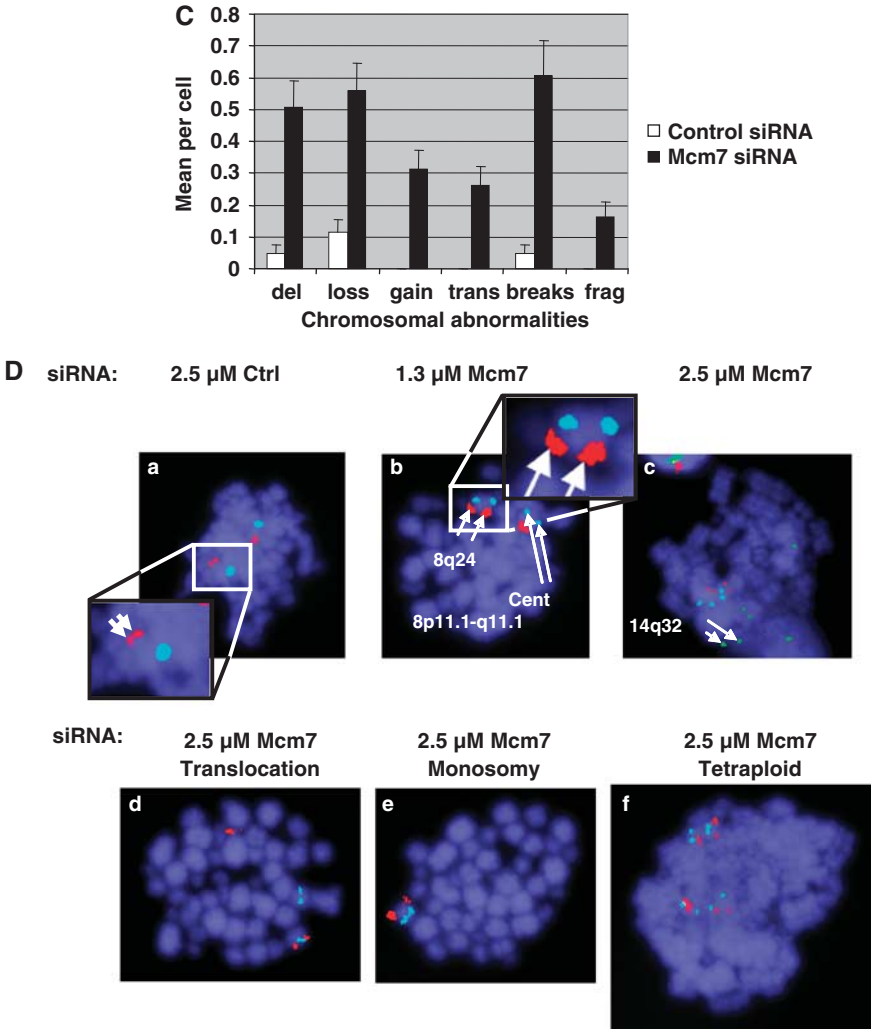

Figure 4 Continued.

11.4 ± 0.9 (mean ± s.e.m.; $P < 2 \times 10^{-5}$) and 12.3 ± 0.6 ($P < 2 \times 10^{-11}$) in T cells transfected with 1.3 and 2.5 μM Mcm7 siRNA, respectively. Quantification of DAPI-stained metaphase spreads showed that PCS occurred in more than one third of Mcm7-depleted cells (18/56 (32.1%) and 20/52 (38.5%): 1.3 and 2.5 μM Mcm7 siRNA), but not in cells transfected with control siRNA (0/50 (0%)). PCS was also observed with each of the four individual Mcm7 siRNA, indicating that it is not due to off-target effects (Supplementary Table S3). These abnormalities are reminiscent of a failure to secure newly synthesized chromatin to the parental template, which normally occurs in S-phase during DNA replication (Nasmyth and Haering, 2005; Moldovan *et al*., 2006).

Both NHEJ and HR are required for Mcm7-depleted cells to enter mitosis

We investigated the mechanisms required for chromosomal abnormalities to occur. DNA damage during S-phase is thought to be repaired predominantly by HR (Arnaudeau *et al*., 2001). Consistent with this, we detected RAD51-foci in cells depleted of Mcm7 or Mcm4 (Figure 3; Supplementary Figure S5A). However, non-homologous end joining (NHEJ) has also been shown to be involved in repairing double-strand breaks caused by stalled replication forks (Lundin *et al*., 2002). We investigated whether NHEJ was also induced by Mcm7 depletion by using two cell-free assays that we employed earlier in studies of chromosome instability syndromes and leukaemias (Gaymes *et al*., 2002a, b). *In vitro* end ligation and plasmid reactivation assays show the formation of dimers in control siRNA-transfected cells because of endogenous levels of NHEJ activity. Depletion of Mcm7 led to an increase in end-ligation activity, shown by an increase in further ligation events, which form trimers and multimers (Figure 5a). The total amount of ligated products was greater in the Mcm7-depleted cells than in controls (Figure 5b) and the frequency of mis-repair was also increased (Figure 5c). In spite of increased NHEJ activity, the expression of the NHEJ DNA ligase IV, XRCC4 (Budman *et al*., 2007) was sometimes reduced after

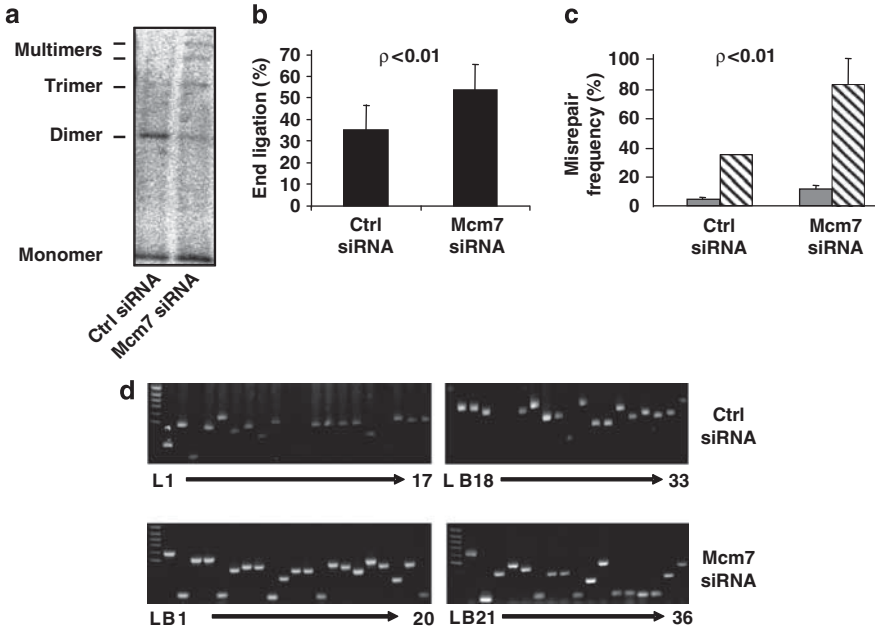

Figure 5 Plasmid DNA end ligation as a measure of NHEJ activity in Mcm7 reduced cells. (**a**) End-ligation assay. Quiescent T cells were transfected with control or Mcm7 siRNA, stimulated with PMA/ionomycin for 72 h, extracts were prepared and incubated with a ^{32}P-labelled pUC18 plasmid cut with *EcoRI* (Gaymes *et al*., 2002a). The ligated plasmid products were separated by gel electrophoresis and visualized by phosphorimaging. Monomer: remaining, unligated, linearized plasmid; dimer, trimer, multimers: ligated plasmid products. (**b**) Quantification of end-ligation activity. End-ligation experiments described in (**a**) were quantified (see Materials and methods) and the mean end-ligation efficiencies in extracts of T cells transfected with Mcm7 or control siRNA are shown (mean ± s.e.m.; $n=3$ experiments). (**c**) Plasmid reactivation assay. Quiescent T cells were transfected with Mcm7 or control siRNA and stimulated for 72 h as for Figure 2a. Nuclear extracts were prepared and incubated with a plasmid cut in the *lacZ* gene. Mis-repair frequency was determined by counting blue/white colonies (grey bars). The percentage of white colonies mis-repaired with large (>30 bp) deletions was determined by colony PCR across the breakpoint as shown in (**d**) (striped bars; mean ± s.e.m.; $n=3$ experiments). (**d**) Plasmid reactivation assay: deletions. The percentage of colonies obtained in (**c**) with >30 bp deletions was determined by colony PCR of randomly picked white colonies. Lanes 1–33 and 1–36: using extracts of control and Mcm7 siRNA, respectively. B, blue colony (correctly repaired, 628 bp); L, DNA ladder.

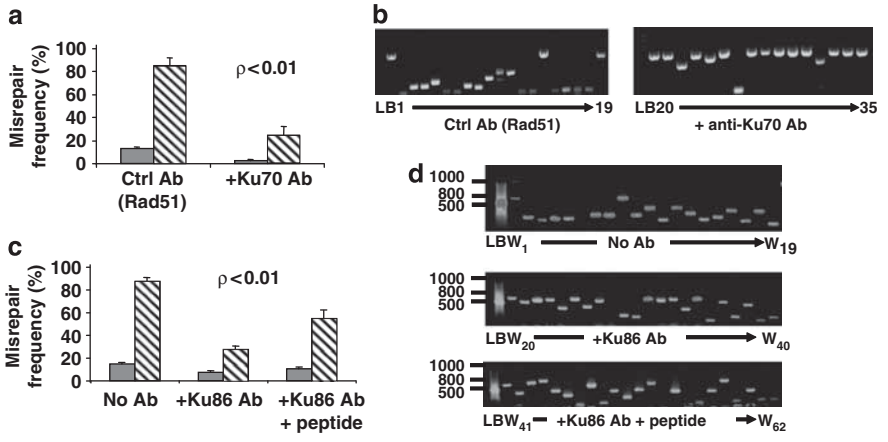

Figure 6 DNA abnormalities in Mcm7-depleted cells are due to increased NHEJ activity (**a**) Mis-repair is reduced by ablating Ku70. T cells were transfected with Mcm7 siRNA and plasmid reactivation assays were carried out as for Figures 5c and d except that the extract was pre-incubated with anti-Ku70 or control antibody (anti-Rad51) before plasmid addition. Grey bars: mis-repair frequency; striped bars: per cent of large deletions (mean ± s.e.m.; $n=3$ experiments). (**b**) Deletions are reduced by ablating Ku70. Colony-PCR of randomly picked white colonies from (**a**). Control antibody: Lanes 1–19; anti-Ku70 antibody: Lanes 20–35; B, blue colony (correctly repaired, 628 bp); L, DNA ladder. (**c**, **d**) Mis-repair is reduced by ablating Ku86. Experiments were carried out as for (**a**) and (**b**) except using anti-Ku86 antibody. Ku86 antibody pre-competed with its cognate peptide was used as the control. Grey bars: mis-repair frequency; striped bars: per cent of large deletions (mean ± s.e.m.; $n=3$ experiments).

Mcm7 depletion (Supplementary Figures S2 and S5E). Mis-repair caused by depleting Mcm7 led to an increase in large deletions (Figures 5c and d). Inhibition of the NHEJ-protein Ku-70, but not the HR-protein RAD51, reduced the mis-repair frequency and the number of large deletions to near normal levels (Figures 6a and b).

Anti-Ku-86 had a similar effect to Ku-70 and this was abrogated by pre-incubating the antibody with its cognate peptide (Figures 6c and d). Depleting XRCC4 with siRNA reduced the repair to zero. These data are consistent with the acquisition of DNA abnormalities because of increased NHEJ mis-repair activity in Mcm7-depleted cells.

To determine whether we could detect chromosomal abnormalities caused by depleting Mcm7 if NHEJ or HR were compromised, we reduced Mcm7 to cause DNA damage and also depleted XRCC4 or Rad51 by co-transfecting both Mcm7 and either XRCC4 or Rad51 siRNAs (Supplementary Figure S5E). No metaphases were obtained when both Mcm7 and XRCC4 or Mcm7 and Rad51 were depleted, indicating that both NHEJ and HR pathways are necessary for cells to progress through to metaphase when Mcm7 is depleted. These data are consistent with earlier reports that HR and NHEJ repair DNA damage caused by replication fork stalling (Arnaudeau *et al*., 2001; Lundin *et al*., 2002), indicating that both pathways are necessary for cells with reduced Mcm7 levels to progress from S-phase through G_2 into M-phase. Flow cytometry of DNA and protein content showed that cells in which Mcm7 and XRCC4 or Rad 51 were depleted were still able to progress through the cell cycle to G_2/M. Although there was a reduction in the proportion of cells in G_2/M in some experiments, this was not statistically significant. Note that depleting XRCC4 or Rad51 alone had no significant effect on cell cycle progression (not shown), indicating that down-regulating NHEJ or HR pathways alone is not sufficient to prevent progression through S-phase to G_2/M in the absence of DNA damage caused by depleting Mcm7.

Discussion

In this study, we have shown that the level of induction of Mcm7, and hence the MCM helicase during the G_0 to G_1 transition, is critical to maintain genome stability in primary human T cells. Even a 50% reduction in Mcm7 levels leads to DNA damage, repair by HR and NHEJ pathways, PCS and leads to severe chromosomal abnormalities typical of genomic instability syndromes.

In yeast and *X. laevis* embryos, MCM proteins are in excess of replication origins, and reducing MCM loading using a ts*Mcm2* mutant causes DNA damage, chromosomal loss and mitotic recombination (Lei *et al*., 1996; Liang *et al*., 1999). In other studies, reducing Mcm4 caused endoreduplication (Coxon *et al*., 1992) and reduction of Mcm2, Mcm4, Mcm6 or Mcm7 caused a block in late S-phase similar to that caused by replication fork collapse, DNA damage and reduced viability (Bailis *et al*., 2008). This study also showed that the MCM complex interacts with the HR protein Rhp51(Rad51) and that loss of HR leads to chromosome mis-segregation after replication fork collapse. The direct interaction of MCM proteins with Rad51 and Rad52 proteins has also been shown to occur in the human cell line HeLa (Shukla *et al*., 2005). Reducing Mcm4 or Mcm7 in HeLa cells caused DNA damage, Rad51 foci, increased apoptosis and a higher proportion of multi-nucleated cells or cells with micronuclei (Bailis *et al*., 2008). Tsao *et al*. (2004) showed that Mcm7 also interacts with Rad17 in human cancer cell lines, including HeLa and that this interaction is required for DNA damage responses triggered by UV irradiation or Aphidicolin. Other studies have shown that the response of human transformed cell lines to a reduction in MCM proteins differs from that of untransformed cell lines. For example, reducing MCM loading in various cancer cell lines impaired replication, caused S-phase arrest and apoptosis, whereas normal cell lines arrested mainly in G_1 (Shreeram *et al*., 2002; Feng *et al*., 2003; Ekholm-Reed *et al*., 2004). Depletion of Mcm7, but not other MCM proteins, also caused S-phase arrest in *Drosophila* S2 cells (Crevel *et al*., 2007). The S-phase arrest is consistent with stalling replication forks, causing engagement of the S-phase DNA damage checkpoint. It has been suggested that the excess of MCM proteins over the replication origins that normally fire may be due to the fact that only some of the possible origins actually fire (Hyrien *et al*., 2003). Indeed, the spacing between active replication origins in human U2OS cells decreases significantly in response to replication stress. This reduction did not occur when MCM levels were decreased, suggesting that dormant origins that are suppressed during a normal S-phase can be activated by the 'excess' MCM proteins after replication fork inhibition, thus allowing completion of DNA replication (Ge *et al*., 2007).

The studies described above have investigated the requirement for MCM proteins in proliferating cells. Earlier work has shown that the levels of MCM proteins reduce significantly in cells exiting the cell cycle (Stoeber *et al*., 2001), and it has been proposed that the presence of licensed origins distinguishes cells in G_1 from G_0 (Blow and Hodgson, 2002). In agreement, our data show that human T cells in G_0 have low levels of most MCM proteins and in particular significantly reduced levels of Mcm7. Mcm7 mRNA and protein are induced as T cells enter G_1 and the levels of all the MCM proteins are significantly higher in continuously proliferating cells. Chromatin has to be in an open conformation to allow transcription factor binding. Such chromatin remodelling is regulated by histone epigenetic modifications. We show elsewhere that, on average, epigenetic signatures at transcriptional start sites, such as H3Ac, are already set at many inducible genes in quiescent T cells before cell stimulation (Smith *et al*., 2009). However, we noted in that study that H3Ac signatures are up-regulated in specific regions of some genes, such as *IRF4* and *RUNX3* when they are induced in G_1. This also occurs for *MCM3* downstream of the transcriptional start site. The minimal *MCM7* promoter contains E2F sites and E2F-1 can induce mRNA encoding several MCM proteins, including Mcm7 (Suzuki *et al*., 1998; Ohtani *et al*., 1999; Bruemmer *et al*., 2003). E2F-1 is not present in T cells in G_0; it is induced in G_1 once cells have passed the $G_0 \rightarrow G_1$ commitment point. The ChIP experiments presented

here show that E2F-1 binds directly to the *MCM7* promoter in G_1, although we cannot rule out that other E2Fs also bind at these sites. Our study showed that in *MCM3*, the E2F site (+38; Taubert *et al*., 2004) is primed in G_0 before cell stimulation, in which it already has a high level of H3Ac. In this context, it may be significant that Mcm3 is already expressed in G_0. In contrast, we show that Mcm7 is present at a very low level in G_0. The E2F sites in the *MCM7* promoter are not primed and H3Ac at this position is induced in cells stimulated to enter G_1. These data suggest that *MCM7* is epigenetically suppressed in G_0 and is regulated by chromatin remodelling, which subsequently allows transcription factor (E2F) access and gene induction.

We also quantified the levels of MCM expression in primary human T cells that had been stimulated to enter the cell cycle. Our data show that there are 2×10^5 Mcm2 molecules per cell, or approximately 2–5 MCM complexes per replication origin. We also determined that there is approximately a 20-fold higher MCM expression in HeLa cells as well as in other cancer cell lines we tested (Orr *et al*., in preparation). HeLa cells have been used in studies by a number of groups and the excess MCM proteins present may have enabled the investigation of aberrant firing at cryptic replication sites. Indeed, Ibarra *et al*., 2008 showed that the excess MCM proteins present in HeLa cells maintain genomic integrity under conditions of replicative stress.

In our study, we investigated the requirement for induction of Mcm7 and Mcm4 during the $G_0 \rightarrow G_1$ transition and to what extent the levels of expression are important for subsequent progression through the cell cycle. In contrast to the cell lines investigated in other studies, a reduction in MCM loading in primary human T cells does not cause cell cycle arrest in G_1 or apoptosis, but induces DNA damage, DNA repair responses (NHEJ and HR) and leads to chromosomal abnormalities. We observed that chromosomal translocations, deletions as well as loss and gain of individual chromosomes can occur separately or all in the same cell and occur within one cell cycle. Our findings are consistent with the fact that replication defects induced by a variety of experimental methods can cause genome instability (Flores-Rozas and Kolodner, 2000; Kolodner *et al*., 2002). As primary human T cells do not have an excess of MCM proteins relative to replication origins, a small decrease in the levels of induction of MCM proteins as cells progress from G_0 through G_1 would have severe consequences. If this occurs in stem cells *in vivo*, it would predispose the cell to further abnormalities, which would accumulate over several replicative cycles and could result in cancer. MCM binding to DNA is decreased by aberrant G_1 CDK activity or overexpression of cyclin E, which occur in many cancers, including breast cancer and acute myeloid leukaemia M4/M5. Furthermore, mice with lower Mcm2 levels develop B- and T-cell lymphomas (Pruitt *et al*., 2007) and chemically induced breast cancers in mice were caused by *MCM4* mutation (Shima *et al*., 2007).

Our study also shows that depletion of Mcm7 causes PCS with a doubling of distances between the centromeres of sister chromatids consistent with a failure to secure sister chromatids during S-phase. Studies on yeast show that replication fork passage is required for establishment of cohesion (Lengronne *et al*., 2006; Shimada and Gasser, 2007) and MCM proteins are required for establishment of cohesion in *X. laevis* egg extracts (Gillespie and Hirano, 2004; Takahashi *et al*., 2004). Taken together, these data suggest that replication fork collapse caused by reducing MCM levels would be expected to reduce chromatid cohesion. The consequences of reduced cohesion are severe *in vivo* and patients with PCS trait have chromosomal abnormalities, exhibit developmental abnormalities and have a high risk of malignancy (Kajii *et al*., 1998, 2001).

We also show that reducing Mcm7 levels results in increased NHEJ activity, and cells with reduced MCM levels do not reach metaphase if both NHEJ and HR pathways are also compromised. NHEJ is a more error-prone pathway than HR and we showed earlier that NHEJ mis-repair is increased in Bloom's chromosomal instability syndrome, as well as in the myelodysplastic syndromes (pre-leukaemic conditions) and in myeloid leukaemias (Gaymes *et al*., 2002a, b). The data here show that the induction of normal levels of MCM proteins during the $G_0 \rightarrow G_1$ transition are critical in maintaining genome stability and a small reduction in MCM levels is sufficient to cause DNA mis-repair, PCS and gross chromosomal abnormalities.

Materials and methods

Routine methods

T-cell isolation, western blotting, immunofluorescence, ChIP and chromosomal analyses were as described earlier (Supplementary Information).

siRNA transfection

Pools of four siRNA to targets described in the text or control siRNA (Smartpool, Dharmacon), or each of the four individual siRNA were transfected into quiescent T cells by nucleofection (Amaxa, Lonza, Berkshire, UK) (siRNA sequences are in Supplementary Information). Cells transfected with siRNA were cultured overnight without stimulus to recover, stimulated with PMA/ionomycin and samples were typically taken 72 h later.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We sincerely thank Hye Kyung Hong, Kai Stoeber and Gareth Williams, UCL for the Mcm2 plasmid and advice on expressing recombinant Mcm2. We also thank our colleagues for helpful comments. This work was supported by grants from the Leukaemia Research Fund (LRF, now Leukaemia and Lymphoma Research (LLR)), Department of Trade and Industry (dti), the British Society for Haematology, the Welch and Packard Foundations and the National Institutes of Health (NIH).

References

Aggarwal P, Lessie MD, Lin DI, Pontano L, Gladden AB, Nuskey B *et al*. (2007). Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication. *Genes Dev* **21**: 2908–2922.

Arnaudeau C, Lundin C, Helleday T. (2001). DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells. *J Mol Biol* **307**: 1235–1245.

Bailis JM, Luche DD, Hunter T, Forsburg SL. (2008). Minichromosome maintenance proteins interact with checkpoint and recombination proteins to promote s-phase genome stability. *Mol Cell Biol* **28**: 1724–1738.

Bell SP, Dutta A. (2002). DNA replication in eukaryotic cells. *Annu Rev Biochem* **71**: 333–374.

Blow JJ, Dutta A. (2005). Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol* **6**: 476–486.

Blow JJ, Hodgson B. (2002). Replication licensing—defining the proliferative state? *Trends Cell Biol* **12**: 72–78.

Bruemmer D, Yin F, Liu J, Kiyono T, Fleck E, Van Herle AJ *et al*. (2003). Rapamycin inhibits E2F-dependent expression of minichromosome maintenance proteins in vascular smooth muscle cells. *Biochem Biophys Res Commun* **303**: 251–258.

Budman J, Kim SA, Chu G. (2007). Processing of DNA for nonhomologous end-joining is controlled by kinase activity and XRCC4/LigaseIV. *J Biol Chem* **282**: 11950–11959.

Chong JP, Blow JJ. (1996). DNA replication licensing factor. *Prog Cell Cycle Res* **2**: 83–90.

Cortez D, Glick G, Elledge SJ. (2004). Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proc Natl Acad Sci USA* **101**: 10078–10083.

Coxon A, Maundrell K, Kearsey SE. (1992). Fission yeast cdc21+ belongs to a family of proteins involved in an early step of chromosome replication. *Nucleic Acids Res* **20**: 5571–5577.

Crevel G, Hashimoto R, Vass S, Sherkow J, Yamaguchi M, Heck MM *et al*. (2007). Differential requirements for MCM proteins in DNA replication in Drosophila S2 cells. *PLoS One* **2**: e833.

Ekholm-Reed S, Mendez J, Tedesco D, Zetterberg A, Stillman B, Reed SI. (2004). Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *J Cell Biol* **165**: 789–800.

Feng D, Tu Z, Wu W, Liang C. (2003). Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. *Cancer Res* **63**: 7356–7364.

Flores-Rozas H, Kolodner RD. (2000). Links between replication, recombination and genome instability in eukaryotes. *Trends Biochem Sci* **25**: 196–200.

Françon P, Mechali M. (2006). DNA replication origins. *Encyclopedia of Life Sciences*, www3.interscience.wiley.com, John Wiley & Sons, Ltd.

Gaymes TJ, Mufti GJ, Rassool FV. (2002a). Myeloid leukemias have increased activity of the nonhomologous end-joining pathway and concomitant DNA misrepair that is dependent on the Ku70/86 heterodimer. *Cancer Res* **62**: 2791–2797.

Gaymes TJ, North PS, Brady N, Hickson ID, Mufti GJ, Rassool FV. (2002b). Increased error-prone non homologous DNA end-joining—a proposed mechanism of chromosomal instability in Bloom's syndrome. *Oncogene* **21**: 2525–2533.

Ge XQ, Jackson DA, Blow JJ. (2007). Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. *Genes Dev* **21**: 3331–3341.

Gillespie PJ, Hirano T. (2004). Scc2 couples replication licensing to sister chromatid cohesion in Xenopus egg extracts. *Curr Biol* **14**: 1598–1603.

Hans F, Dimitrov S. (2001). Histone H3 phosphorylation and cell division. *Oncogene* **20**: 3021–3027.

Howell RT, Taylor AMR. (1992). Chromosomal instability syndromes. In: Rooney DE, Czepulkowski, BH (eds). *Human Cytogenetics. A Practical Approach*. Oxford University Press: Oxford. pp 209–234.

Hua XH, Yan H, Newport J. (1997). A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. *J Cell Biol* **137**: 183–192.

Hyrien O, Marheineke K, Goldar A. (2003). Paradoxes of eukaryotic DNA replication: MCM proteins and the random completion problem. *Bioessays* **25**: 116–125.

Ibarra A, Schwob E, Mendez J. (2008). Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. *Proc Natl Acad Sci USA* **105**: 8956–8961.

Kajii T, Ikeuchi T, Yang ZQ, Nakamura Y, Tsuji Y, Yokomori K *et al*. (2001). Cancer-prone syndrome of mosaic variegated aneuploidy and total premature chromatid separation: report of five infants. *Am J Med Genet* **104**: 57–64.

Kajii T, Kawai T, Takumi T, Misu H, Mabuchi O, Takahashi Y *et al*. (1998). Mosaic variegated aneuploidy with multiple congenital abnormalities: homozygosity for total premature chromatid separation trait. *Am J Med Genet* **78**: 245–249.

Kolodner RD, Putnam CD, Myung K. (2002). Maintenance of genome stability in Saccharomyces cerevisiae. *Science* **297**: 552–557.

Lea NC, Orr SJ, Stoeber K, Williams GH, Lam EW, Ibrahim MA *et al*. (2003). Commitment point during G0→G1 that controls entry into the cell cycle. *Mol Cell Biol* **23**: 2351–2361.

Lei M, Kawasaki Y, Tye BK. (1996). Physical interactions among Mcm proteins and effects of Mcm dosage on DNA replication in Saccharomyces cerevisiae. *Mol Cell Biol* **16**: 5081–5090.

Lengronne A, McIntyre J, Katou Y, Kanoh Y, Hopfner KP, Shirahige K *et al*. (2006). Establishment of sister chromatid cohesion at the S. cerevisiae replication fork. *Mol Cell* **23**: 787–799.

Liang DT, Hodson JA, Forsburg SL. (1999). Reduced dosage of a single fission yeast MCM protein causes genetic instability and S phase delay. *J Cell Sci* **112**(Part 4): 559–567.

Lundin C, Erixon K, Arnaudeau C, Schultz N, Jenssen D, Meuth M *et al*. (2002). Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells. *Mol Cell Biol* **22**: 5869–5878.

Mailand N, Diffley JF. (2005). CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. *Cell* **122**: 915–926.

Moldovan GL, Pfander B, Jentsch S. (2006). PCNA controls establishment of sister chromatid cohesion during S phase. *Mol Cell* **23**: 723–732.

Nakanishi M, Shimada M, Niida H. (2006). Genetic instability in cancer cells by impaired cell cycle checkpoints. *Cancer Sci* **97**: 984–989.

Nasmyth K, Haering CH. (2005). The structure and function of SMC and kleisin complexes. *Annu Rev Biochem* **74**: 595–648.

Niida H, Nakanishi M. (2006). DNA damage checkpoints in mammals. *Mutagenesis* **21**: 3–9.

Ohtani K, Iwanaga R, Nakamura M, Ikeda M, Yabuta N, Tsuruga H *et al*. (1999). Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. *Oncogene* **18**: 2299–2309.

Pruitt SC, Bailey KJ, Freeland A. (2007). Reduced Mcm2 expression results in severe stem/progenitor cell deficiency and cancer. *Stem Cells* **12**: 3121–3132.

Shima N, Alcaraz A, Liachko I, Buske TR, Andrews CA, Munroe RJ *et al*. (2007). A viable allele of Mcm4 causes chromosome instability and mammary adenocarcinomas in mice. *Nat Genet* **39**: 93–98.

Shimada K, Gasser SM. (2007). The origin recognition complex functions in sister-chromatid cohesion in Saccharomyces cerevisiae. *Cell* **128**: 85–99.

Shreeram S, Sparks A, Lane DP, Blow JJ. (2002). Cell type-specific responses of human cells to inhibition of replication licensing. *Oncogene* **21**: 6624–6632.

Shukla A, Navadgi VM, Mallikarjuna K, Rao BJ. (2005). Interaction of hRad51 and hRad52 with MCM complex: a cross-talk between recombination and replication proteins. *Biochem Biophys Res Commun* **329**: 1240–1245.

Smith AE, Chronis C, Christodoulakis M, Orr SJ, Lea NC, Twine NA *et al*. (2009). Epigenetics of human T cells during the G0->G1 transition. *Genome Res* **19**: 1325–1337.

Stoeber K, Tlsty TD, Happerfield L, Thomas GA, Romanov S, Bobrow L *et al*. (2001). DNA replication licensing and human cell proliferation. *J Cell Sci* **114**: 2027–2041.

Suzuki S, Adachi A, Hiraiwa A, Ohashi M, Ishibashi M, Kiyono T. (1998). Cloning and characterization of human MCM7 promoter. *Gene* **216**: 85–91.

Takahashi TS, Yiu P, Chou MF, Gygi S, Walter JC. (2004). Recruitment of Xenopus Scc2 and cohesin to chromatin requires the pre-replication complex. *Nat Cell Biol* **6**: 991–996.

Tanaka S, Diffley JF. (2002). Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. *Genes Dev* **16**: 2639–2649.

Taubert S, Gorrini C, Frank SR, Parisi T, Fuchs M, Chan HM *et al*. (2004). E2F-dependent histone acetylation and recruitment of the Tip60 acetyltransferase complex to chromatin in late G1. *Mol Cell Biol* **24**: 4546–4556.

Tsao CC, Geisen C, Abraham RT. (2004). Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling. *EMBO J* **23**: 4660–4669.

Williams GH, Stoeber K. (2007). Cell cycle markers in clinical oncology. *Curr Opin Cell Biol* **19**: 672–679.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)