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ATR Regulates Fragile Site Stability

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Summary

Conditions that partially inhibit DNA replication induce expression of common fragile sites. These sites form gaps and breaks on metaphase chromosomes and are deleted and rearranged in many tumors. Yet, the mechanism of fragile site expression has been elusive. We demonstrate that the replication checkpoint kinase ATR, but not ATM, is critical for maintenance of fragile site stability. ATR deficiency results in fragile site expression with and without addition of replication inhibitors. Thus, we propose that fragile sites are unreplicated chromosomal regions resulting from stalled forks that escape the ATR replication checkpoint. These findings have important implications for understanding both the mechanism of fragile site instability and the consequences of stalled replication in mammalian cells.

Introduction

Common fragile sites are loci that exhibit gaps and breaks on metaphase chromosomes of cells that have been cultured under conditions of replicative stress, such as folate deficiency or treatment with aphidicolin (Glover et al., 1984). Unlike rare fragile sites, which result from expanded di- or trinucleotide repeat mutation (Sutherland et al., 1998), common fragile sites do not contain such repeats and are a normal component of chromosome structure. The exact number of common fragile sites that exist is a matter of interpretation, and 75 aphidicolin-induced common fragile sites are listed in Genbank. Increasing the stress placed on DNA replication leads to the expression of increasing numbers of fragile sites, until replication stops altogether. However, gaps and breaks at just 20 fragile sites represent over 80% of all lesions observed in lymphocytes following treatment with low doses of aphidicolin (Glover et al., 1984). FRA3B at 3p14.2 stands out as the most "fragile" site in the genome, followed by 16q23 (FRA16D), 6q26 (FRA6E), 7q31.2 (FRA7G), and Xp22.3 (FRAXB).

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Common fragile sites are normally stable in cultured human cells. However, following induction with replication inhibitors, these sites are "hot spots" for increased sister chromatid exchanges (SCE), translocations, and deletions (Glover and Stein, 1987, 1988; Wang et al., 1997). They are preferred sites of plasmid integration (Rassool et al., 1991) and may be favored targets for DNA virus integration in vivo (Thorland et al., 2000; Wilke et al., 1996). Fragile sites may also play a role in some gene amplification events, both in vitro and in tumor cells, by triggering a breakage-fusion-bridge cycle (Coquelle et al., 1997). All of these chromosomal alterations are preceded by a DNA double-strand (DS) break, leading to the conclusion that DS breaks are sometimes associated with fragile site expression.

Five fragile sites have been cloned and characterized and have been found to extend over hundreds of kilobases, with gaps or breaks on metaphase chromosomes occurring throughout these regions (Glover, 1998; Huebner and Croce, 2001). Numerous studies have shown that these sites are unstable in tumor cells. For example, *FHIT*, the gene spanning FRA3B, is often rearranged or partially deleted in many tumors, including lung, breast, ovarian, cervical, and esophageal (Druck et al., 1997; Michael et al., 1997; Mimori et al., 1999). Investigation of chromosome 3 homologs from tumor cell lines showed multiple variable deletions within FRA3B, suggesting ongoing instability in the region (Corbin et al., 2002). *WWOX*, the gene at FRA16D, shows loss of heterozygosity and deletions in various cancers, as do FRA7G, FRA7H, and FRAXB (Arlt et al., 2002; Huang et al., 1999; Paige et al., 2000; Ried et al., 2000). Through functional studies, both *FHIT* and *WWOX* have been identified as tumor suppressors (Bednarek et al., 2001; Dumon et al., 2001; Paige et al., 2001). However, the genes identified at FRAXB are not involved in tumor progression, indicating that fragile site instability in tumors is not driven solely by associated gene function. All of these results support the hypothesis that fragile sites are involved in chromosome rearrangements in cancer.

Determining the mechanism of common fragile site expression is important in understanding a normal component of chromosome structure and function and in understanding the instability found at fragile sites in tumor cells. Beyond the knowledge that partial inhibition of DNA synthesis is involved, little is known about the mechanism of common fragile site expression. Sequence analyses have not readily revealed why the sites are unstable; however, all fragile sites studied to date are relatively AT-rich (Arlt et al., 2002; Ried et al., 2000; Shiraishi et al., 2001) and contain more areas of high flexibility than non-fragile regions (Mishmar et al., 1998). Studies of replication timing at FRA3B have found this site to be late replicating (Le Beau et al., 1998; Wang et al., 1999), as had previously been shown for the fragile X site (FRAXA) (Hansen et al., 1997). Following addition of aphidicolin, FRA3B replicates even later, and in some

cells may remain unreplicated in G2. Replication timing at FRA7H has also been studied and a detailed analysis indicated significant differences in the replication timing of adjacent segments with some segments replicating late in S phase, a pattern that was exaggerated by the addition of aphidicolin (Hellman et al., 2000). These results suggest that late replication may play a role in fragile site expression.

Based on the occurrence of DNA breaks and chromosome rearrangements at fragile site loci and the possible role of replication fork stalling at these sites, we hypothesized that the S phase and G2/M cellular checkpoint proteins Ataxia-Telangiectasia Mutated (ATM) and Ataxia-Telangiectasia and Rad3-Related (ATR) are involved in fragile site maintenance and stability. Recent findings show that ATM and ATR act in distinct but partially overlapping pathways in response to specific types of DNA damage during cellular replication (Abraham, 2001; Durocher and Jackson, 2001). ATM has been studied in cell lines derived from AT patients and is activated by DNA DS breaks. Thus, AT cells are sensitive to ionizing irradiation but not UV-light (Pincheira et al., 2001). ATR has been more difficult to study, as knockout mice die in early embryogenesis, and cells lacking this protein are inviable within a few cell divisions (Brown and Baltimore, 2000). In the past few years, it has been shown that ATR is required for checkpoint responses after treatment of cells with UV light and agents that block replication fork progression, such as hydroxyurea and aphidicolin (Cliby et al., 1998; Cortez et al., 2001; Nghiem et al., 2001) and, more recently, hypoxia (Hammond et al., 2002). A major outcome of ATR deficiency found with these approaches was extreme chromosome fragmentation following treatment of cells with high concentrations of aphidicolin or hydroxyurea (Nghiem et al., 2001). The current model is that ATM and ATR perform critical early functions to activate the replication checkpoints in response to DNA DS breaks or stalled replication forks, respectively.

We have investigated the role of ATM and ATR in the maintenance of fragile site stability. We found that 2-aminopurine (2-AP), an inhibitor of ATM and ATR kinases (Dimitrova and Gilbert, 2000; Sarkaria et al., 1999), increases fragile site expression. We then used AT cell lines to study ATM and three methods to study ATR: (1) a dominant-negative approach in stably transfected cell lines; (2) cre-lox mediated ATR deficiency in cell lines with lox P-flanked ATR; and (3) RNAi using siRNA duplexes directed against ATR. We found that fragile site expression is unchanged in AT cells, indicating that ATM is not required for the maintenance of fragile site stability. In contrast, partial inhibition of ATR caused a 5- to 20-fold increase in aphidicolin-induced fragile site expression compared to control cells. Furthermore, we found that fragile sites are expressed in ATR-deficient cells without addition of replication inhibitors. These results demonstrate that the ATR checkpoint pathway is critical for the maintenance of stability at common fragile sites. This finding is an important advance in understanding the mechanism of fragile site maintenance in normal cells and chromosome rearrangements at fragile sites in tumor cells.

Results

2-AP, an Inhibitor of ATM and ATR, Increases Fragile Site Expression

We first tested the hypothesis that ATM and ATR have a role in the maintenance of fragile site stability by investigating the effect of 2-AP, a kinase inhibitor of both ATM and ATR (Dimitrova and Gilbert, 2000; Sarkaria et al., 1999), on fragile site expression. Caffeine, which also inhibits the function of both ATM and ATR (Sarkaria et al., 1999), has previously been observed to enhance fragile site expression (Glover et al., 1986; Yunis and Soreng, 1984), suggesting that these checkpoint proteins could be involved in fragile site recognition or repair. Thus, we predicted that 2-AP, like caffeine, would also increase fragile site expression. We compared the effect of treating human lymphocytes with 2-AP to treatment with caffeine. Metaphase chromosomes were trypsin banded to determine whether the chromosomal gaps and breaks observed after treatment corresponded to the cytogenetic location of recognized fragile sites. Few chromosomal gaps or breaks were observed with 2-AP alone, but six of the ten total gaps/breaks from two individuals observed at the 5 mM treatment level were at FRA3B ($n = 50$ cells per individual). When 2-AP is added with aphidicolin, there is a dramatic rise in average overall chromosomal gaps and breaks that escalates with increasing 2-AP concentrations (Figure 1A). From banded chromosomes, we determined that $>90\%$ of the gaps and breaks occurred at recognized fragile sites (data not shown). The expression of a specific fragile site, FRA3B, correlates with these data (Figure 1B). These results are similar to those observed with caffeine (Figures 1C and 1D). Thus, both caffeine and 2-AP increase the expression levels of common fragile sites. As caffeine and 2-AP inhibit ATM and ATR, these data support the hypothesis that one or both of these protein kinases are involved in the maintenance of fragile site stability.

ATM Is Not Required for the Maintenance of Fragile Site Stability

In order to differentiate between the relative contributions of ATM and ATR to fragile site stability, we studied cell lines deficient in either ATM or ATR activity. We investigated ATM by comparison of fragile site expression in seven AT lymphoblast cell lines to that in four normal control human lymphoblast lines following aphidicolin treatment. Four of the seven AT lines studied have known truncating mutations in the *ATM* gene (Telatar et al., 1996; Wright et al., 1996), and analysis of protein expression by Western blot demonstrates that none of the seven AT lines have detectable ATM expression (Figure 2A). Following aphidicolin treatment, we found an average of 1.21 gaps and breaks per cell in normal lines, compared to an average of 1.16 gaps and breaks per cell in AT lines ($P = 0.526$) (Figure 2B). Analysis of average breaks at FRA3B per cell also indicated insignificant differences between normal and AT lines ($P = 0.856$) (Figure 2B). These results suggest that the role of ATM in fragile site expression is negligible.

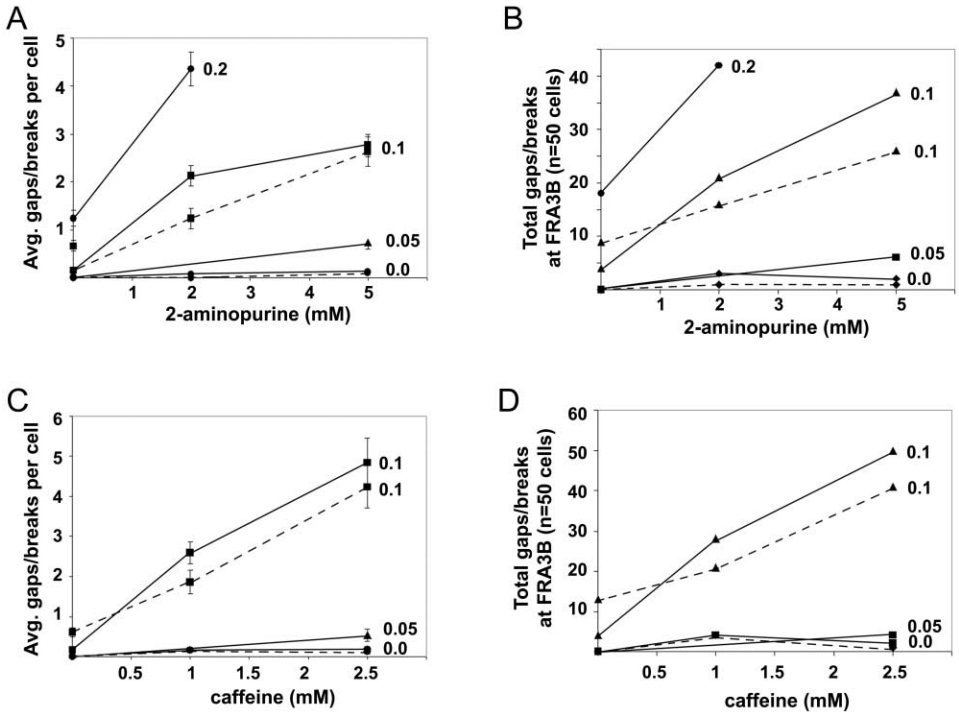

Figure 1. 2-Aminopurine Increases Common Fragile Site Expression

(A) Average overall chromosome gaps and breaks per cell in normal human lymphoblasts after aphidicolin and 2-AP treatment (n = 50 metaphases).

(B) Total gaps and breaks at FRA3B in normal human lymphoblasts after aphidicolin and 2-AP treatment (n = 50 metaphases).

(C) Average overall chromosomal gaps and breaks per cell in normal human lymphoblasts after aphidicolin and caffeine treatment (n = 50 metaphases).

(D) Total gaps and breaks at FRA3B in normal human lymphoblasts after aphidicolin and caffeine treatment. (n = 50 metaphases). Values next to plotted lines indicate μM aphidicolin treatment.

(A–D) Solid and dashed lines indicate results from two individuals.

ATR Is Critical for the Maintenance of Fragile Site Stability

We next studied the role of ATR in fragile site stability. As animals and cells lacking ATR are inviable (Brown and Baltimore, 2000), we used three methods to overcome this problem: (1) a dominant-negative approach using stable cell lines expressing either ATR-wt or ATR-kd; (2) cre-lox mediated ATR deficiency using $ATR^{flox/-}$ cell lines allowing for removal of lox-P flanked ATR; and (3) RNAi using siRNA duplexes directed against ATR.

We first tested the effect of inducing fragile sites with aphidicolin in U2-OS (human osteosarcoma) cells stably transfected with doxycycline-inducible wild-type (ATR-wt) or dominant-negative kinase-dead ATR (ATR-kd) expression constructs (Nghiem et al., 2001). These lines had undetectable expression of the FLAG-tagged ATR-wt and ATR-kd proteins in the absence of doxycycline. Dox induction resulted in greatly increased levels of ATR-wt or ATR-kd as monitored by Western blotting (Figure 3A). After the addition of aphidicolin, average overall chromosomal gaps and breaks were increased >20-fold in ATR-kd expressing cells, as compared to ATR-wt expressing cells (Figure 3B). ATR-kd expressing cells were much more sensitive to aphidicolin than ATR-wt expressing cells. Aphidicolin concentrations that produce few to no fragile sites in normal cells and cells expressing ATR-wt result in very high levels of gaps and breaks in ATR-kd expressing cells. It is known that most chromosomal gaps and breaks following aphidicolin treatment occur at fragile sites (Glover et al., 1984). However, to verify that the increase in gaps and breaks in cells with ATR deficiency is not due to global instability at random sites, we measured expression at specific common fragile sites using FISH approaches with BAC and YAC probes specific to FRA3B, FRA16D, and FRA7H. We did not observe typical expression of FRA3B or FRA16D under any treatment condition, perhaps due to rearrangements in this tumor cell line. However, typical fragile site expression was observed at FRA7H, thus allowing its analysis. Comparison of breaks and gaps at this site showed a 10-fold increase in FRA7H expression in cells expressing ATR-kd after aphidicolin treatment as compared to ATR-wt controls, which is in agreement with our results from total gaps and breaks (Figure 3C).

In a second approach, we inactivated ATR in $ATR^{flox/-}$ cells. In this cell line, derived from HCT116 (human mismatch repair-deficient) cells, both alleles of ATR have been stably modified such that one allele is interrupted by insertion of the *neo*^{r} gene and the other allele contains lox P sites flanking exon 2 (Cortez et al., 2001). ATR expression in $ATR^{flox/-}$ cells is less than half that of the parent HCT116 cells, and adenovirus mediated cre-lox removal of the remaining allele of ATR results in undetectable ATR expression by three days post infection, as

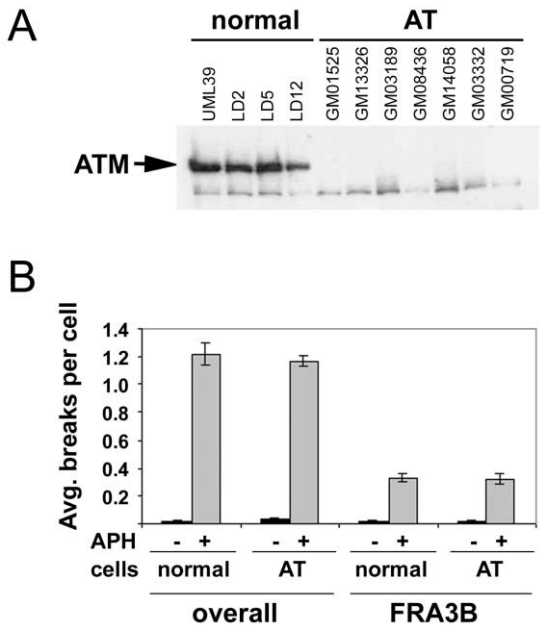

Figure 2. ATM Deficiency Does Not Affect Common Fragile Site Expression

(A) Western blot probed with α-ATM showing lack of ATM protein expression in cell lines from AT patients. Non-specific bands indicate protein loading.

(B) Average total chromosomal gaps and breaks per cell and average gaps and breaks at FRA3B per cell in four normal lymphoblast lines (UML39, LD2, LD5, and LD12) versus seven AT lymphoblast lines (GM08436, GM13326, GM01525, GM03189, GM00719, GM03332, and GM14058); n = 50 metaphases for each cell line and condition. For fragile site induction, 0.4 μM aphidicolin was added 24 hr before harvesting. APH: aphidicolin.

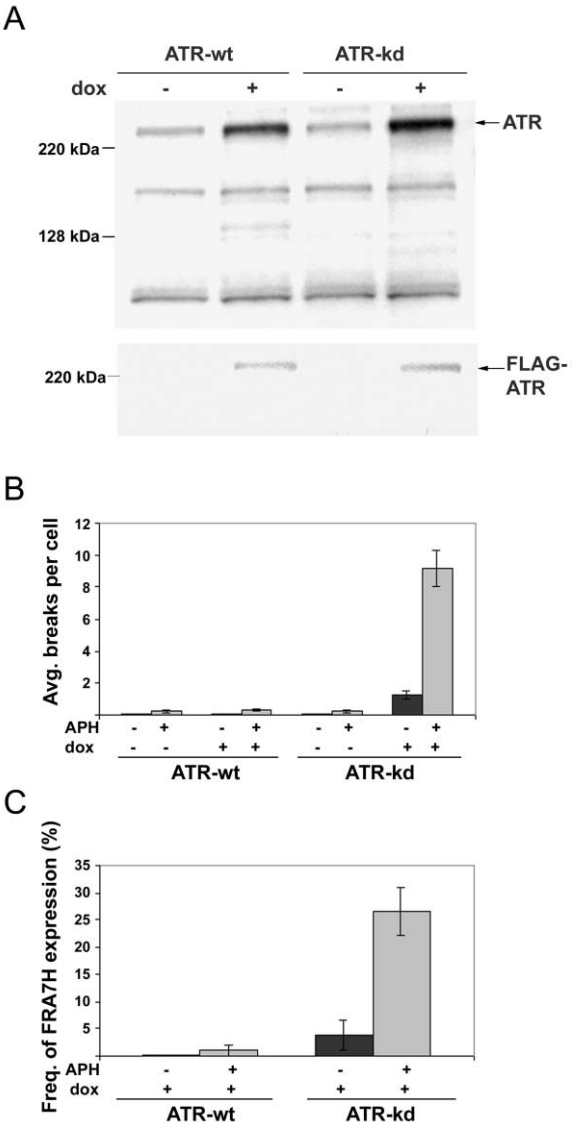

Figure 3. Cells Expressing ATR-kd Have Increased Common Fragile Site Expression

(A) Western blot probed first with α-ATR, then stripped and reprobed with α-FLAG antibodies, showing specific, highly inducible expression of FLAG-tagged ATR-wt or ATR-kd in stably transfected U2-OS cells. Non-specific bands indicate even loading. Whole-cell lysate was collected 24 hr after dox induction.

(B) Average overall chromosomal gaps and breaks in ATR-wt or ATR-kd expressing cells; n = 50 metaphases for each condition.

(C) Frequency of FRA7H expression in ATR-wt or ATR-kd expressing cells; n = 100 hybridization signals for each condition. Induction of ATR-wt or ATR-kd expression was achieved by addition of 1 μg/ml doxycycline 48 hr before harvest. For fragile site induction, 0.05 μM aphidicolin was added 24 hr before harvest.

observed by Western blots (Figure 4). PCR with primers flanking the lox P sites also indicated the expected deletion product following AdCre1 infection (data not shown). We were able to achieve infection levels of 90% in this line as monitored by infection with adenovirus-expressing GFP (data not shown). We found that average overall chromosomal gaps and breaks were increased in lines lacking ATR expression, as compared to the parent line after treatment with aphidicolin (Figure 4B). This increase was relatively linear with increasing aphidicolin treatment levels. Compared to controls, fragile site expression was increased 5-fold in floxed cells after treatment with 0.05 μM aphidicolin, while after treatment with 0.1 μM aphidicolin, fragile site expression was increased 10-fold (Figures 4B and 4D). At the 0.1 μM aphidicolin treatment level, there was a wide range of fragile site expression, with a few cells expressing so many gaps and breaks that chromosomes had a shattered appearance, similar to the previously observed premature chromatin condensation (PCC) phenotype reported in ATR deficient cells that had been treated with much higher aphidicolin concentrations (Nghiem et al., 2001). Similar to the overall gaps and breaks, cells lacking ATR had a 7- to 8-fold increase in FRA3B and FRA16D expression as measured by FISH using probes specific to these fragile sites (Figure 4C).

RNA interference (RNAi) directed against ATR was used as a third independent method to explore the relationship between ATR function and the maintenance of fragile site stability. Successful employment of RNAi in mammalian cells has recently been achieved through the use of short interfering RNAs (siRNAs) (Elbashir et al., 2001; Harborth et al., 2001). These siRNAs are duplexes of 21 nt RNAs with 2 nt 3′ overhangs, matching unique sequence in the 5′ region of the targeted mRNA.

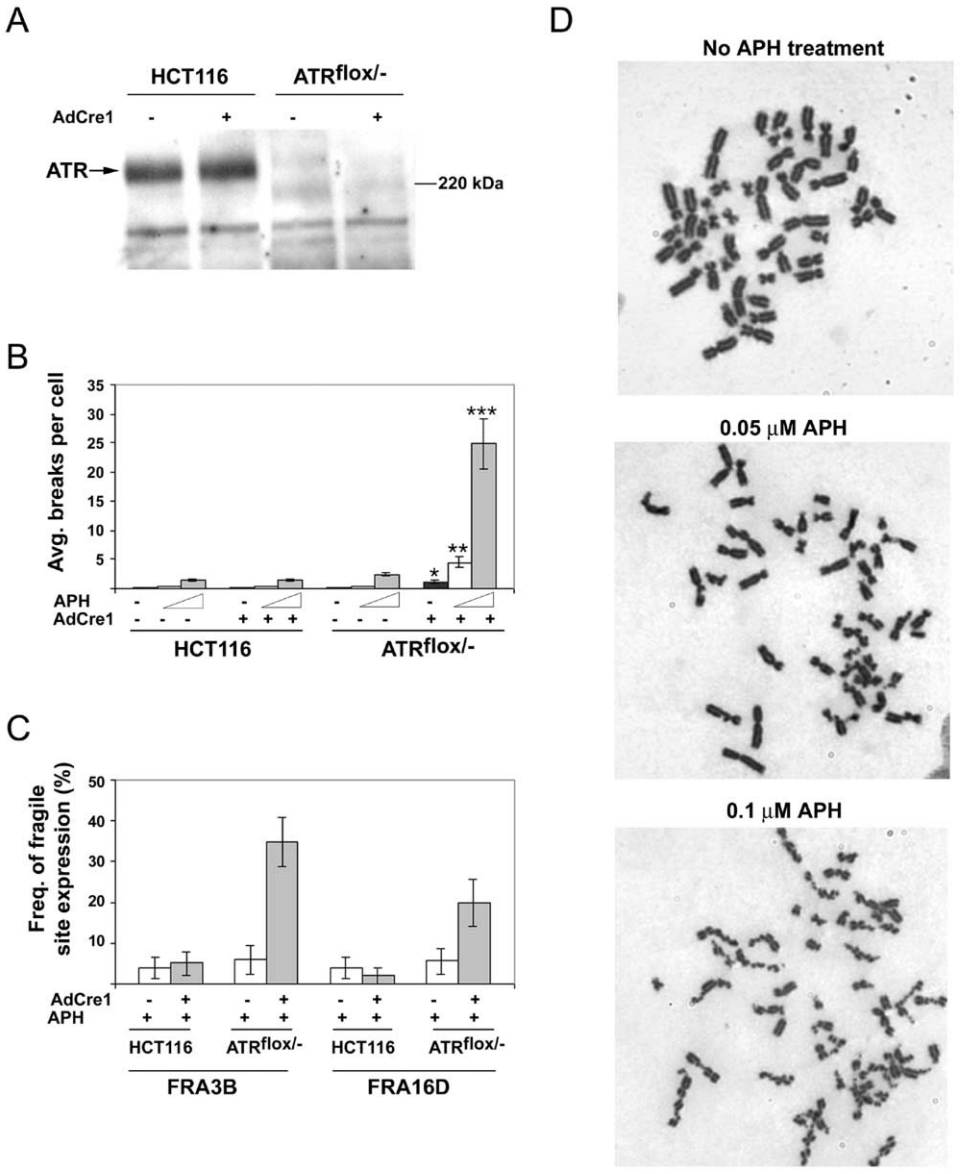

Figure 4. Cells with Cre-Lox Inactivation of ATR Have Increased Common Fragile Site Expression

(A) Western blot probed with α-ATR shows severely reduced ATR expression in $ATR^{flox/-}$ cells as compared to HCT116 cells and complete loss of ATR expression in $ATR^{flox/-}$ cells 72 hr after AdCre1 infection. Non-specific bands indicate even loading. Whole-cell lysate was collected 72 hr after AdCre1 infection.

(B) Average overall chromosomal gaps and breaks in HCT116 cells compared to $ATR^{flox/-}$ cells; n = 50 metaphases for each condition. As indicated on the graph, *6 of 50, **7 of 50, and ***29 of 50 metaphases analyzed had a shattered appearance similar to the PCC phenotype (Nghiem et al., 2001) and thus were not included in calculation of average breaks per cell. Cre-lox inactivation of ATR was achieved by AdCre1 infection 96 hr before harvest. For fragile site induction, 0.05 μM or 0.1 μM aphidicolin was added 24 hr before harvest.

(C) Frequency of FRA3B and FRA16D expression in HCT116 cells compared to $ATR^{flox/-}$ cells; n = at least 50 hybridizations for each condition. For fragile site induction, 0.05 μM aphidicolin was added 24 hr before harvest.

(D) Representative metaphases of $ATR^{flox/-}$ cells after cre-lox inactivation of ATR and addition of increasing concentrations of aphidicolin. See text for details. Cre-lox inactivation of ATR was achieved by AdCre1 infection 96 hr before harvest.

Two siRNA sequences directed against ATR (siRNA-1 and siRNA-2) and matching single-stranded RNA sense controls were constructed. These RNAs were transfected into HCT116 and HeLa cells. Transfection levels of a fluorescently conjugated (Cy-3) control RNA were 95% and 90% in these lines, respectively (data not shown). Western blots from cells two days after transfection indicate that siRNA-2 is more efficient at inhibiting ATR protein levels than siRNA-1. No decrease in ATR protein level was observed after transfection with single-stranded, sense control RNAs (Figure 5A). In agreement with the Western blot data, we found that the increase in gaps and breaks in cells transfected with siRNA-1 was not as pronounced as the increase observed in cells transfected with siRNA-2. Compared to controls, gaps and breaks increased 3-fold and 9-fold

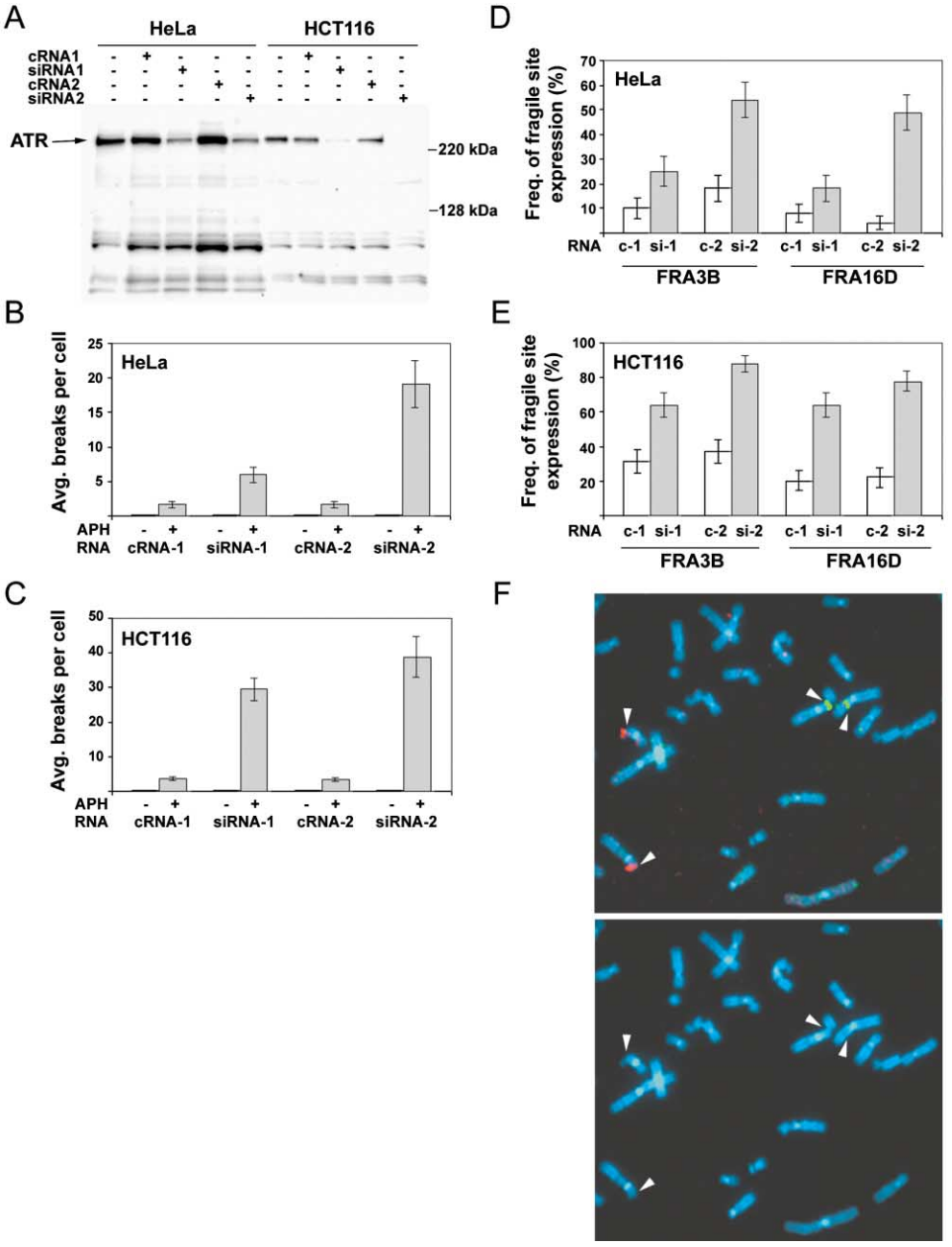

Figure 5. Cells with siRNA Inactivation of ATR Have Increased Common Fragile Site Expression

(A) Western blot probed with α-ATR showing reduced ATR expression in HCT116 and HeLa cells transfected with siRNA-1 or siRNA-2. Whole-cell lysate was collected 24 hr after transfection.

(B) Average overall chromosomal gaps and breaks in HeLa cells after transfection with siRNA-1, siRNA-2, or control RNAs; n = 20 metaphases for each condition. Inactivation of ATR was achieved by siRNA transfection 48 hr before harvest. Fragile site induction was achieved by addition of 0.3 μM aphidicolin 24 hr before harvest.

(C) Average overall chromosomal gaps and breaks in HCT116 cells after transfection with siRNA-1, siRNA-2, or control RNAs; n = 20 metaphases for each condition. Inactivation of ATR was achieved by siRNA transfection 48 hr before harvest. Fragile site induction was achieved by addition 0.3 μM aphidicolin 24 hr before harvest.

(D) Frequency of FRA3B and FRA16D expression in HeLa cells after transfection with siRNA-1, siRNA-2, or control RNAs; n = at least 50 hybridizations for each condition. Inactivation of ATR was achieved by siRNA transfection 48 hr before harvest. Fragile site induction was achieved by addition of 0.3 μM aphidicolin 24 hr before harvest.

(E) Frequency of FRA3B and FRA16D expression in HCT116 cells after transfection with siRNA-1, siRNA-2, or control RNAs; n = at least 50 hybridizations for each condition. Inactivation of ATR was achieved by siRNA transfection 48 hr before harvest. Fragile site induction was achieved by addition of 0.3 μM aphidicolin 24 hr before harvest.

(F) Partial metaphase showing an example of FISH on a siRNA-1 transfected HCT116 cell after 0.3 μM aphidicolin treatment. FRA3B is indicated by hybridization with YAC probe 850A6 (green); one homolog is broken. FRA16D is indicated by hybridization with BAC probe 264L1 (red); one homolog is broken. The G-banding karyotype of HCT116 cells is 45,X,-Y,t(8;16)(q13;p13), add (10q), add (18p) (Masramon et al., 2000).

Table 1A. Fragile Sites Are Observed in ATR-kd Expressing Cells without Prior Aphidicolin Induction

Cell line	Treatment	Avg. gaps Breaks per cell (n = 50 cells)	% FRA7H loci with a break	% of total breaks attributable to FRA7H
ATR-wt	+ dox +APH	0.26	1% (1/100)	7.7%
	+ dox −APH	0.08	0% (0/100)	0%
	− dox −APH	0.08	0% (0/100)	0%
ATR-kd	+ dox +APH	9.16	26% (26/100)	5.7%
	+ dox −APH	2.86	7% (7/100)	4.7%
	− dox −APH	0.10	1% (1/100)	20%

APH, aphidicolin (0.05 μm)

in aphidicolin-treated HeLa cells transfected with siRNA1 and siRNA2, respectively (Figure 5B). Data from transfected HCT116 cells showed a similar pattern, with gaps and breaks increased 6-fold and 8-fold in siRNA1 and siRNA2 transfected cells, respectively (Figure 5C). FISH on both HCT116 and HeLa cells with probes to FRA3B and FRA16D indicated a similar increase in gaps and breaks at these specific loci in cells transfected with siRNA, as compared to control transfections (Figures 5D, 5E, and 5F).

Fragile Sites Are Observed in ATR-Deficient Cells without Aphidicolin Induction

Our results, together with the known essential replication checkpoint role of ATR, suggest that replication stalling at fragile sites could be a normal occurrence during cellular replication. Thus, we hypothesized that fragile site expression would be observed in ATR-deficient cells after extended culture times, even without aphidicolin induction. We tested this hypothesis in ATR-kd and ATR-wt expressing cells and in $ATR^{flox/-}$ cells. Dox induction of ATR-wt and ATR-kd expression was maintained for five days before harvest, and no aphidicolin was added to cultures. We observed a 30-fold increase in average chromosomal gaps and breaks in ATR-kd expressing cells, as compared to control cells, and FISH with a probe to FRA7H indicates that the expression of this fragile site correlates with these data. Although the total gaps and breaks per cell is greater in aphidicolin-treated cells, the percentage of total breaks attributable to FRA7H ATR-kd expressing cells without aphidicolin treatment (4.7%) is similar to that observed in aphidicolin-treated cells (5.7%) (Table 1A). In $ATR^{flox/-}$ cells, ATR was inactivated by adenovirus-mediated cre expression four days prior to harvest, and no aphidicolin was added to cultures. We observed an 18-fold increase in total gaps and breaks in cells lacking ATR, as compared to control cells, and FISH with probes to FRA3B and FRA16D indicated that these sites are also broken more often in ATR null cells (Table 1B).

Discussion

We have demonstrated that ATR, but not ATM, is critical for the maintenance of common fragile site stability. We found that 2-AP, like caffeine, increases aphidicolin-induced fragile site expression. Both 2-AP and caffeine inhibit the kinase function of ATM and ATR. Thus, these results support the hypothesis that these cellular checkpoint proteins have a role in the maintenance of fragile site stability. We then differentiated between the relative contributions of ATM and ATR. Using AT cell lines, we determined that ATM deficiency has no effect on fragile site expression. As the ATM/CHK2 pathway is known to be necessary for response to DS breaks during S phase, it is therefore unlikely that DS breaks are a primary cause of fragile site expression, although they can sometimes occur as a secondary event at these sites and give rise to chromosomal rearrangements.

We used three independent approaches to study ATR, including dominant-negative kinase-dead ATR expression, cre-lox mediated deletion of ATR, and RNAi directed against ATR. Our results from all three approaches clearly demonstrate that ATR is critical for the

Table 1B. Fragile Sites Are Observed in $ATR^{flox/-}$ Cells without Prior Aphidicolin Induction

Cell line	Treatment	Avg. gaps, Breaks per cell (n = 50 cells)	% FRA3B loci with a break	% of total breaks attributable to FRA3B	% FRA16D loci with a break	% of total breaks attributable to FRA16D
HCT116	+cre +APH	0.46	5% (3/59)	22%	2% (1/50)	9%
	+cre −APH	0.04	-	-	-	-
	−cre −APH	0.04	-	-	-	-
$ATR^{flox/-}$	+cre +APH	4.5[a]	35% (22/63)	15.5%	20% (10/50)	8.8%
	+cre −APH	0.73[b]	4.2% (6/122)	11.5%	1% (2/122)	2.8%
	−cre −APH	0.04	-	-	-	-

[a] 7 metaphases with shattered chromosomes (similar to PCC phenotype) were not included in count of total gaps/breaks or analysis of fragile site expression frequency.

[b] 2 metaphases with shattered chromosomes (similar to PCC phenotype) were not included in count of total gaps/breaks or analysis of fragile site expression frequency.

APH, aphidicolin (0.05 μM)

maintenance of fragile site stability, as cells with ATR deficiency are particularly sensitive to aphidicolin-induced gaps and breaks at fragile sites. Furthermore, we have found that fragile sites are expressed in cells with ATR deficiency after several generations without prior addition of aphidicolin or other replication inhibitors and that ATR deficiency results in gaps and breaks at fragile sites. These results are important, as they indicate that replication fork stalling that can result in fragile site expression is a normal occurrence during unchallenged cellular replication, and that ATR recognizes and responds to stalled or incomplete replication at these sites. The association of this pathway with the maintenance of genome stability at fragile sites has important implications for understanding the consequences of stalled replication in mammalian cells.

ATM and ATR are key members of the intra-S phase, or replication, and G2/M checkpoint pathways (Abraham, 2001). ATM has been extensively studied, and is known to respond to DNA DS breaks, such as those produced by ionizing irradiation (Pincheira et al., 2001). *ATR* was identified in 1996 as a member of the *PI3K* family (Bentley et al., 1996; Cimprich et al., 1996). Homologs of *ATR* are present in all eukaryotic cells examined to date, including budding and fission yeast. Studies of the related *MEC1* in *S. cervisiae* and *rad3* in *S. pombe* indicate that these genes are necessary for both the DNA damage-induced G2 checkpoint and the intra-S phase checkpoint, which suppresses late origin firing in the presence of replication inhibitors and prevents cells from entering mitosis in the presence of unreplicated DNA (Bentley et al., 1996; Santocanale and Diffley, 1998; Weinert et al., 1994). In aphidicolin-arrested CHO cells, exposure to caffeine or 2-AP results in initiation of replication at late replicating origins and loss of the ability to reinitiate replication at earlier initiated sites (Dimitrova and Gilbert, 2000). ATR is likely to be the relevant target of caffeine and 2-AP in this pathway, as shown by recent studies where catalytically inactive versions of ATR result in loss of S phase checkpoint responses after treatment of mammalian cells with agents that block replication fork progression, such as hydroxyurea, aphidicolin, and hypoxia (Cliby et al., 1998; Cortez et al., 2001; Hammond et al., 2002; Nghiem et al., 2001). Mammalian cells lacking ATR function are also slightly radiosensitive (Cliby et al., 1998), and chromatin immunoprecipitation studies in *S. cervisiae* of ATR homolog Mec1 have shown this protein to be recruited to a site-specific DS break at the mating type (MAT) locus (Kondo et al., 2001), suggesting that the role of ATR may be broader than presently indicated.

Downstream targets of ATR include CHK1, BRCA1, p53, and H2AX (Liu et al., 2000; Nghiem et al., 2002; Tibbetts et al., 1999, 2000; Ward and Chen, 2001; Zhao and Piwnica-Worms, 2001). All of these targets can be examined using approaches similar to those used here for ATR. Based on checkpoint function, we propose that CHK1 and BRCA1 are likely to be involved in fragile site stability. Like ATR, BRCA1 activates CHK1 (Yarden et al., 2002), which regulates the activity of the CDC25 and WEE1 kinases. These proteins regulate the CDC2/cyclin B complex, and thus the G2/M checkpoint and progression into mitosis. The exact mechanisms of ATR control of the S phase checkpoint are unclear, but appear to involve stabilization of existing replicons and inhibition of late firing origins (Dimitrova and Gilbert, 2000), possibly through recruitment of repair proteins or DNA polymerases (Lopes et al., 2001; Marchetti et al., 2002).

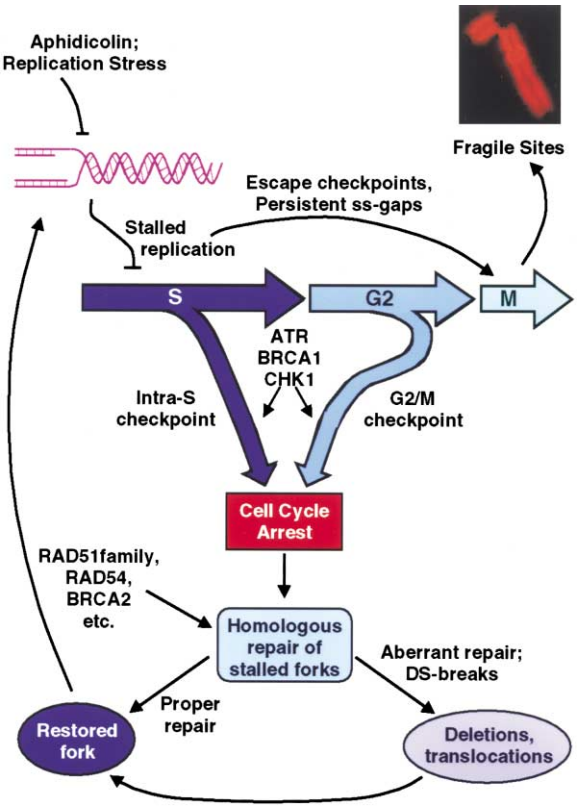

Figure 6. Model of Common Fragile Site Expression
See text for details.

The current model is that ATM and ATR are critical early activators of cellular checkpoints in response to DNA damage during replication. ATM responds primarily to DS breaks, and acts at the G1 or later checkpoints (Abraham, 2001). ATR responds primarily to replicative stress, such as caused by aphidicolin and hydroxyurea, and functions during the intra S and G2/M checkpoints to stabilize, and later restart, stalled replication forks (Lopes et al., 2001; Tercero and Diffley, 2001), inhibit late origin firing in the presence of stresses (Tercero and Diffley, 2001), and block cell cycle progression into mitosis before replication is complete (Nghiem et al., 2001).

A Model for Common Fragile Sites

Our recent data, combined with findings we and others have made over the past twenty years, allow us to create a model for common fragile sites (Figure 6). We propose that fragile sites represent single-stranded (unreplicated) regions caused by stalled or collapsed replication forks following partial inhibition of DNA replication. For unknown reasons, likely related to sequence and/or replication dynamics, fragile site regions are significantly more sensitive to replication fork delay or arrest than other regions in the genome. Fragile sites are recognized

by the S phase and/or G2/M checkpoints in which ATR plays a key role. However, the appearance of fragile sites on metaphase chromosomes in normal cells suggests that some proportion of these lesions can escape checkpoint controls. Expressed fragile sites thus support classic suggestions that chromosome gaps represent single-stranded, unreplicated regions. Repair of such structures usually requires DNA-directed homologous repair. Fragile sites are frequently accompanied by a sister chromatid exchange, thus supporting this view (Glover and Stein, 1987). Deletions or translocations at fragile sites can result from DNA DS breaks caused by aberrant processing of Holliday junctions at damaged forks or illegitimate recombination. Such breaks should be recognized by ATM. Thus, the deletions seen in tumor cells are proposed to arise from unequal or faulty homologous repair of stalled forks or from mutations in the replication checkpoint or associated repair pathways. In this way, deletions at fragile sites in tumors are "signatures" of stalled replication forks. This model predicts that tumor cells with mutations or alterations in replication checkpoint or associated homologous repair genes will show increased chromosomal rearrangements at fragile sites.

Common fragile sites appear to be conserved in primates (Smeets and van de Klundert, 1990), the mouse (Glover et al., 1998; Shiraishi et al., 2001), and likely other mammals, suggesting a conserved function such as mediating chromosome replication events during late S phase. It has recently been shown that Mec1 deficiency in *S. cerevisiae* results in non-random chromosome breakage within ~10 kb "replication slow zones" which occur, on average, every ~60 kb in chromosome III (Cha and Kleckner, 2002). While their size and distribution indicates a more simple structure, these regions may be functionally analogous to mammalian common fragile sites. It is of interest in this regard that at least two human fragile sites have been shown to be late replicating. However, it is unclear from these studies whether the late replication observed in fragile site regions is the result of late origin activation, slow replication initiated at earlier activated origins, replication fork stalling, or a combination of these factors.

Replication forks are routinely arrested by a variety of stresses, and checkpoint recognition and the associated repair of these sites is extremely important in genome maintenance. Currently, much more is known about the checkpoint response to DNA DS breaks than to stalled forks due to replication inhibition. Additionally, prior studies of these checkpoint and repair pathways have used high concentrations of replication inhibitors, such as aphidicolin and hydroxyurea, which would normally completely block replication. Fragile sites are induced at much lower concentrations that only partially inhibit replication and are more relevant to natural genotoxic exposure levels. Thus, fragile site expression provides a novel cytological assay for these checkpoint and associated DNA repair pathways in mammalian cells and can provide insight, not only into the nature of fragile sites, but also the broader consequences of stalled replication and its repair due to partial inhibition of DNA synthesis.

Experimental Procedures

Cell Culture and Fragile Site Analysis

Whole blood cultures from normal volunteers were grown in RPMI 1640 medium + 15% FBS with PHA stimulation for 2-AP and caffeine treatment. AT lymphoblast cell lines GM08436, GM13326, GM01525, GM03189, GM00719, GM03332, and GM14058 were obtained from Coriell Cell Repositories (Camden, NJ). Cell lines GM08436, GM13326, GM01525, and GM03189 contain identified truncating mutations in the *ATM* gene (Telatar et al., 1996; Wright et al., 1996). Normal control lymphoblast cultures UML39, LD2, LD5, and LD12 were established by Epstein-Barr virus transformation. All lymphoblastoid cell lines were diploid. Cells were maintained in RPMI 1640 medium + 15% FBS. ATR-wt and ATR-kd cells that allow doxycycline-inducible expression of FLAG-tagged wild-type or dominant-negative kinase-dead ATR were maintained in MEMα medium + 10% FBS + 200 μg/ml G418 and 200 μg/ml hygromycin B. Induction of ATR-wt or ATR-kd was achieved by addition of 1 μg/ml doxycycline 48 hr prior to harvest. ATR$^{flox/-}$cells, which allow for cre-lox mediated removal of ATR, were obtained from Dr. Stephen J. Elledge (Baylor College, Houston, TX) (Cortez et al., 2001) and maintained in MEMα medium + 10% FBS + 200 μg/ml G418. Expression of cre recombinase in these cells was accomplished through infection with adenovirus AdCre1, which was obtained from Dr. Frank Graham (McMaster University, Hamilton, Ontario, Canada) (Anton and Graham, 1995).

Fragile sites were induced by aphidicolin using the concentrations and times indicated in the figures and tables. Cells were harvested for chromosome preparations using standard conditions of a 45 min colcemid treatment (50 ng/ml) followed by an 18 min incubation in 0.075 M KCl at 37°C and multiple changes of Carnoy's fixative (3:1 methanol:acetic acid). Cells were dropped onto slides and slides were baked overnight at 60°C before Giemsa banding or FISH protocols.

YAC and BAC probes crossing or within fragile site regions were used for FISH analysis. YAC 850A6 was used for FRA3B, BAC264L1 (RP-11) for FRA16D, and BAC 36B6 (RP-11) for FRA7H. Probes were labeled with biotin-14-dATP or digoxigenin-11-dUTP. Probe hybridization and immunologic detection were performed according to standard protocols (Wilke et al., 1996). Biotin-labeled probes were detected with avidin-FITC followed by anti-avidin-FITC, and digoxigenin-labeled probes were detected with rhodamine-conjugated antibody followed by Texas Red anti-goat. For both single-color and two-color FISH experiments, chromosomes were counterstained with DAPI (4′, 6-diamidino-2-phenylindole). A Zeiss Axioscope epifluorescence microscope and digital image acquisition were used for analysis.

siRNA

Two siRNA sequences directed against ATR were constructed and ordered from Dharmacon Research, Inc. (Lafayette, CO). Both siRNAs are duplexes of 21 nt RNAs with a 2 nt 3′ overhang; the siRNA-1 target sequence is AAGCCAAGACAAATTCTGTGT and the siRNA-2 target sequence is AACCTCCGTGATGTTGCTTGA. Selection of sequence for these siRNAs was based on Elbashir et al. (2001) and Harborth et al. (2001) and on guidelines posted on the Tuschl lab website, http:/a/www.mpibpc.gwdg.de/abteilung/en/1001/105/sirna.html. Matching single-stranded sense control RNA sequences were constructed for each of the two siRNAs. Oligofectamine (Invitrogen, Carlsbad, CA) was used for transfection of these RNAs into HCT116 and HeLa cells, according to protocols provided by Dharmacon. Fragile site expression was induced by the addition of the indicated concentrations of aphidicolin for 24 hr two days posttransfection.

Western Blots

5% stacking, 8% resolving polyacrylamide gels were used for protein separation for detection of ATR. 4% stacking, 6% resolving polyacrylamide gels were used for protein separation for detection of ATM. 50 μg whole-cell lysate was loaded for all lanes. Gels were transferred to PVDF membrane, and antibody hybridization and chemiluminescence detection were performed according to standard protocols. ATR was detected with a rabbit polyclonal antibody

generated against amino acids 1–20 of ATR. FLAG-tagged ATR was detected with an anti-FLAG M5 antibody from Sigma (St. Louis, MO). ATM was detected with a rabbit polyclonal antibody from Novus Biologicals (Littleton, CO). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Amersham (Piscataway, NJ).

Statistical Analysis

For comparisons of average overall gaps and breaks and comparisons the frequency of gaps and breaks at specific fragile sites (i.e., fragile site expression), the Student's t test was employed.

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