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SMC1 involvement in fragile site expression

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Common fragile sites have been involved in neoplastic transformation, although their molecular basis is still poorly understood. Here, we demonstrate that inhibition of the *SMC1* by RNAi is sufficient to induce fragile site expression. By investigating normal, ATM- and ATR-deficient cell lines, we provide evidence that the contribution of *SMC1* in preventing the collapse of stalled replication fork is an Atr-dependent pathway. Using a fluorescent antibody specific for γ-H2AX, we show that very rare discrete nuclear foci appear 1 and 2 h after exposure to aphidicolin and/or RNAi-*SMC1*, but became more numerous and distinct after longer treatment times. In this context, fragile sites might be viewed as an *in vitro* phenomenon originating from double-strand breaks formed because of a stalled DNA replication that lasted too long to be managed by physiological rescue acting through the Atr/Smc1 axis. We propose that *in vivo*, following an extreme replication block, rare cells could escape checkpoint mechanisms and enter mitosis with a defect in genome assembly, eventually leading to neoplastic transformation.

INTRODUCTION

Common fragile sites have drawn considerable attention for their involvement in human chromosome remodeling and cell transformation. Indeed, they are hot spots for intra- and inter-chromosomal recombination (1,2) and they are preferential sites for both viral and plasmid integration (3,4). All these chromosome rearrangements require DNA double-strand breaks (DSBs), suggesting that fragile sites are associated with DSBs. The involvement of fragile sites in tumorigenesis is supported by the observations that some of them map to cancer breakpoints (5,6) and are general targets of many mutagens and carcinogens (7,8). Deletion in the FRA3B, the most common fragile sites among humans, are observed in several different solid tumors, whereas FRA16D shows loss of heterozygosity and deletions in breast and ovarian cancers (reviewed in 9). Through functional studies, both *FHIT* and *WWOX*, at FRA3B and FRA16D, respectively, have been identified as tumor suppressor genes (10,11).

The majority of common fragile sites are induced by aphidicolin, an inhibitor of DNA polymerase α, ε and δ (12–14) and their frequency increase after caffeine or campthotecin treatment, the latter being able to also induce new fragile sites (15). Five fragile sites, namely, FRA3B, FRA6E, FRA7G, FRA7H and FRA16D, have been cloned and characterized and they span from hundreds of kilobases to 4 Mb (9,16).

Understanding the mechanisms of common fragile site expression is pivotal for clarifying their role in both chromosome instability and tumorigenesis. Fragile sites are conserved among primates (17) and a majority of the breakpoints observed in the evolution of primates chromosomes fall at or near known fragile sites (18). These observations suggest that their chromatin is conserved in structure and function. One of the hypotheses suggests that rearrangements at fragile sites arise as a result of replication failure at sequences unusually sensitive to interference during DNA synthesis (19–21), which escape the *ATR* replication checkpoints (22,23).

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The identification of the structural maintenance of chromosome (SMC) family of ATPases has provided an important molecular clue to our understanding of a higher order of chromosome dynamics. In eukaryotes, the Smc protein containing complexes condensin and cohesin regulate chromosome condensation and sister chromatid cohesion, respectively. Condensin is a five subunit complex composed of two Smc subunits (Smc2 and Smc4) and three non-Smc subunits (CAP-D2, -G and -H). The cohesin complex consists of Smc1, Smc3 and two non-Smc subunits (Scc1 and Scc3). The Smc1–Smc3 heterodimer has also been found to promote repair of gaps and deletions (24,25) and cohesin complex is required for postreplicative DSBs repair in *Saccharomyces cerevisiae* (26). Finally, Smc1 is a component of the DNA damage response and participates in the cellular response to DNA damage through its phosphorylation on Ser 966 and 957 (27,28).

Recently, we showed that the antisense oligonucleotide inhibition of *SMC1* led to chromosomal aberrations in normal human fibroblasts (29). Because of functional links of cohesin subunits, Smc1 and Smc3, to chromosome dynamics and DNA repair, it was particularly attractive to investigate the possibility that Smc1 and Smc3 are involved in fragile site expression. Here, we show that inhibition of *SMC1* and, to a lesser extent, *SMC3* by RNA interference (RNAi) or antisense oligonucleotide is sufficient to induce chromosomal aberrations in normal human fibroblasts, most of which are located at fragile site chromosome bands. Aphidicolin plus *SMC1* inhibition treatment increased aberrations frequency with enhanced intensity, due to the synergistic effect of treatments, whereas aphidicolin plus *SMC3* inhibition resulted in an additive effect. To gain further insight into fragile site expression, we analyzed cellular response to aphidicolin treatment. We observed an increase in Smc1 synthesis after aphidicolin treatment, and enhanced DSBs induction, as visualized by γ-H2AX foci formation when treated cells were analyzed by immunohistochemistry. Smc1 is known to be phosphorylated on both Ser957 and 966 by Atm following DNA damage induced by irradiation (27,28). We found that following exposure of cells to aphidicolin, Smc1 is phosphorylated on Ser966 but not on Ser957 by an Atr-dependent, Atm-independent pathway. Our work suggests that Smc1, phosphorylated on Ser966 by Atr, is involved in fragile site expression and provide, for the first time, a role for *SMC1* in chromosomal instability in human cells.

RESULTS

SMC1 and *SMC3* inhibition is sufficient to induce chromosomal aberrations and fragile site expression

Recently, we showed that the *SMC1* antisense oligonucleotide inhibition led to chromosome aberrations (29), suggesting that cohesin subunits may play a role in chromosomal stability. As knock out mouse or cells lacking *SMC1* and *SMC3* are not available, we used two different approaches, RNAi and antisense oligonucleotides, to study *SMC1* and *SMC3* involvement in chromosomal stability. Two different concentrations, 20 and 100 nM, of RNAi against *SMC1* and *SMC3* were used in separate experiments with primary normal human fibroblasts and their effects were evaluated after 24 h of treatment. Transfection levels of FITC-conjugated control RNAi were 90 and 87% for *SMC1* and *SMC3*, respectively (data not shown). Western blots showed that at both doses a specific inhibition of Smc1 (Fig. 1A) and Smc3 (Fig. 1B) syntheses was obtained. No decrease in either Smc1 or Smc3 synthesis was observed with control scrambled RNAi (Figs 1A and B).

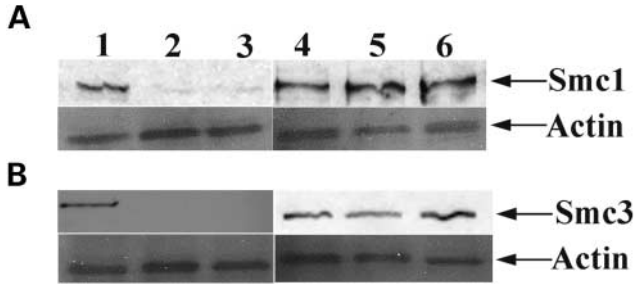

Figure 1. Effects of RNAi treatments in normal human fibroblasts. (**A**) Western blotting showing downregulation of Smc1 protein in normal human fibroblasts treated with 20 nM RNAi-*SMC1* (lane 2), 100 nM RNAi-*SMC1* (lane 3) compared with untreated cells (lane 1) and cells treated with 20 nM scrambled RNAi-*SMC1* (lane 5) or 100 nM scrambled RNAi-*SMC1* (lane 6). Lane 4 represents untreated cell used as control in scrambled RNAi experiments. (**B**) Western blotting showing downregulation of Smc3 protein in normal human fibroblasts treated with 20 nM RNAi-*SMC3* (lane 2), 100 nM RNAi-*SMC3* (lane 3) compared with untreated cells (lane 1) and cells treated with 20 nM scrambled RNAi-*SMC3* (lane 5) and 100 nM scrambled RNAi-*SMC3* (lane 6). Lane 4 represents untreated cell used as control for scrambled RNAi treatment.

The inhibition of *SMC1* and *SMC3* induced 0.26 ± 0.5 and 0.10 ± 0.3 aberrations per cell, respectively. Metaphase chromosomes were G-banded by trypsin to verify co-location of the detected chromosomal aberrations with fragile sites. Using this analysis, we found that 18 out of 26 aberrations (69%) were mapped to published fragile sites after *SMC1* inhibition. The number of observed aberrations occurring at fragile sites is higher than expected (18 versus 8.7, $P < 0.01$) suggesting that, after *SMC1* inhibition, aberrations fall preferentially at known fragile sites. In addition, the most involved bands were 3p14 (Fig. 3A),16q23 and Xp22 (Table 1), which contain the most common fragile sites, in agreement with our conclusion that inhibition of *SMC1* and *SMC3* induce non-random breakages at these sites. This conclusion was further corroborated by a stringent statistical analysis (30), showing that the number of aberrations in these three bands known to contain fragile sites is higher than expected ($P < 0.001$). Two aberrations in untreated cells and two and three aberrations in control RNAi-treated cells were observed. None of them was located on fragile sites.

In a second approach, we inactivated *SMC1* and *SMC3* by antisense oligonucleotides. Through this technique a downregulation of proteins synthesis was obtained, as shown by western blotting with specific antibodies (Fig. 2). As a result of this inhibition, we found 0.18 and 0.07 chromosomal aberrations per cell after *SMC1* and *SMC3* inhibition, respectively. G-banded chromosome analysis showed that 3p14 and 16q23 chromosomal bands were again the most involved in chromosome aberrations (data not shown). Untreated and scrambled oligonucleotide antisense-treated cells showed two

Table 1. Location of chromosomal aberrations after RNAi-*SMC1* and -*SMC3* inhibition

Treatment	Chromosomal band	Number of aberrations	*P*
SMC1	1p36[a]	1	
	1p13	1	
	1q41	1	
	3p14[a]	4	<0.001
	4q32	1	
	5p15	1	
	5q31[a]	1	
	6q26[a]	2	
	7p22[a]	1	
	7q31[a]	2	
	10q24	1	
	13q12	1	
	14q24[a]	1	
	16q23[a]	3	0.017
	17q21	1	
	18q23	1	
	Xp22.3[a]	3	0.017
SMC3	2p25	1	
	3p14[a]	3	<0.001
	7q31[a]	2	
	8q23	1	
	11q22	1	
	15q24	1	
	19p12	1	

[a]Chromosomal band with published fragile sites.

aberrations, not located at fragile sites. These results suggest that the inhibition of cohesin subunits, *SMC1* and *SMC3*, is sufficient to induce the cytogenetic expression of fragile sites.

Combined treatment (aphidicolin plus RNAi) led to a synergistic effect on fragile site expression after *SMC1* inhibition and an additive effect following *SMC3* inhibition

We next studied the effect of a combined treatment, aphidicolin plus RNAi against *SMC1* or *SMC3* on fragile site expression. As expected, aphidicolin alone induced fragile site expression, with a mean of 0.74 ± 0.84 aberrations per cell and 58 out of the 74 aberrations (78%) were mapped to known fragile sites. Aphidicolin plus RNAi-*SMC1*-mediated inhibition showed a synergistic effect of the two treatments, because the mean number of aberrations per cell increased to 1.79 ± 1.39; 159 out of 179 aberrations (89%) were mapped to known fragile sites. This increase occurs principally in the form of gaps, whereas the number of breaks is not affected; an increase in both the number of aberrations per cell and the number of aberrant cells (from 55 to 81%) was seen (Supplementary Material, Table 1). On the other hand, aphidicolin plus RNAi-*SMC3*-mediated inhibition results in only an additive effect. Moreover, the combined treatment induced 0.91 ± 0.8 aberrations per cell, a value very similar to the sum of the individual treatments.

A detailed analysis shows that, again, the aberration events occurring after the combined aphidicolin plus RNAi-*SMC1* treatment focused to a limited number of bands, where known fragile sites map because 49% of these aberrations occurred in 3p14, Xp22.3, 16q23 and 7q31 (Table 2).

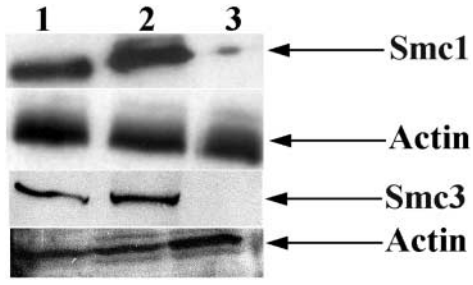

Figure 2. Effect of antisense oligonucleotide treatment on *SMC1* and *SMC3* expression. Western blotting with Smc1- and Smc3- specific antibodies performed on untreated cells (lane 1), control scrambled oligonucleotide-treated cells (lane 2) and *SMC1*- or *SMC3*- antisense-treated cells (lane 3).

No aberration mapped at these sites in untreated and scrambled RNAi-treated cells. This observation indicates that known fragile sites are the major targets of *SMC1* inhibition. To confirm that aberrations do involve fragile sites, a fluorescent YAC probe for the most common fragile site, FRA3B, was hybridized to metaphase spreads (Fig. 3B and C). With this probe, the signal bridged the aberration site, confirming that the additional Smc1-dependent aberrations map to the canonical common 3p14 fragile site. Taken together, these results show that the additional abnormalities caused by the combined treatment target bona fide fragile sites.

Aphidicolin treatment leads to an increase in Smc1 synthesis, its phosphorylation on Ser966 through Atr kinase action and DSBs formation

The functional consequences of aphidicolin treatment for cellular responses were explored next. Normal human primary fibroblasts were treated with aphidicolin and protein extracts were collected 6, 16 and 26 h after the beginning of the treatment and analyzed by western blotting. We found that a strong increase of Smc1 synthesis occurs during the aphidicolin treatment time course analysis. Only faint bands were seen when protein extracts were analyzed with an Smc3 antibody (Fig. 4). To investigate whether this cellular response to aphidicolin is a general phenomenon, we repeated the above analysis in both HeLa and an A-T cell line. We found that after 6 h of treatment, Smc1 is increased when compared with untreated control cells (Fig. 4), and that this increase is maintained at 16 and 26 h, although Smc1 synthesis at these time points is also increased in untreated cells, although to a lesser extent than in aphidicolin-treated cells. It is likely that this increase occurs in all cell types in relation to cell proliferation. Exposure of cells to ionizing irradiation and caffeine had no effect on Smc1 synthesis while a weak increase was found in response to MMC when compared with control cells (data not shown). These findings suggest that the level of aphidicolin-induced Smc1 synthesis increases specifically after DNA replication inhibition and DNA polymerase stalling.

Recently, it has been shown that *SMC1* is a component of the DNA damage response pathway and that, following ionizing radiation, phosphorylation on Ser957 and Ser966 occurs (27,28). To investigate whether Smc1 is phosphorylated in response to aphidicolin treatment, we examined the

Table 2. Number of chromosomal aberrations observed at the most frequent fragile sites

Treatment	Fragile sites			
	3p14	7q31	16q23	Xp22.3
Control	0	0	0	0
Scrambled RNAi-*SMC1*	0	0	0	0
RNAi-*SMC1*	4	2	3	3
Aphidicolin	12	6	6	5
Aphidicolin + RNAi-*SMC1*	38	14	18	19

phosphorylation of these two residues by rabbit polyclonal antibodies that specifically recognize Smc1 when either of these serines are phosphorylated. An increase in the amount of Ser966 phosphorylated Smc1 was evident in all cell lines, although with different kinetics. On the contrary, no difference between control and treated cells was found on Ser957 (Fig. 4). Recent results have demonstrated that Smc1 protein was phosphorylated at both Ser966 and Ser957 in an Atm-dependent manner following irradiation (27,28). However, the observation that Smc1 can become phosphorylated on Ser966 in A-T cell lines suggests that kinases other than Atm are responsible for phosphorylation after aphidicolin treatment. On the basis of the occurrence of rearrangements at fragile sites and the possible role of replication fork stalling at these sites, we hypothesized that Atr might play a role in Smc1 phosphorylation. To investigate this possibility, cell lines were treated with aphidicolin for 16 h and their protein extracts were co-immunoprecipitated with the Smc1 antibody, blotted and analyzed with an Atr antibody. We repeated this analysis by Atr co-immnunoprecipation followed by Smc1 western blotting, providing further evidence of this association (Fig. 5A, lanes 1–4). On the contrary, no band was visible in co-immunoprecipitation experiments performed in untreated cells (Fig. 5A, lanes 5–8). An irrelevant antibody did not co-immnunoprecipitate Atr (data not shown). In addition, no increase in SMC1 synthesis occurred in aphidicolin-treated DK0064, an ATR-deficient cell line (Fig. 5B). These results suggest that aphidicolin induces both the increase of Smc1 synthesis and its Atr-dependent phoshorylation at Ser966, strengthening the idea that Smc1 is involved in fragile sites stability.

Fragile sites are preferential sites of both chromosome recombination and viral integration. All of these processes are preceded by DSBs, suggesting that fragile sites are in some way associated with DSBs. As the exact nature of fragile sites is unknown, we also investigated whether they involve DSB formation by examining γ-H2AX foci formation in primary human fibroblasts as cellular response to aphidicolin treatment. Using a fluorescent antibody specific for γ-H2AX, discrete nuclear foci were absent or very rare after 1 and 2 h (data not shown) but could be visualized 6 h after aphidicolin treatment and became more numerous and distinct after longer treatment times (Figs. 6A–C). The mean values were 6.2 ± 3.56, 29.26 ± 8.68 and 34.31 ± 9.43 foci per cell at 6, 16 and 26 h, respectively (Fig. 6E). Statistical analysis performed by Student's *t*-test showed significant differences between treatment at 6 h in comparison with treatments at 16 and 26 h ($P < 0.00001$). On the contrary,

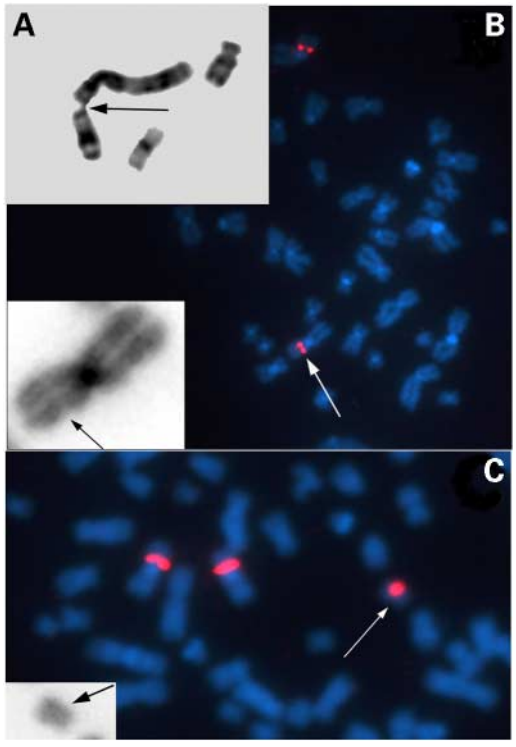

Figure 3. Chromosomal aberrations in the FRA3B region. (**A**) Partial G-banded metaphase showing a chromosomal aberration at 3p14.2 (arrow). (**B** and **C**) FISH experiments with the 850A6 YAC clone showing that the probe maps to the site of a gap (B, white arrow; compare with insert, black arrow) or to a chromosomal fragment originating from breakage at 3p14 band (C, white arrow; compare with insert, black arrow).

clear, discrete foci were present after a few minutes in irradiated cells (data not shown). We next investigated the effect of aphidicolin plus RNAi-*SMC1* combined treatment. The mean was 39.6 ± 8.52 foci per cell, significantly different from individual treatments (Fig. 6D–E). Sporadic foci were visualized in control RNAi-treated and untreated cells (data not shown).

These observations suggest that aphidicolin treatment induces DSBs only after a long latency and that the kinetics of their repair is quite different from that occurring after DSBs-irradiation induced damage. In fact more time is required to activate enzymatic complexes responsible for processing and resolving DNA damage, probably because DSB accumulates much more slowly in the case of replication fork stalling than after irradiation.

DISCUSSION

Understanding the molecular mechanisms of DNA damage repair provides insights into processes involved in both cell survival and transformation. Smc1 and Smc3 are components of the cohesin complex, which is necessary not only for sister chromatid cohesion but also for DNA repair and is required for post-replicative DSBs in *S. cerevisiae* (26). This evidence suggests that Smc1 and Smc3 play a role in chromatin and DNA dynamics and make them particularly interesting

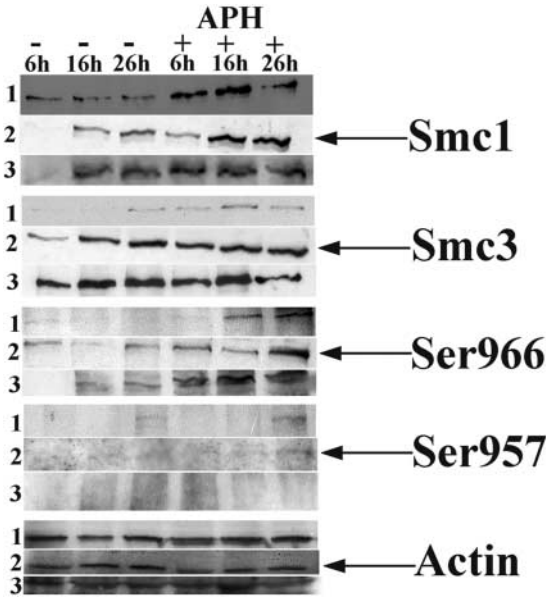

Figure 4. Cellular response to aphidicolin treatment in different cell lines. Time course (6, 16 and 26 h) of Smc1, Smc3, Ser966 and Ser957 in normal human fibroblasts (1), HeLa (2) and 252RM (3) with (+) and without (−) aphidicolin treatment.

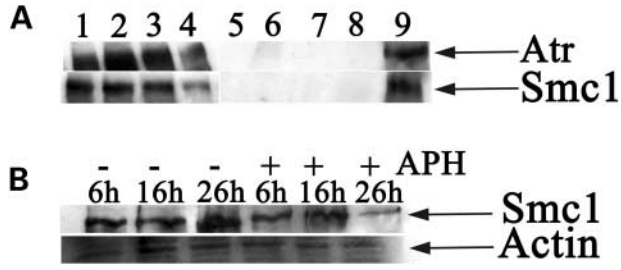

Figure 5. Smc1 associates with Atr in response to aphidicolin treatment. (**A**) Co-immunoprecipitation with Smc1 and western blotting with Atr antibody and vice versa performed in cell lines (normal human fibroblasts, lane 1; Hela, lane 2; ATGS, lane 3 and 252RM, lane 4) treated (+) with 16 h of aphidicolin and untreated cell lines (normal human fibroblasts, lane 5; Hela, lane 6; ATGS, lane 7 and 252RM, lane 8) Lane 9 represents positive control. (**B**) Time course (6, 16 and 26 h) of Smc1, in DK0064, an ATR-deficient cell line, with (+) and without (−) aphidicolin treatment.

players in chromosome stability, deserving further investigation. In this paper, we show the involvement of *SMC1* in the pathway leading to fragile site expression through an *ATR*-dependent mechanism. We used two different approaches, RNAi and antisense oligonucleotides, to investigate the involvement of Smc1 and Smc3 cohesin-subunits in fragile site expression. Our results show that the inhibition of Smc1 alone is sufficient to induce chromosome breaks and that most of them co-localize with fragile sites. The aphidicolin plus RNAi-*SMC1* combined treatment led to a synergistic effect with an enhancement of fragile site expression. Inhibition of *SMC3* led to a lower frequency of aberrations and the combined treatment (aphidicolin plus RNAi-*SMC3*) caused only an additive effect. The molecular characterization of processes involved in cellular response to aphidicolin showed that Smc1 synthesis increases during a time course analysis. This effect is extremely specific. In fact, although Smc1 phosphorylation occurs after irradiation, no Smc1 increase is seen following irradiation, confirming previous results (31), or when cells are treated with caffeine or MMC. Furthermore, we found that Smc1 is phosphorylated on Ser966 following aphidicolin treatment while no effect was found on Ser957. Therefore, the observed increase in fragile site expression could be linked to Smc1 role in the DNA protection. Smc1 is a downstream effector of Atm, acting through phosphorylation on Ser957 and Ser966, in the activation of the irradiation-induced S phase checkpoint (28). In addition to irradiation, data presented here show that Smc1 is required for chromosome stability also after aphidicolin treatment. It might play this role through two different pathways. First, an increase in Smc1 synthesis can stabilize sister chromatid cohesion, improving the recruitment of DNA repair enzymes. Second, its phosphorylation on Ser966 may be required for activation of the S phase checkpoint as already shown to occur in irradiated cells (28). Under our experimental conditions, as *SMC1* inhibition leads to S phase checkpoint inactivation, the high level of fragile site expression seems to be the consequence of cells arriving at mitosis with a number of regions still replicating. It is worthy to note that in MEF the lack of Smc1 serines phosphorylation lead both to a defective S phase checkpoint and to a high level of spontaneous chromosomal aberrations and their level increase following *in vitro* treatment (32).

The phosphorylation of Smc1 in A-T cells suggests that kinases other than Atm are responsible for phosphorylation following aphidicolin treatment. Recently, it has been shown that Atr plays an essential role in preventing fragile site expression (22), although its molecular targets have yet to be identified. To delineate the possible interrelationship between Smc1 and Atr, we immunoprecipitated Smc1 from normal fibroblasts, HeLa and A-T cell lines nuclear extracts and detected Atr by western blotting (Fig. 5). By this approach, we showed that Atr interacts with Smc1. These findings make Atr the first candidate for Smc1 phosphorylation in the cellular response to aphidicolin. The lack of requirement for Atm following aphidicolin treatment is not surprising as Atm seems to be specifically involved in response to other kinds of cellular stresses, such as irradiation (27,28). This is in agreement with recent studies in yeast, which suggest that Atr is involved in stabilizing stalled DNA replication forks. As a matter of fact, Mec1, the Atr ortholog in *S. cerevisiae*, plays a role in checkpoint signaling leading to the stabilization of stalled replication forks and prevention of the generation of DSBs (33,34). No increase in SMC1 synthesis occurred in aphidicolin-treated DK0064, an *ATR*-deficient cell line, further suggesting a role of SMC1 in chromosome stability. This is supported by the observation that patients affected by Seckel syndrome, caused by mutation in *ATR* gene, showed increased fragile sites expression (35).

H2AX histone is phosphorylated on Ser139 (γ-H2AX) in response to DSBs formation and is a reliable indicator of the occurrence of DSBs (36,37). Using a fluorescent antibody specific for γ-H2AX, discrete nuclear foci can be visualized after protracted aphidicolin treatment, which become more

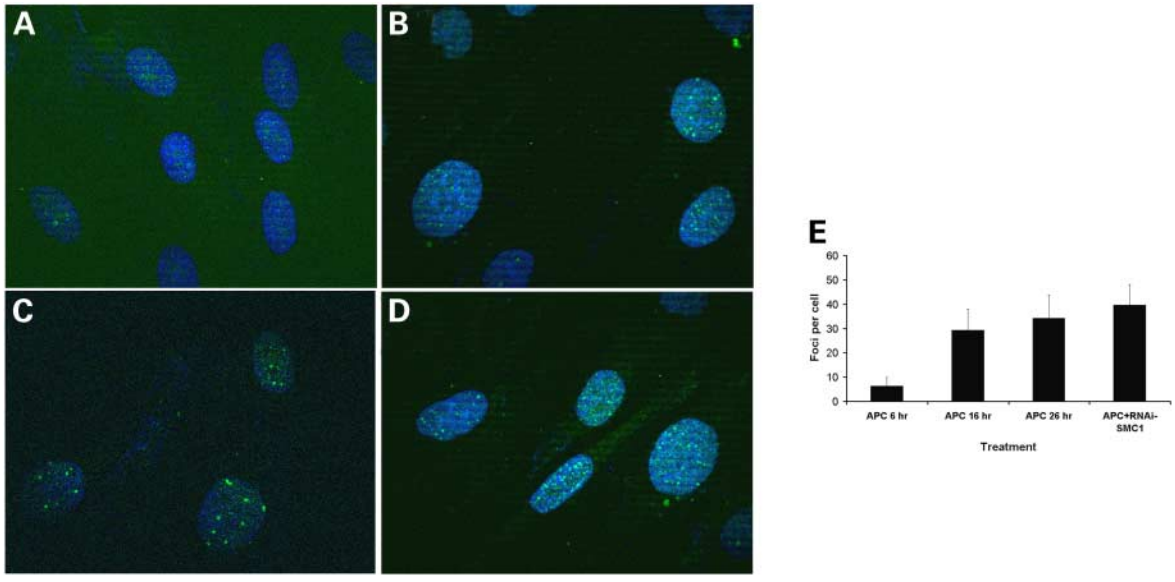

Figure 6. Time course of DSBs induction in normal human fibroblasts. (**A**) γ-H2AX foci in cells treated with aphidicolin for 6 h, (**B**) 16 h, (**C**) 26 h or (**D**) with combined treatment (aphidicolin plus RNAi-*SMC1*) and (**E**) mean number of foci for various times and type of treatments.

numerous and distinct after longer exposure and after combined treatments. This suggests that fragile site expression is directly associated with DSBs. As the Atm checkpoint is involved in DSBs repair and Atm deficiency has no effect on fragile site expression, a recent hypothetical model excluded that DSBs were the primary cause of fragile site expression (22). However, several experimental data support an association between ATR, fragile sites and DSBs. An increase in H2AX phosphorylation occurs in wild-type cells upon aphidicolin treatment (38) and a robust increase was observed in $ATR^{\Delta/-}$ cells after treatment with aphidicolin, suggesting that ATR is required to prevent the formation of DSBs (39). However, we cannot exclude that DSBs are secondary events occurring during fragile site formation. We suggest that the checkpoint responsible for cell cycle blockade is monitored by at least two different pathways. Irradiation treatment promotes the phosphorylation of Smc1 on Ser957 and Ser966 by Atm while aphidicolin treatment leads to phosphorylation on Ser966 through the kinase activity of Atr. As a result, the cell cycle is blocked for DNA damage repair in both pathways. Therefore, our data confirm that different biochemical pathways are used that specifically trigger a response to different events that challenge genome integrity.

Our data support a model in which *SMC1* is required to prevent fragile site expression (Fig. 7). In our model, cohesin contributes to DNA replication by holding together the replicating strands. Stalled replication forks challenging the chromosome integrity occur physiologically and this activates Atr, which in turn phosphorylates Smc1. This modification allows the DNA strand to remain unchanged until the replication block is relieved. If this occurs, replication resumes. In the presence of aphidicolin, replication forks stall in regions that normally replicate late and, as the block is not eliminated, permanent damage ensues and the fragile site is expressed, manifesting itself in the late replicating regions, since they have not yet replicated. On the contrary, it is conceivable that during normal unperturbed DNA replication, stalled forks occur for unknown reasons, but do not last long; in this setting Smc1 phosphorylation gives the cell enough time to override the temporary block and resume replication with high fidelity. In this context, fragile sites might therefore be an *in vitro* phenomenon originating from DSBs formed as a result of a stalled DNA replication that lasted too long to be managed by physiological rescue mechanisms acting through the Atr/Smc1 axis. Following an extreme block which probably does not happen *in vivo*, cells exposed to aphidicolin escape checkpoint mechanisms and enter mitosis with a defect in genome assembly, whose nature is not completely clear at the moment, but which is probably related to DSBs that form at same point during stalled replication. Recently, it has been shown in *Xenopus* egg extracts that aphidicolin treatment triggers adaptation, a phenomenon that allows escape from the checkpoint arrest in yeast so that mitosis occurs despite the presence of unrepaired DNA (40). In this regard, although a role for adaptation in mammalian cells is still debatable, fragile sites could represent a manifestation of such phenomenon. This could at least in part explain the cancer proneness, which has been associated to fragile sites.

As most fragile sites are conserved in evolution as well as among cells from different tissues, it is likely that these late replicating regions represent the physiological sequence of replication of a large genome such as the human genome. These regions could include specific structures such as replicons closure regions (15). In this regard it is interesting that all sequenced common fragile sites are AT rich (41) and that Smc1 preferentially binds to AT rich regions (42). It is therefore possible that Smc1 is absolutely required for the correct completion of the last steps of DNA replication. Over recent years, starting from cytogenetic observations,

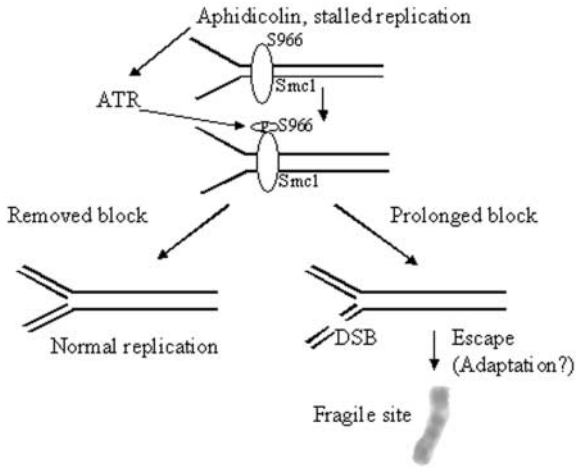

Figure 7. Model for the cytogenetic appearance of fragile sites.

fragile sites studies have provided new insights into cell cycle regulation and this paper highlights new relationships among fragile site expression, DNA repair and genome stability.

MATERIALS AND METHODS

Cell culture

Normal primary human fibroblasts, A-T cell lines from ataxia-teleangectasia patients (252RM, ATGS), HeLa cells and DK0064, an ATR-deficient cell line (kindly provided by P. Jeggo), were grown in Dulbecco's minimal essential medium (Gibco BRL) supplemented with 10% fetal calf serum and antibiotics in a humidified 5% CO_2 atmosphere.

RNAi synthesis and cell treatment

RNAi corresponding to *SMC1* and *SMC3* mRNAs were designed as recommended (43) with two base overhangs. The following gene-specific sequences were used: RNAi-*SMC1* 5′-AUC UCA UGG AUG CCA UCA G dTT-3′, RNAi-*SMC3* 5′-CAG CGG UUG GCU UUA UUG C dTT-3′. Scrambled RNA, *SMC1* 5′-UGA CAA UUG CCU AGC UAC G dTT-3′, *SMC3* 5′-UGA CCG UUG GAU UUC UGC G dTT-3′, was constructed for each of the two RNAi as control. Cells (at 40–60% confluence) were transfected with 100 and 20 nM RNAi by using siPort Amine (Ambion).

Antisense oligonucleotide treatment

The general experimental approach to antisense has been described (29). Briefly, cells were treated with 40 μg/ml of each antisense and control oligonucleotides for 24 h and additional 20 μg/ml for a further 24 h.

Treatment for fragile site expression

Cells were treated with aphidicolin (0.4 μM) for 26 h, alone or in combination with *SMC1* and *SMC3* inhibitor.

Cell treatments

Cells were treated with Mytomicin C (MMC) 5 μM for the last 2 h of cell culture, caffeine 1 mM for 24 h, or irradiated with 2 and 10 Gy by a linear accelerator Philips 75-5 with a 6 MV photon energy source. Protein extracts were collected 6, 12 and 24 h or 1, 12 and 24 h after caffeine and irradiation treatments, respectively.

Immunoprecipitation

Immunoprecipitation with Atr (Santa Cruz Biotechnology) and Smc1 (Bethyl) antibodies was performed according to a published protocol (29). Briefly, a specific antibody was incubated at 4°C with protein extracts for 1 h. Protein A-agarose (Santa Cruz Biotechnology) was added overnight, followed by four washings with buffer lysis.

Western blotting

Western blotting studies used the following antibodies: Smc1, Smc3, Smc1-Ser957p and Smc1-Ser966p, which were purchased from Bethyl; Atr and Atm from Santa Cruz Biotechnology. Samples were boiled in sample buffer and separated by SDS–PAGE (4–12% according to protein weight). The proteins were transferred to nitrocellulose membrane (Amersham) and incubated with the primary antibody (1:250 to 1:15 000 dilution). After removal of the unbound primary antibody, membranes were incubated with secondary antibody–peroxidase conjugate (Sigma) and processed for detection by chemiluminescence (Amersham) and imaged on Biomax film (Kodak). Actin antibody (Santa Cruz Biotechnology) was used as internal controls.

Cytogenetic analysis

Exponentially growing fibroblasts were treated with colcemid (0.05 μg/ml, Gibco BRL), harvested, incubated with KCl 0.075 M, and fixed in methanol–acetic acid 3:1. Chromosome preparations were G-banded according to the trypsin digestion procedure and scored by direct microscopic examination. Metaphase spreads were scored for gaps; break and band locations were assigned according to ISNC recommendation.

Fluorescence *in situ* hybridization (FISH)

The YAC clone 850A6, encompassing FRA3B, was obtained from the YAC Screening Center (Milan). This probe was labeled by nick translation using digoxigenin-11-dUTP (Roche) and was detected by rhodamine-conjugated antibody in standard FISH experiments. Nuclei were counterstained with DAPI. Further details for FISH protocol can be found at http://www.riedlab.nci.nih.gov/.

Visualization of γ-H2AX foci

Visualization of γ-H2AX by immunohistochemistry was performed according to a published protocol (44). Cells were fixed in 2% paraformaldehyde for 15 min, washed three times in PBS, permeabilized for 5 min on ice in 0.2% Triton

X-100 and blocked in PBS with 1% BSA for 30 min at room temperature. The coverslips were incubated with anti-γ-H2AX antibody (Trevigen) for 1 h, washed in PBS, 1% BSA and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) for 1 h at room temperature. Cells were washed in PBS and mounted by using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories). Specimens were examined using a ViCo Video-Confocal Microscope (VCM; Biomedica Mangoni) in which an arc lamp is used as a multi-point excitation source and a CCD camera as an image detector to achieve high spatial resolution and spectral flexibility, even in the presence of UV-excited nuclear stains (45). DAPI and FITC fluorescence were detected in sets of high-resolution optical sections taken at focal steps of 500 nm, using an oil-immersion objective (Leica, Pl Apo 63X, 1.40 NA) thus permitting us to obtain, by maximum projection, extended-focus images representative of an optical thickness of 5–10 μm.

Fragile sites and statistical analysis

For fragile site analysis, the chromosomal bands involved in aberrations after *SMC1* and *SMC3* inhibition were matched to the location of published fragile sites. In addition, a stringent method was used to distinguish fragile sites from random breakage events after *SMC1* and *SMC3* inhibition (30). It is based on the study of the expected random distribution, assumed to be equal to a Poisson distribution, with the expected value of events per band as the mean. It has been suggested as the best statistical test for fragile sites analysis (46).

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