

# Aberrant chromatin resolution in G2/M leads to chromosome instability

Lora Boteva<sup>1</sup>, Ryu-suke Nozawa<sup>1</sup>, Catherine Naughton<sup>1</sup>, Kumiko Samejima<sup>2</sup>, William C Earnshaw<sup>2</sup>, Nick Gilbert<sup>1\*</sup>

<sup>1</sup>MRC Human Genetics Unit, The University of Edinburgh, Crewe Rd South, Edinburgh, EH4 2XU, UK

<sup>2</sup>Wellcome Centre for Cell Biology, University of Edinburgh, Michael Swann Building, Max Born Crescent, Edinburgh, EH9 3BF, UK

\*Correspondence

Nick.Gilbert@ed.ac.uk

+44 131 651 8551

Interphase to mitosis transition is integral to cellular life and requires extensive large-scale chromatin remodelling. Cells coordinate compaction but recurrent cytogenetic lesions appear at common fragile sites (CFSs) in a tissue-specific manner following replication stress, marking regions of genome instability in cancer. Current views suggest lesion formation is dependent on late replication, but instead we report that replication stress causes changes in replication dynamics and origin firing efficiency. We show that CFSs are characterised by impaired compaction, manifested either as cytogenetic abnormalities or as disrupted mitotic figures on cytogenetically normal chromosomes. Chromosome condensation assays reveal that compaction-resistant chromatin lesions persist at CFSs throughout the cell cycle, due to faulty condensin loading at CFS region and a defect in condensin I mediated compaction. Our data supports a model for CFS formation where aberrant replication dynamics lead to faulty condensin I recruitment and mitotic misfolding, mitotic DNA synthesis, and subsequent chromosomal instability.

## Introduction

The folding of chromosomes in preparation for mitosis is the most profound structural change the genome undergoes throughout a cell's lifetime (Antonin and Neumann, 2016). Mitotic condensation is linked to successful cell division and cell cycle progression in a functional and regulatory manner and its failure can be costly, leading to lagging chromosomes and aneuploidy (Gordon et al., 2012; Zhu et al., 2018). Much effort has been made to define the molecular basis of the condensation process and bridge the cytogenetic features of mitotic chromosomes with molecular-level understanding of the chromatin and scaffolding proteins that comprise them. As a result, it is now accepted that a fully folded, cytogenetically normal metaphase chromosome is the product of successful and timely completion of inter-connected processes including replication, sister chromatid separation and chromatin condensation (Gibcus et al., 2018; Ono et al., 2013; Wechsler et al., 2011). Consequently, the cytogenetic integrity of chromosomes is affected by the disruption of these processes; common fragile sites (CFSs) (Durkin and Glover, 2007), regions of the genome known for forming lesions on metaphase chromosomes when cells are challenged with replication stress (Zeman and Cimprich, 2014), are a prominent example. Illustrating the importance of mitotic compaction for genome stability, these sites overlap with recurrent cancer deletions and tumour suppressor genes frequently lost in cancer (Bignell et al., 2010; Le Tallec et al., 2013). Sequencing of cancer genomes reveals that instability of CFSs is an early event in cancer development and therefore, interest in the mechanistic processes is high. Unlike constitutively fragile locations such as Fragile X, CFSs form in a cell type specific manner, leading to suggestions that an epigenetic component plays a role in their fragility (Le Tallec et al., 2011; Letessier et al., 2011). However, the mechanistic basis for their fragility is yet unknown; a number of shared, but not fully deterministic factors have been identified, including late replication timing, presence of long genes and features of the underlying sequence (Fungtammasan et al., 2012; Helmrich et al., 2011; Le Tallec et al., 2014). Their cell-type specificity also limits the potential use of available data and they have to be cytogenetically defined, for each cell type under study, in a laborious and low-resolution process. Consequently, many suggested models of CFS lesion formation are the result of extrapolating from observations at a small set of fragile locations. CFSs have also been identified as regions where active DNA synthesis is apparent on mitotic chromosomes in a process dependent on the Mus81 nuclease. The exact steps involved in triggering synthesis remain unknown but the process may be active at regions of the genome that are not completely condensed in mitosis (Minocherhomji et al., 2015). Although failure of condensation at CFS is an attractive model, and has been recently suggested for HR-deficiency mediated mitotic lesions, it has not been convincingly demonstrated (Chan et al., 2018). The effectors of such condensation failures are likely to be proteins that drive mitotic folding such as the condensin I and II complexes which are crucial for chromosome compaction (Gibcus et al., 2018; Lipp et al., 2007; Samejima et al., 2012). Furthermore, mechanisms established in yeast show that condensin loading is inhibited in slow replicating zones, which resemble CFS, by the ATR homologue Mec1 (Cha and Kleckner, 2002; Hashash et al., 2012).

Given the close relationship between replication and mitotic compaction (Ono et al., 2013), we hypothesised that replication stress can lead to disrupted mitotic folding, particularly at CFS regions. We fully characterised the CFS repertoire in two distinct cell types and find evidence that fragility at these sites is associated with failure of local chromatin to compact for mitosis: this is the case at cytogenetic lesions but also when the sites appear cytogenetically normal, where we demonstrate a previously unknown propensity for smaller scale lesions (100 kb), visible only at the molecular and not the cytogenetic level. Furthermore, we show this molecular instability at CFSs is mechanistically dependent on a failure to recruit condensin and remodel chromatin at the G2/M boundary to facilitate mitotic folding. Our data indicates that the condensin I complex, rather than condensin II, is the effector of disrupted mitotic compaction at CFS. Surprisingly, this is not associated with cell-type specific or aphidicolin-induced delays in replication timing, indicating that a simple model of delayed replication cannot fully explain CFS instability. We propose that non-fragile regions

undergo structural and compositional priming of chromatin in preparation for mitosis, while this process is deficient at CFS locations. Our analysis suggests that CFSs are regions of the genome where chromatin is inefficiently primed for mitotic compaction even in unperturbed conditions, leading to cytogenetic lesions in the extreme conditions of replication stress.

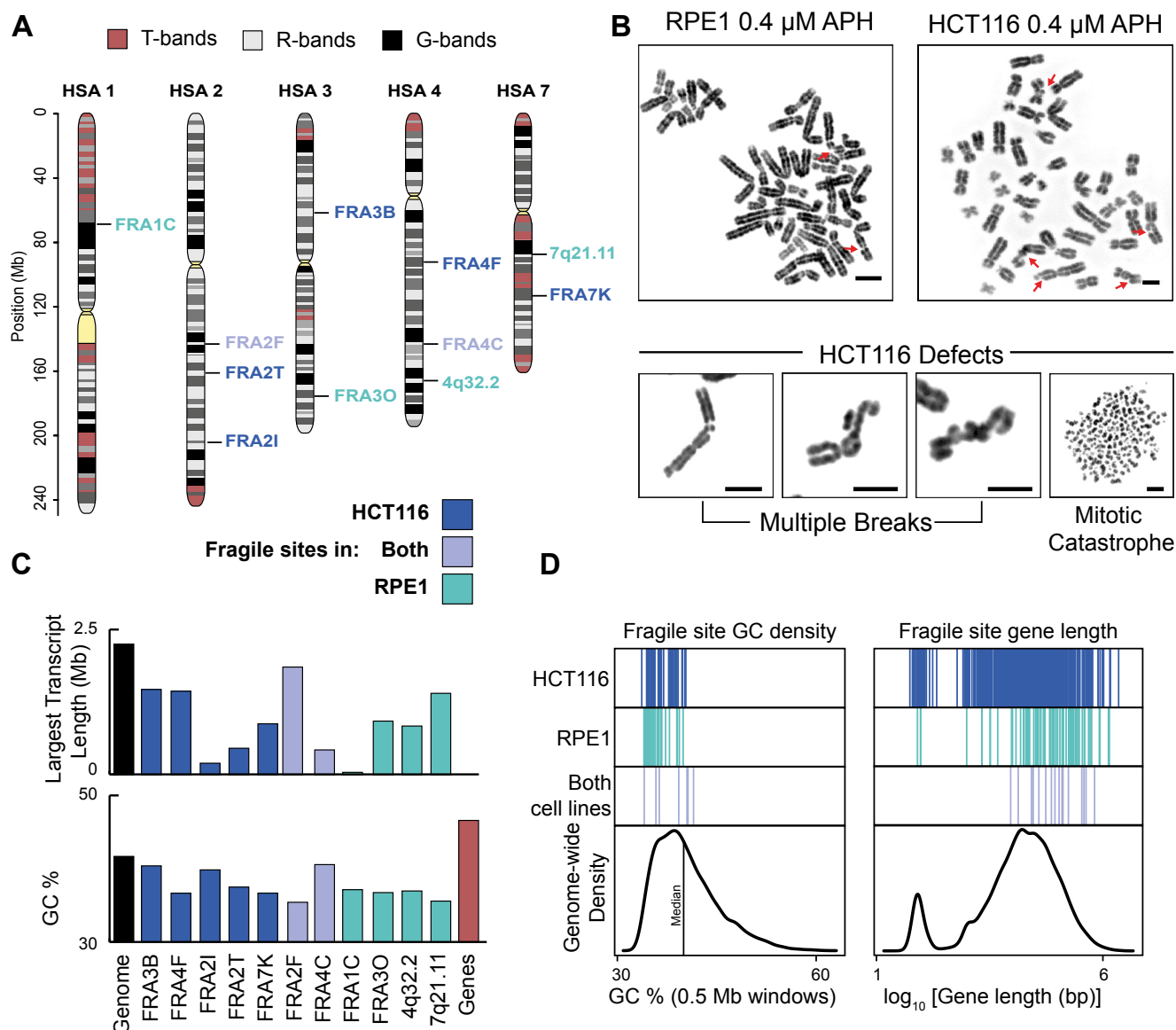
## Results

### Genomic features of the CFS repertoire in RPE1 and HCT116 cells

To analyse the relationship between genomic features and CFS fragility we characterised the CFS repertoire in two epithelial chromosomally normal diploid cell lines (HCT116 and RPE1) after inducing replication stress with aphidicolin (APH). We analysed 372 lesions across 371 metaphases for APH concentrations ranging from 0.1 to 0.6  $\mu$ M, showing that higher APH concentration led to increased rate of breakage and more severe CFS phenotypes but did not cause cell cycle arrest (Supplementary Figure 1 A-C). Cytogenetic lesions were then scored in HCT116 (n= 157 lesions) and RPE1 (n = 62 lesions) cells following 24 hour treatment with 0.4  $\mu$ M APH (Figure 1A-B, Supplementary Figure 1D-E, Supplementary Table 1). Despite both cell lines being of epithelial origin, the CFS repertoire differed significantly: FRA3B was the most fragile site in the HCT116 line (23% of all breaks), followed by locations on chromosome 2 (FRA2I, 2q33.2) and chromosome 4 (FRA4F, 4q22.1). In contrast, the most fragile location in the RPE1 cell line, FRA1C on 1p31.2 was only weakly fragile in HCT116 (18.6% of all breaks in RPE1; 5.8% in HCT116); additionally, two of the most common break sites (approx. 10% of all breaks) in the RPE1 cell type have not been previously identified as CFS locations: 4q32.2 and 7q21.11.

It is unknown whether different cell types form CFS lesions at the same frequency. Here, we found the tumour-derived HCT116 cell line displayed a more severe response to APH treatment than the RPE1 cell line (0.98 breaks/metaphase in RPE1 vs 1.88 breaks/metaphase in HCT116, Welch's t-test  $p < 1 \times 10^{-5}$ ). In addition, more severe cytogenetic abnormalities were observed in the HCT116 cell line, including multiple breaks per chromosome and mitotic catastrophe (Figure 1B). The increase in fragility in the HCT116 cell line can be accounted for by the presence of endogenous replication stress in this tumour-derived background and is consistent with previous work showing that although HCT116 cells do not display structural instability in normal conditions, replication stress and nucleotide deficiency induce chromosomal abnormalities (Burrell et al., 2014).

CFSs are reported to share a number of characteristics: the presence of long genes, AT-rich sequences and late replication timing, although it is not known if any of these characteristics are necessary for fragility (Arlt et al., 2009; Fungtammasan et al., 2012; Wilson et al., 2015). The genomic features of the sites we identified were consistent with these trends, although a genome-wide comparison found that CFSs do not contain either the most GC-poor regions in the genome or the longest genes, confirming previous observations that such features are not fully determinant of fragility (Figure 1C-D). Among the most fragile locations in our study, 9 out of 11 overlapped with genes larger than 0.3 Mb including FRA3B (FHIT), FRA4F (GRID2, CCSER), 4q32.2 (MARCH1, 0.85 Mb; FSTL5, 0.78 Mb) and 7q21.11, which spans MAGI2 (1.4 Mb). While the frequent FRA1C site in the RPE1 cell line does not overlap with any long genes, it is in close proximity with LRRC7 (0.32 Mb) and 2.5 Mb away from NEGR1 (0.89 Mb). COSMIC mutation data showed that, as expected, the majority of the most active CFS sites (8 out of 11) overlapped with recurrent cancer deletion clusters. Exceptions to these generalised trends, such as FRA3B, which had GC content close to the genome average (40 and 41.6 %, respectively), confirm that while certain genomic features may contribute to CFS fragility, none are necessary or uniquely deterministic.



**Figure1: Location and genomic features of CFSs do not determine fragility.**

A. Ideograms showing fragile locations in RPE1 and HCT116 epithelial cells, after aphidicolin treatment with locations cytogenetically scored. CFSs specific to HCT116 cells (blue), RPE1 (green) and both (mauve) are shown.

B. Representative metaphase spreads from RPE1 (left) and HCT116 (right) cell lines, showing CFS fragility (red arrows) following aphidicolin treatment (top); Bottom, extreme chromosomal defects in HCT116 cells (reverse DAPI staining); Scale bar, 10  $\mu$ m.

C. Length of largest transcript (top) and GC content (bottom) at sites fragile in HCT116 (blue), RPE1 (green) or both cell lines (mauve).

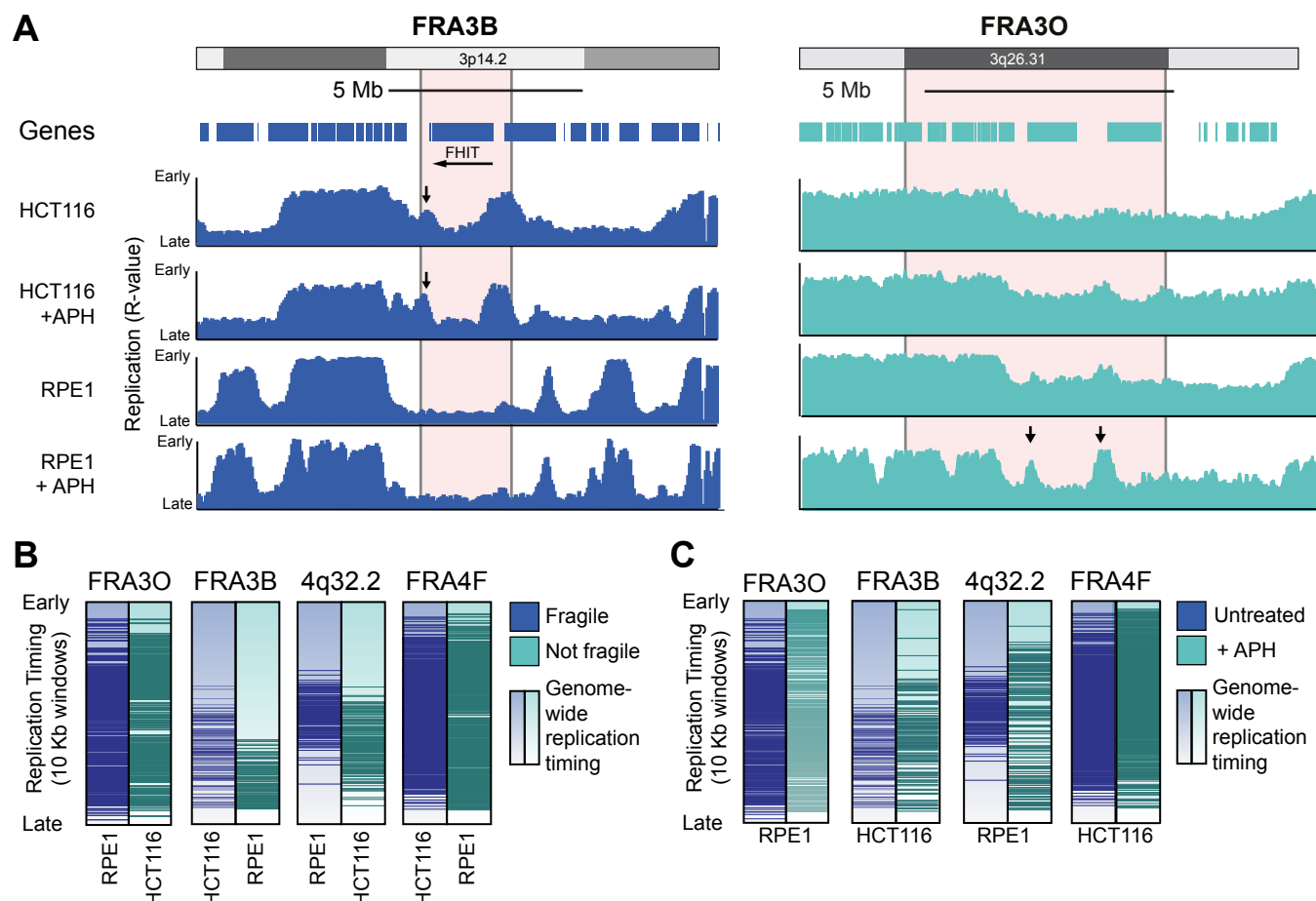
D. Left, genome-wide GC (% in 0.5 Mb windows) density plot with GC density at CFSs in HCT116 (blue), RPE1 (green) or both cell lines (mauve). Right, genome-wide gene length (for NCBI genes) density plot with gene length of genes encompassed within CFSs in HCT116 (blue), RPE1 (green) or both cell lines (mauve);

## Replication timing delays are not a universal determinant of CFS fragility

Replication programmes are cell-type specific, with up to 50% of the genome showing differential replication timing across cell lines (Ryba et al., 2010). Although fragility at CFSs is thought to be the result of late replication, methods for mapping replication timing across the genome, such as Repli-seq, have only been applied to a limited set of CFS loci (Hansen et al., 2010). Investigation of replication dynamics at three CFSs (FRA3B, 3q13.3 and 1p31.1) revealed they show specific, origin-devoid replication profiles in cell types where they are fragile (Le Tallec et al., 2011; Letessier et al., 2011), but this analysis has not been extended to other CFS locations. To examine replication dynamics across multiple CFS regions, we developed and applied an improved approach called Repli-click (see Methods) in the RPE1 and HCT116 cells. Unlike previous studies, which examined replication dynamics in the absence of replication stress, we mapped replication timing both in non-perturbed cells and following aphidicolin treatment. Repli-click mapping in the two cell types indicated that CFSs occur predominantly, but not exclusively, in late-replicating regions of the genome (Supplementary Figure 2A). We compared replication dynamics across multiple CFS regions to determine if they conform to the previously proposed replication timing signature of CFS fragility, consisting of long domains devoid of origins which are replicated from forks travelling from the nearest initiation zones. Some of the sites, such as FRA1C, showed a pattern consistent with this previously proposed origin paucity model (Letessier et al., 2011): the fragile core of this site is devoid of origins and is replicated in late S phase from forks initiating approximately 2 Mb away in early S (Figure 2A). In HCT116 cells, where the site is only weakly fragile, initiation zones (indicated by peaks in the Repli-click profile) appeared to be located closer to the fragile region and forks could be seen to move faster along FRA1C, as indicated by the earlier replication timing of the site in the HCT116 cell line. However, the origin paucity model is inconsistent with replication dynamics at FRA3B, the most fragile location in the HCT116 cell line. FRA3B displayed a characteristic late-replicating profile in the RPE1 cell line, where it was never cytologically unstable; however, in HCT116, FRA3B had an earlier replication timing than in RPE1 and spanned peaks indicative of origin activity (Figure 2A). Replication dynamics at other CFSs in both RPE1 and HCT116 cell lines (FRA4F, FRA2F, FRA3O and 4q32.2) were also inconsistent with the origin paucity hypothesis (Supplementary Figure 2A). Furthermore, analysis of replication timing across CFS regions showed that while these regions were found predominantly in late-replicating regions, there was no clear tendency for CFSs to be later replicating in the cell line in which they were fragile (Figure 2B and Supplementary Figure 2B).

Remarkably, our analysis of population-level replication timing following induction of replication stress indicated that not all CFS regions experience delays in replication timing upon aphidicolin treatment (Figure 2A and C, Supplementary Figure 2A and C). Contrary to previously proposed models of CFS formation, at multiple sites, including FRA3B, FRA4F and FRA3O, peaks in the replication timing profile, indicative of mid-S origins, gained efficiency upon replication stress induction. While surprising, this finding is consistent with a recently described tendency for gains in origin efficiency upon oncogene induced replication stress (Macheret and Halazonetis, 2018). In summary, our extensive analysis of CFS replication behaviour across two cell types strongly indicates that fragility at these regions is not due to insufficient origin activation as previously proposed and CFSs are not the latest regions in the replication timing programme of a given cell type. Our data also suggests for the first time that changes in replication dynamics, rather than a shift towards later replication, underlies replication-stress induced fragility at CFS.





**Figure 2: Replication dynamics do not determine fragility.**

A. Replication timing in the absence (top) or presence (bottom) of aphidicolin (Repli-click; R-values, see methods) at the most fragile locations (pink box) in HCT116 (FRA3B, left panel) and RPE1 (FRA30, right panel) cells; arrows indicate locations of origins within the fragile sites.

B. Ranked replication timing (R-values) for 10 kb windows across the genome (blue or green gradient) in HCT116 and RPE1 cells, superimposed with replication timing at specific CFS regions, where the site is more (blue) or less (green) fragile.

C. Ranked replication timing (R-values) for 10 kb windows across the genome (blue or green gradient) in untreated (blue) or aphidicolin-treated (green) cells, superimposed with replication timing at specific CFS regions; data is presented only for the cell line where the site is fragile (bottom label).

## CFS regions have disrupted chromatin structure on cytogenetically normal chromosomes

Consistent with the variance of genomic features and replication timing profiles observed across CFSs, cytogenetic mapping revealed a range of lesion phenotypes at mitosis: chromatid breaks and gaps, chromosome gaps, concatenations and other complex abnormalities. There was no association between particular locations and the type of abnormality observed: different lesion phenotypes could be observed at each site (Supplementary Figure 1E). To interrogate the chromatin state of fragile regions we pursued a FISH-based strategy by hybridising fluorescently labelled BAC probes to CFS regions identified in our cytogenetic screen. We applied this approach to chromosomes showing CFS lesions as well as cytogenetically normal chromosomes.

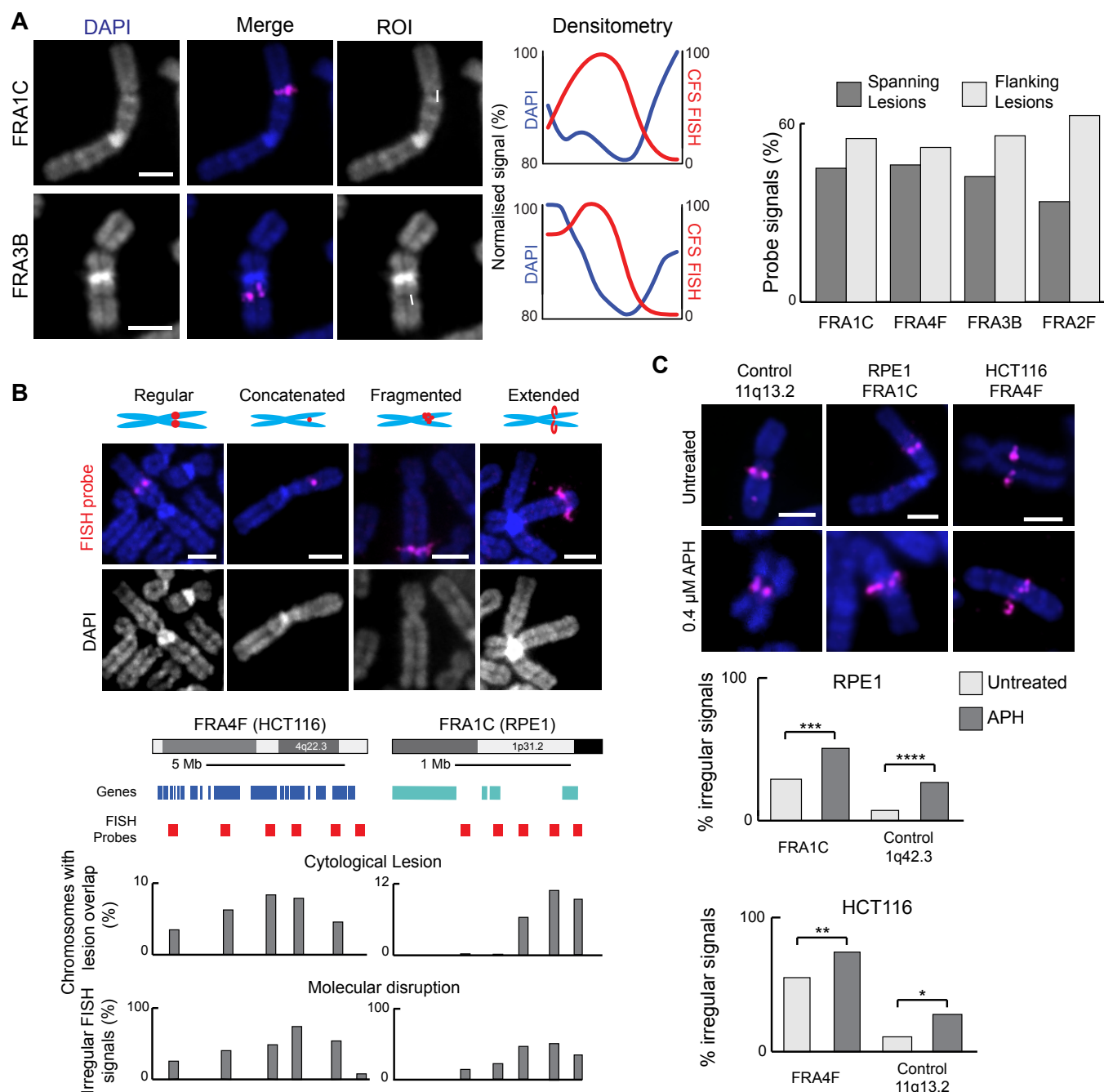
The position of a BAC probe at CFS lesions is indicative of the underlying chromatin state: if lesions coincide with physical breaks along the chromosome arm, the probe signal would be expected to flank the abnormality; in contrast, probe signal spanning a lesion would indicate that DNA is present within the lesion. We found that BAC signals would often span the CFS lesions, with the fluorescence intensity of the probes peaking over the DAPI – faint regions marking the CFS, consistent with DNA being present within the cytogenetically visible breaks (Figure 3A and Supplementary Figure 3A). BAC signals spanning lesions were more common among the less severe cytogenetic lesions; at more extreme lesions, BAC probes tended to partially overlap, rather than span the lesion, which may indicate that the severity of lesion corresponds to the size of the affected genomic region. At FRA1C, FRA3B and FRA4F, the BAC probe would span the cytogenetic lesion 40-50 % of the time, while at FRA2F, the frequency of these events was 35% (Figure 3A).

As previous work on chromosome fragility only explored CFS in the context of cytogenetic lesions, we extended our study to investigate CFS chromatin state on cytogenetically normal mitotic chromosomes. Since cytogenetic mapping provides relatively low-resolution information on the molecular location of a fragile site lesion, we devised a BAC-walking strategy to fine-map two cytogenetically identified CFS regions: FRA1C, which is active in RPE1 cells and FRA4F in the HCT116 cell line (Figure 3B and Supplementary Table 1). We selected probes spanning the two sites (7 BACs spanning 10.8 Mb at FRA4F and 5 BACs spanning 3.2 Mb at FRA1C) and quantified the frequency of chromosomes showing cytogenetic lesions overlapping with the probes (Figure 3B). We observed that rather than always occurring at the same location, breaks appeared to cluster along both CFS sites: a high frequency of breaks was observed at a fragile “core” region, which then tailed off at BACs located upstream or downstream (80% break overlap at the core of the sites reduced to 33% at the flanks). Having defined the molecular boundaries of fragility at the two sites, we proceeded to characterise their chromatin state, using the FISH signal from the BAC probes as a marker for chromatin condensation at a molecular level. Focusing on chromosomes which were exposed to aphidicolin, but did not show a cytogenetic lesion at the corresponding CFS site, we found that probes within CFS regions showed a propensity to form atypical FISH signals (Figure 3B and Supplementary Figure 3C). Rather than twin symmetrical foci characteristic of mitotic chromosomes, CFS spanning probes frequently formed multiple, asymmetric spots, or appeared as a single spot sitting between the two chromatids. However, the most extreme of these atypical signals was a phenotype in which BACs were extending away from the chromosome, spreading far beyond the DAPI-dense area. These signals are reminiscent of abnormal FISH signals formed at telomeres in response to replication stress termed “fragile telomeres” (Sfeir et al., 2009) and are indicative of problems with mitotic condensation and decatenation. To investigate how these atypical signals related to fragility at the sites, we quantified the frequency of chromosomes showing such signals for each of the BAC probes at FRA1C and FRA4F. This fine-mapping of the molecular-level misfolding phenotype (irregular FISH signals) revealed that the frequency of atypical signals showed similar probabilistic distribution along the CFS regions as the cytogenetic breaks. At both FRA1C and FRA4F, atypical signals most frequently appeared at BACs that also showed the highest overlap with cytological breaks. The frequency of

abnormal compaction signals was reduced at BACs that did not frequently overlap with breaks, however the misfolding extended beyond the region most affected by cytogenetic lesions. This analysis reveals for the first time that CFS regions, while highly prone to forming cytogenetic abnormalities, are also characterised by an additional level of instability at the molecular level, suggesting a defect in mitotic condensation.

We next aimed to establish the mechanisms underlying the compaction defects at the sites. Instructively, we observed atypical signals across two fragile sites, which have very different features (Figure 2 and Supplementary Figure 2). As we originally observed the misfolding in chromosomes exposed to replication stress, we wanted to determine if such signals were still present in unperturbed cells, which do not show cytogenetic lesions. We examined the signal phenotypes for two BACs at FRA1C and at FRA4F. Surprisingly, molecular misfolding was still occasionally present on chromosomes even in the absence of replication stress, although, as expected, the frequency of atypical signals was significantly higher in the presence of replication stress (Figure 3C). This was particularly pronounced in HCT116 cells, where 60% of chromosomes carried disruptions in the FRA4F region in the absence of replication stress. The high frequency of these events excludes the possibility that fragility is triggered by rare replication events, which would not be visible in population level Repli-click data. In order to determine if mitotic condensation defects were specific to CFS regions, we also examined the signal phenotypes for two control regions, located on HSA 1q42.3 and 11q13.2 that were never seen to overlap with cytogenetic lesions. Notably, the frequency of atypical signals also increased at these non-fragile loci in the presence of replication stress, but remained much lower compared to CFS regions (7 to 27% in RPE1, 11% to 28 % in HCT116). These results suggest that replication stress can lead to an increase in the frequency of mitotic condensation defects at non-fragile locations.





**Figure 3: CFS regions have disrupted chromatin structures on cytogenetically normal chromosomes.**

A. Left, representative images showing FISH probes spanning lesions (red) at FRA1C (RPE1 cells) and FRA3B (HCT116 cells), hybridised to metaphase spreads from cells treated with aphidicolin, counterstained with DAPI. Scale bar, 5  $\mu$ m. Intensity profiles of the regions spanning cytogenetic lesions (marked with white lines) at FRA1C (top) and FRA3B (bottom); Right, quantification of FISH signal relative to cytogenetic lesions ( $n = 10$ , each) in RPE1 (FRA1C and FRA2F) and HCT116 (FRA4F and FRA3B) cells.

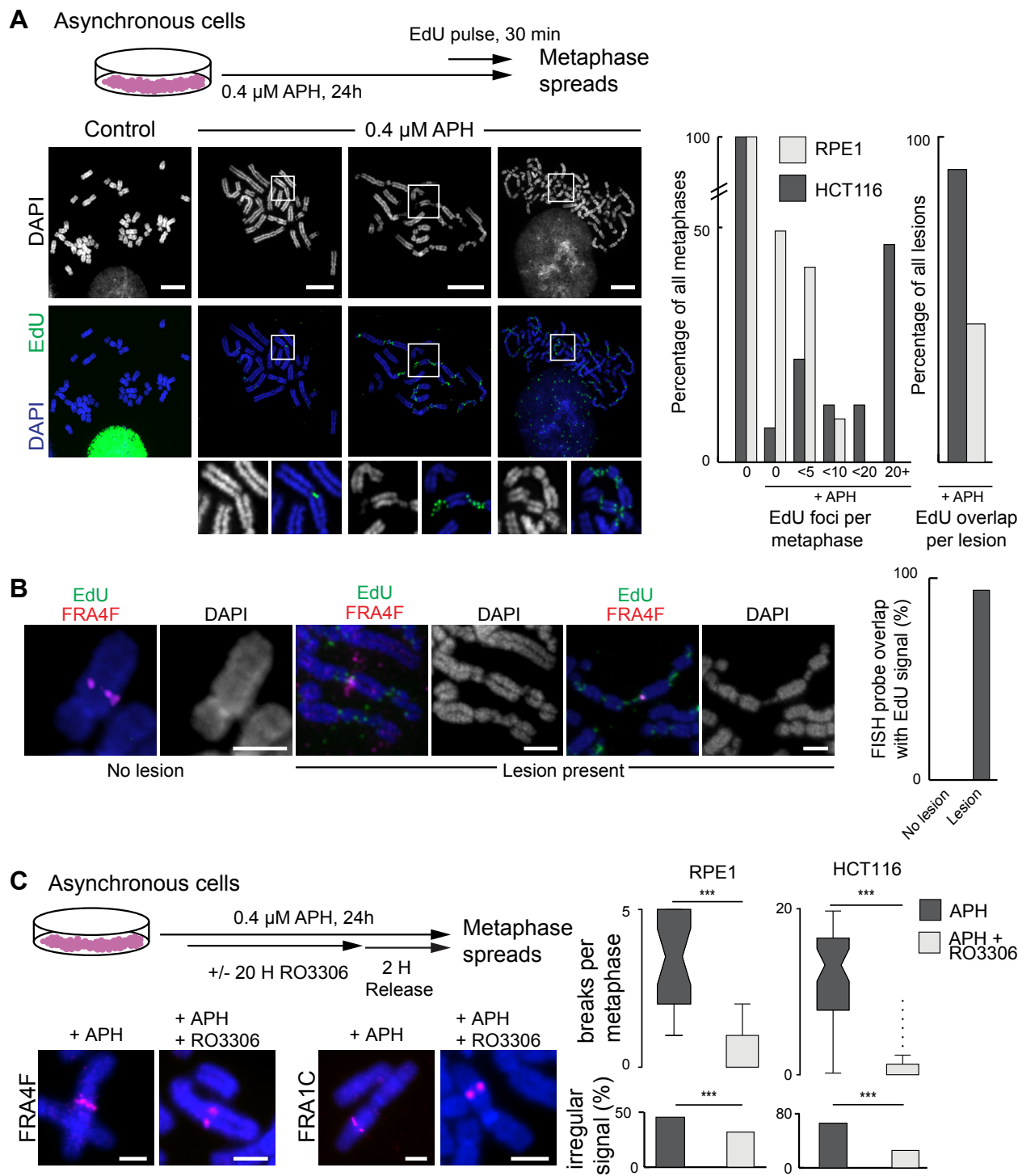
B. Representative FISH probe phenotypes at CFS loci on cytogenetically normal chromosomes. Regular - symmetrical, round signals; Concatenated - a single signal sitting between the two sister chromatids; Fragmented - multiple, asymmetric signals; Extended - a signal extending beyond the DAPI stained chromosome area. Below, fine-mapping cytogenetic lesions and atypical probe signals marking molecular disruptions using FISH probes (magenta) across FRA4F (HCT116 cells,  $n = 439$ ) and FRA1C (RPE1 cells,  $n = 180$ ). Percentage of chromosomes showing probe overlap with cytogenetic lesions (top) and irregular FISH signals (bottom) for each probe.

C. Representative chromosomes from untreated cells (top) or cells treated with aphidicolin (APH, bottom), to induce replication stress, hybridised to FISH probes for a non-fragile locus 11q13.2, or fragile loci FRA4F (HCT116 cells) and FRA1C (RPE1 cells). Bottom, quantification of atypical signals in the presence and absence of APH.

## Extending G2 reduces cytogenetic lesions and molecular defects at CFS

CFS regions were recently identified as sites of DNA synthesis on metaphase chromosomes: by utilising a short pulse with the thymidine analogue EdU in mitosis, DNA synthesis foci can be observed at cytogenetic CFS lesions (Minocherhomji et al., 2015). We performed similar labelling to characterise the relationship between MIDAS, cytological lesion formation and molecular misfolding in our cell lines. We found that mitotic DNA synthesis (MIDAS) occurred at DAPI-faint regions and on many occasions could be seen bridging gaps in chromosomes (Figure 4A and Supplementary Figure 4A). In a subset of metaphases showing extensive damage and wide-spread under condensation, mitotic synthesis foci joined chromosome fragments, overlapping with regions of under-condensation. Consistent with the severity of cytogenetic phenotypes, mitotic synthesis was significantly more frequent in HCT116 cells than in RPE1: mean number of foci per metaphase was 23.2 and 1.53, respectively (Mann-Whitney U test  $p < 2.2 \times 10^{-16}$ , Figure 4A). We also observed that mitotic synthesis was very frequently associated with cytogenetically visible lesions, especially in the HCT116 cell line (91% of EdU foci coincided with lesions, Figure 4A) indicating that mitotic DNA synthesis preferentially occurs in the context of under-condensed mitotic chromatin. We also examined the concurrence between molecular-scale misfolding and mitotic synthesis at the FRA4F site by combining FISH with MIDAS labelling. We found that at this site mitotic synthesis foci rarely appeared on cytogenetically normal regions of the chromosome, even if chromatin at the site showed small scale molecular disruption indicated by an abnormal FISH signal (Figure 4B). Strikingly, this is similar to observations of MIDAS at telomeres, where the fragile telomere phenotype was found not to correlate to the appearance of MIDAS foci (Özer et al., 2018). This observation suggests that unlike cytogenetic disruptions, molecular level misfolding is not accompanied by MIDAS, but raises the possibility that the misfolding phenotypes represent structures that have not yet undergone repair. Eliminating ongoing mitotic synthesis using a high dose of aphidicolin during mitosis did not abolish decondensation, indicating that the mitotic condensation defects are not caused by the ongoing process of mitotic synthesis (Supplementary Figure 4B).

Although the structures underlying both cytogenetic lesions and molecular lesions are unclear, we wanted to examine whether they represent intermediates that can be resolved or permanent defects in mitotic chromatin structure. Utilising the CDK1 inhibitor RO3306, we artificially prolonged the duration of G2 following induction of replication stress, allowing potential intermediates to be repaired and resolved prior to releasing the cells into mitosis (Figure 4C). We found that the frequency of both cytological lesions and molecular misfolding was reduced following RO3306 treatment, indicating that the structures underlying these phenotypes are subject to repair during G2 (Student's t-test  $p < 2.2 \times 10^{-16}$ , Figure 4C).



**Figure 4: Large-scale chromatin disruptions at cytogenetic CFS lesions are coincident with mitotic DNA synthesis (MIDAS).**

A. Top, Staining procedure for MIDAS visualised using EdU labelled with FITC-azide. Bottom left, representative metaphase spreads prepared from cells treated with or without aphidicolin. Insets show MIDAS (green signal) and widespread chromosome compaction defects. Right, quantification of MIDAS in RPE1 (n = 65) and HCT116 (n = 82) metaphase spreads from APH treated cells and overlap between mitotic DNA synthesis and cytogenetic lesions HCT116 (n = 1622 foci) and RPE1 (n = 96 foci). Scale bar, 40  $\mu$ m.

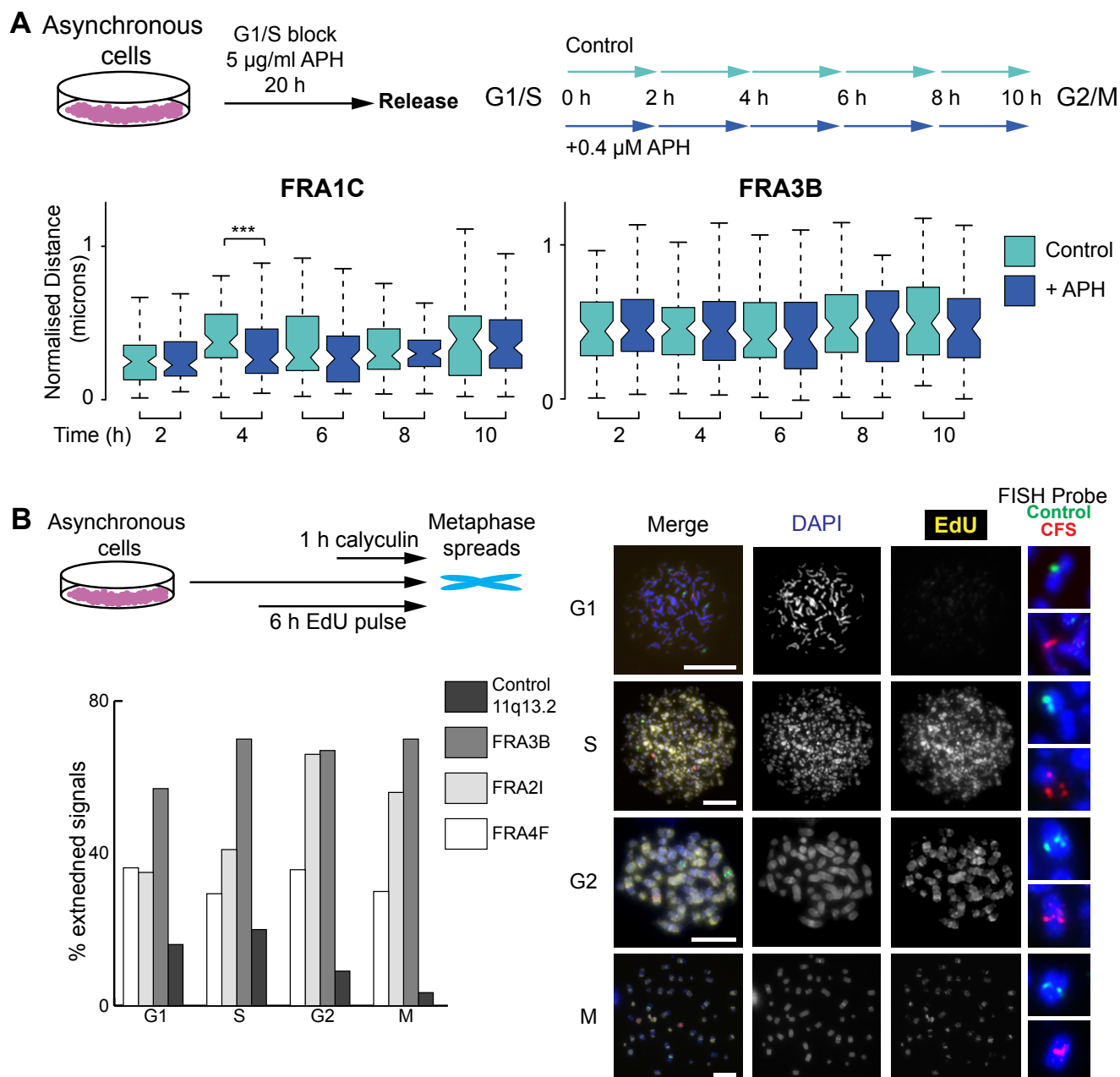
B. Atypical FISH signals, cytogenetic lesions and mitotic DNA synthesis (MIDAS) at the FRA4F locus in HCT116 cells. Representative chromosomes are shown, with increasing degrees of lesions and aberrant condensation. Right, graph showing overlap frequency of FRA4F probe with MIDAS foci on chromosomes in the presence or absence of cytogenetic lesions. Scale bars, 10  $\mu$ m.

C. Top, treatment conditions for extending G2 following induction of replication stress. Bottom left, representative images of FISH signals at the FRA4F (HCT116) and FRA1C (loci) following aphidicolin treatment followed by a normal duration (left) or extended (right) G2. Right, quantification of the frequency of cytological breaks (boxplots) and abnormal FISH signals (bar charts) in the two cell lines after aphidicolin treatment followed by a normal duration (dark grey) or extended (light grey) G2. Scale bars, 10  $\mu$ m.

## Chromatin at CFS regions is not primed for mitosis

We were interested in the possibility that the aberrant configurations observed at metaphase might have arisen at an earlier stage in the cell cycle. We therefore considered that differences in interphase chromatin structure caused by replication stress could result in mitotic condensation problems. To test this, we used a two-probe FISH approach to examine chromatin compaction across two fragile sites: FRA3B (HCT116 cells) and FRA1C (RPE1 cells), in the presence and absence of aphidicolin (Figure 5A) and analysed synchronised cell populations, at different time points throughout the transition from the G1/S boundary through to G2 and mitosis (Figure 5A and Supplementary Figure 5A). No replication-stress induced changes in interphase chromatin structure were observed in FRA3B and FRA1C post-replication, but there was a change in compaction in FRA1C (replicates at 4 h) in early to mid S-phase ( $p < 0.01$ ) which is likely to reflect changes in replication dynamics at the site. Our data therefore indicated that replication-stress per se does not induce chromatin structure changes which could explain mitotic condensation failure observed at these sites.

Compaction for mitosis involves many compositional and structural changes, which are required to prepare and prime chromatin for condensation. We speculated that this process was disrupted at CFS regions. To assess the frequency of misfolding lesions at CFS loci throughout the cell cycle, we utilised a premature chromosome condensation assay at three CFSs and a control, non-fragile region on chromosome 11q13.2. Cells were treated with the phosphatase inhibitor calyculin A, which causes chromosome condensation, irrespective of cell cycle stage (Figure 5B). This results in the formation of prematurely condensed chromosomes with morphologies that are indicative of the cell cycle stage they are derived from: thin and zig-zag shaped in G1; fragmented chromatin in S-phase; cross shaped chromosomes with fuzzy boundaries in G2 cells and typical metaphase chromosomes in mitotic cells (Achkar et al., 2005; Ono et al., 2013). To assess the condensation capacity of FRA4F, FRA2I, FRA3B and the control location at different phases of the cell cycle, we hybridised FISH probes mapping to the three locations and characterised the morphology of the FISH signals on chromosomes showing morphologies characteristic of G1, S, G2 and M stages in the absence of replication stress. To verify the accuracy of our approach, we quantified the frequencies of one-spot (unreplicated) and two-spot (replicated) signals throughout the different cell cycle stages and found that, as expected, one-spot signals were more common at the early stages of the cell cycle and two-spot signals were more frequent in G2 and mitotic chromosomes, especially at the control location (Supplementary Figure 5B). Our analysis revealed contrasting dynamics in chromatin competence for condensation at the CFS sites and the control region (Figure 5B). At the control locus, we observed the frequency of atypical signals decreased in the later phases of the cell cycle, with a very small proportion of signals retaining the misfolded phenotype in G2 and M chromosomes. This indicated that chromatin at the locus acquires competency for mitotic compaction as the cell cycle proceeds. In contrast, at the three CFSs, the atypical FISH signals persisted throughout the cell cycle and remained high in mitotic chromosomes, indicating that the process which allows non-fragile locations to re-set their chromatin environment and compact for mitosis may be disrupted at CFSs. These results also confirmed that the molecular –level lesions we observe at CFS regions are initiated at earlier cell cycle stages and not acquired during mitosis.



**Figure 5: Chromatin at CFS is not primed for mitosis.**

A. Top, model detailing experimental strategy to analyse chromatin folding at CFS regions. Cells progressing synchronously through the cell cycle were harvested every two hours. Bottom, boxplot of normalised inter-fosmid distances between pairs of probes flanking FRA1C ( $n > 60$  RPE1 nuclei) or FRA3B ( $n > 60$  HCT116 nuclei). P-values are for a Wilcoxon test.

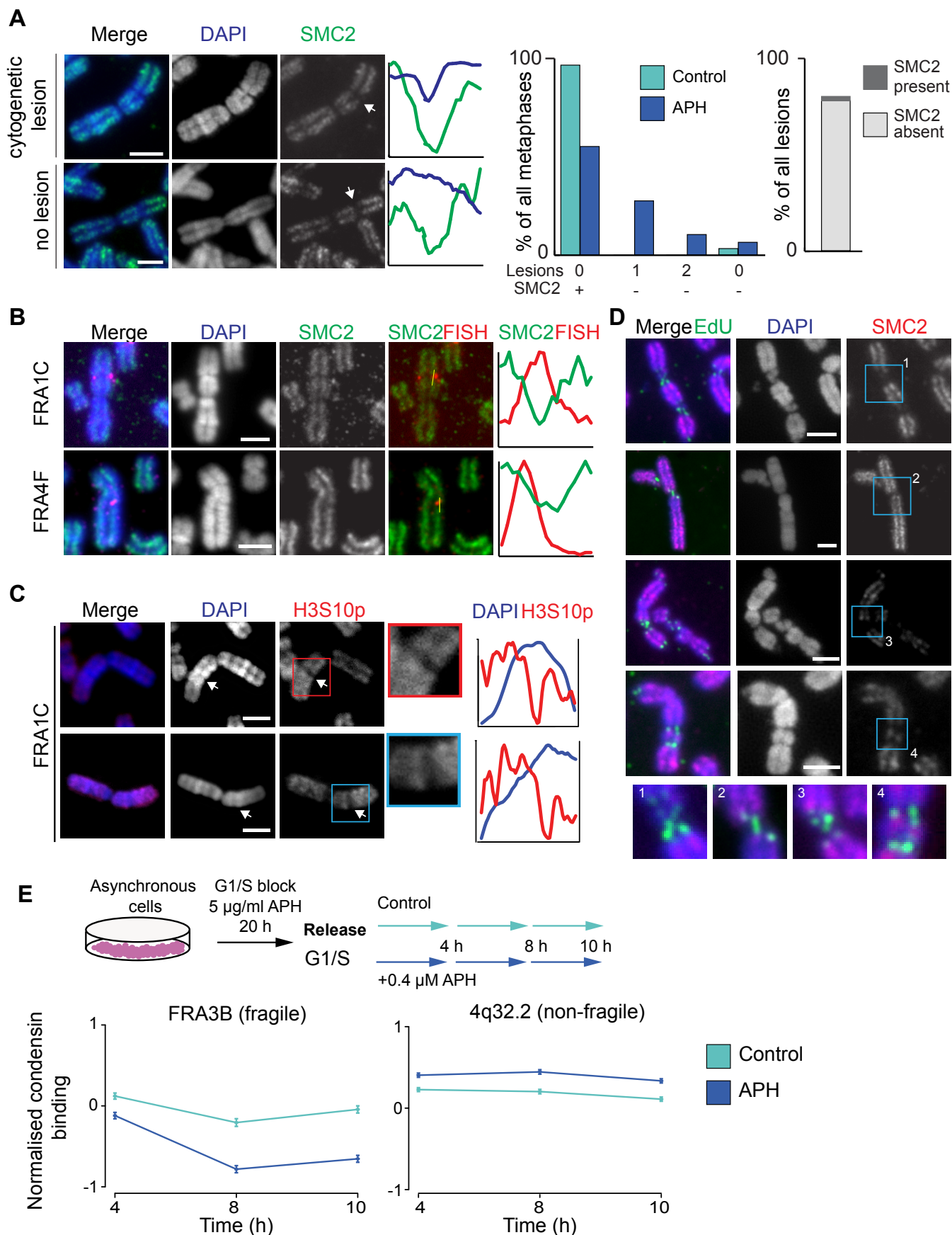
B. Depiction of premature chromosome condensation (PCC) assay (see methods) in HCT116 cells. Cells labelled with EdU (5 h) were condensed using calyculin (1 h), harvested and hybridised to FISH probes for a control locus (11q13.2) and CFSs (FRA4F and FRA2I). Right, representative chromosomes and left, quantification of the proportion of extended probe signals at FRA2I ( $n = 136$  chromosomes), FRA4F ( $n = 119$ ) and 11q13.2 control probe ( $n = 116$ ).



## Condensin loading in mitosis is defective at CFS regions

We considered the potential mechanisms that may affect mitotic compaction at CFS loci. Previous work in yeast has indicated that recruitment of condensin is blocked at slow replication zones to prevent break formation (Hashash et al., 2012). To determine if the yeast model is applicable to mammalian cells we examined condensin localisation to cytogenetic breaks at CFS loci. Using an antibody against SMC2, a component of both condensin complexes active in mammalian cells, we found that CFS cytogenetic lesions are always depleted of condensin (Figure 6A, Supplementary Figure 6A-B). Furthermore, the region of condensin depletion frequently appeared to encompass a larger area than the cytogenetic break (Figure 6A, Supplementary Figure 6A-B). Surprisingly, on a small proportion of chromosomes, large regions of SMC2 depletion could be observed in the absence of a cytogenetic break, at cytogenetic locations that were consistent with frequent CFSs, such as FRA1C. To verify that regions of SMC2 depletion in the absence of cytogenetic abnormalities occur at CFSs, we combined SMC2 immuno-fluorescence with FISH using probes for the FRA1C and FRA4F CFS regions and confirmed that SMC2 depletions occur at CFS regions (Figure 6B, Supplementary Figure 6C). Consistently, staining for the H3 serine 10 phosphorylation mark, which is acquired on chromatin in preparation for mitotic folding, also showed a depletion at FRA1C on cytogenetically intact mitotic chromosomes (Figure 6C). As condensin phosphorylation by Cdk1 (Abe et al., 2011) and Chk2 (Zhang et al., 2016) is necessary for chromosome compaction we speculated that failure of condensin loading at CFSs could be triggered by ATM or ATR signaling (Casper et al., 2002). ATM inhibition did not affect the presence of SMC2 lesions (Supplementary Figure 6D), whilst ATR inhibition caused widespread chromosome shattering, not limited to common fragile sites. This phenotype resembled calyculin-induced chromosome condensation in replicating nuclei and is likely caused by chromosomes initiating condensation too early in the cell cycle (Supplementary Figure 6E). As CFS lesions are restricted to specific genomic locations and do not result in checkpoint activation their molecular basis might be distinct from damage typically signaled through ATR. Nevertheless, condensin depletion appeared to be essential for repair processes at CFSs, as MIDAS labelling with SMC2 immunostaining revealed that synthesis only occurs in regions of mitotic chromosomes depleted of condensin, confirming that uncondensed chromatin is a necessary condition for mitotic DNA synthesis (Figure 6D).

The cytogenetic observation of SMC2 depletion indicated that it may be possible to observe reduction of SMC2 binding at CFS loci using a molecular approach. The binding of condensin complexes to chromatin is known to be dynamic and dependent on cell cycle stage (Ono et al., 2013). To quantify condensin depletion at CFS loci, SMC2 levels were analysed at CFS locations using ChIP-seq in unperturbed cells and following aphidicolin treatment. To avoid confounding effects resulting from altered cell cycle distributions, the experiments were performed in synchronised HCT116 cell populations at three distinct time points, including mid-S (4 H), late-S (8 H) and G2 (10 H). Despite only a small proportion of cells showing fragility (Figure 6A) there was a pronounced reduction in condensin binding at FRA3B and FRA4F sites (Figure 6E and Supplementary Figure 6F-G) at the 8 H and 10 H time points, suggesting that defects at condensin loading at the locus emerge as cells progress through S-phase and into G2 when they have already replicated. In contrast, this was not apparent at the 4q33.2 and FRA3O CFSs which are not active in the HCT116 cell line (Figure 6E and Supplementary Figure 6F-G), indicating that condensin depletion is a feature of active CFSs. Since the reduction in condensin binding appears to be present at both the 8 hour and 10- hour time points, this data supports a model of defective condensin recruitment rather than targeted removal of condensin for repair.



**Figure 6: SMC2 depletion underlies CFS lesions and MIDAS on mitotic chromosomes.**

**Figure 6: SMC2 depletion underlies CFS lesions and MIDAS on mitotic chromosomes.**

A. Representative images showing regions of SMC2 depletion at cytogenetic lesions (top) and on cytogenetically normal chromosomes (bottom) with intensity profiles of DAPI and SMC2 (regions of interest are shown in Supplementary Figure 6A); scale bars, 2.5  $\mu$ m. Right, quantification of the frequency of cytogenetic lesions and lesion-free SMC2 depletions in the presence or absence of aphidicolin. Far right, quantification of SMC2 occupancy at cytogenetic lesions in the RPE1 cell line.

B. Representative immuno-FISH images showing immunofluorescence for SMC2 and FISH probes for the FRA1C (RPE1, top) and FRA4F (HCT116, bottom) sites. overlap with regions of SMC2 depletion on metaphase chromosomes. Right, intensity profiles across the region of interest indicated by white line. Scale bars, 2.5  $\mu$ m.

C. Representative immunofluorescence staining for H3S10 phosphorylation on mitotic chromosomes at the FRA1C locus after replication stress. Scale bars, 5  $\mu$ m.

D. Representative images of EdU incorporation marking MIDAS on chromosomes co-stained for SMC2. Scale bars, 5 $\mu$ m.

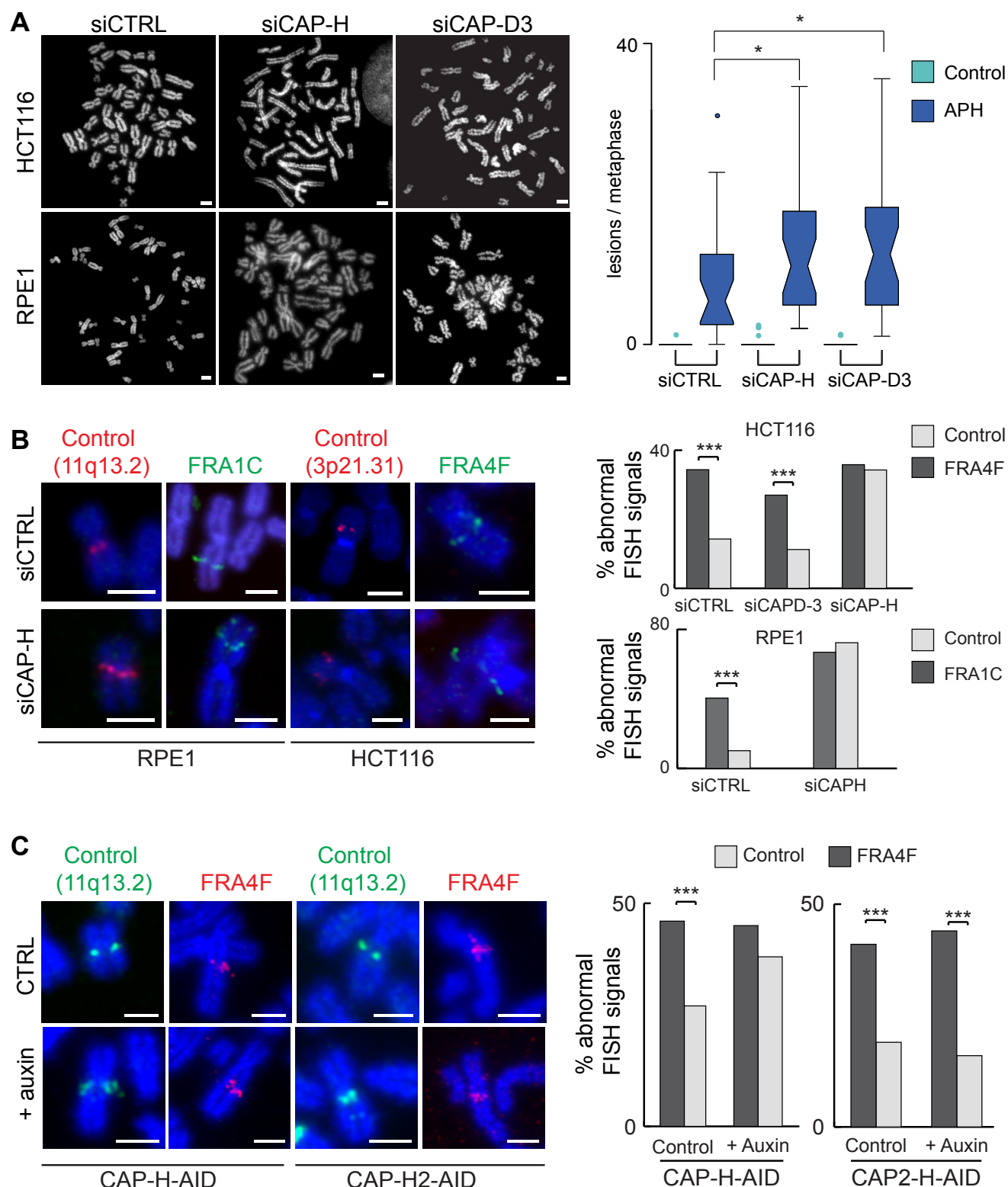
E. Top, model detailing experimental strategy to analyse condensin recruitment at CFS regions in synchronised cell populations by ChIP. Bottom, line graphs showing mean normalised condensin binding in 1kb windows at the FRAB and 4q32.2 sites at different time points in the absence (green) or presence (blue) of aphidicolin.

## Condensin I depletion causes CFS mitotic folding defects at non-fragile locations

As condensin depletion appears to be a major driver of CFS misfolding in mitosis, we sought to examine the effects of global depletion of the condensin complexes. We used siRNAs against the condensin components CAP-H and CAP-D3 to induce depletion of the condensin I and condensin II complexes, respectively, which resulted in defects in mitotic chromosomes as previously described (Green et al., 2012) (Figure 7A, Supplementary Figure 7A). Scoring of cytogenetic lesions and MIDAS foci in these chromosomes revealed that although CAP-H and CAP-D3 depletion did not induce lesion formation in unperturbed conditions, there was a significant increase in the frequency of CFS lesions in the condensin-depleted chromosomes once replication stress was induced (Supplementary Figure 7B). This is consistent with replication stress and condensin depletion being both necessary factors for lesion formation at CFS.

We next examined the effect of condensin depletion on the mitotic misfolding phenotype at CFS and control, non-fragile locations. As expected, the frequency of misfolding at the CFS locations was significantly higher than control loci in siCTRL-treated cells in both the RPE1 and HCT116 cell line (Figure 7B). However, in cells depleted of CAP-H, the frequency of misfolding was similar across CFSs and control-loci, suggesting that depletion of condensin I is sufficient to recapitulate the misfolding phenotype characteristic of CFS sites at a non-fragile location. In contrast, depletion of CAP-D3 (condensin II) did not affect the frequency of misfolding at non-fragile locations, indicating that the condensin I complex is the primary effector of mitotic misfolding at CFS locations.

To further investigate the role of condensin in an independent system, we used HCT116 cell lines in which both copies of CAP-H or CAP-H2 were fused to an AID tag, which enabled rapid and complete depletion of either condensin I or II upon addition of auxin (Takagi et al., 2018). Similar to using siRNAs misfolding of a control locus was also observed in a cell line in which CAP-H degradation was triggered using an auxin inducible degron tag (Figure 7C), indicating that defects in condensin I loading post-replication underlie mitotic misfolding and that the process is not specific to CFSs but is important genome-wide. In contrast, CAP-H2 degradation did not lead to an increase in misfolding at the control locus. These results were further supported using an HCT116 cell line in which both copies of SMC2 were fused to an AID tag. The HCT116-SMC2-AID cell line showed severe defects in mitotic chromosome structure upon SMC2 degradation: individual chromosomes could not be distinguished and metaphases appeared as a mass of condensed fragments instead (Supplementary Figure 7C). This phenotype was visible following 6-hour SMC2 degradation, suggesting that the presence of SMC2 during S/G2 is essential for mitotic chromosome folding. MIDAS foci could still be observed in SMC2 depleted metaphases, showing condensin is not required for mitotic synthesis (Supplementary Figure 7C), whilst FISH analysed showed a significant increase in molecular misfolding at both control and fragile sites (Supplement Figure 7D).



**Figure 7: Impaired recruitment of condensin I underlies CFS defects in mitosis.**

A. Left, representative images of chromosomal defects in the HCT116 and RPE1 cell lines following depletion of the condensin components CAP-H (condensin I) and CAP-D3 (condensin II). Scale bars, 10  $\mu$ m. Right, quantification of the number of cytogenetic lesions per metaphase following condensin depletion in the HCT116 cell line in the absence (green) or presence (blue) of aphidicolin.

B. Left, representative images of chromosomal defects visualised by FISH in the HCT116 and RPE1 cell lines following depletion of the condensin components CAP-H and CAP-D3. Scale bars, 5  $\mu$ m. Right, quantification of the number of cytogenetic lesions per metaphase following condensin depletion in the HCT116 cell line.

C. Representative images showing FISH probes at two CFS regions (FRA1C and FRA4F) and two non-fragile regions (11q13.2, 3p21.31) following depletion of the condensin I component CAP-H. Scale bars, 5  $\mu$ m. Right, quantification of the frequency of irregular FISH signals, indicative of mitotic misfolding, at FRA1C, FRA4F and the two non-fragile locations following depletion of CAP-H and CAP-D3.



## Discussion

Replication stress entails the genome-wide slowing down of individual forks, leading to activation of extra origins, changes in origin efficiency and potentially, altered replication dynamics (Courbet et al., 2008; Macheret and Halazonetis, 2018). A number of factors can trigger replication stress: oncogene activation, misincorporation of nucleotides or replication-transcription conflicts (Helmrich et al., 2011; Hills and Diffley, 2014; Reijns et al., 2015). An often overlooked aspect of replication stress is the local chromatin environment. DNA supercoiling or catenanes (Naughton et al., 2013), paucity of active chromatin marks and unusual DNA structures such as R loops and G-quadruplexes have all been shown to interfere with replication dynamics, suggesting that features of the underlying chromatin environment could be a critical factor linking replication stress to genome instability (Comoglio et al., 2015).

CFSs best illustrate the relationship between replication stress and chromosomal fragility. While traditional models envision this relationship is mediated through replication dynamics, we show that defects in the processing of post-replicative chromatin, resulting in disrupted mitotic folding, also play a role. Most genomic regions undergo compositional and structural priming of chromatin to permit condensation in mitosis, but our data indicates that CFSs are inefficiently primed and remain refractory to compaction as a result of defective recruitment of condensin complexes. Our data shows that this defective condensin recruitment is essential for a range of CFS-specific phenotypes, such as cytogenetic lesions and MIDAS.

Cytological lesions are one outcome of inefficient compaction but we now report a new layer of instability at CFSs, visible at the molecular but not the cytogenetic level. These aberrant structures bear similarity to phenotypes previously seen at telomeres following replication stress and recently, at centromeres (Sfeir et al., 2009). At telomeres, lesions are thought to result from replication problems such as fork collapses or G-quadruplex structures formed by GC-rich telomeric repeats; however, CFSs are not composed of repetitive sequences and it is unclear how small-scale events can lead to fragility and failure of mitotic compaction on such a large genomic scale, suggesting additional factors like chromatin structure or epigenetics play a role. The similarity between CFS phenotypes support the idea that mitotic misfolding is a universal feature of CFSs and potentially, other difficult to replicate regions. While classic cytogenetic lesions that characterise CFSs cannot be observed in the absence of aphidicolin, this newly characterised low level of instability, apparent only using molecular techniques, is present at these loci at a low frequency even when cells undergo normal replication. This finding indicates the inherent fragility present at CFS regions even in the absence of exogenous replication stress and implicates a model for their instability in physiological contexts such as during tumour development (Alexandrov et al., 2013).

In addition to inherent low level misfolding, mitotic DNA synthesis (MIDAS) is a frequent feature of CFSs. Mitotic misfolding appears to promote a permissive environment for the MIDAS process, and most synthesis occurs in the context of uncondensed chromatin, which is free of condensin. It has been previously proposed that the process constitutes a repair pathway for lesions prior to the completion of mitosis (Min-oherhomji et al., 2015; Naim et al., 2013) but our observations suggest that altered chromatin compaction at CFS could also aid their repair. Remarkably, under-condensation in mitosis at unresolved homologous recombination intermediates was found to aid cell division, although these mitotic structures represented a distinct phenotype from CFS lesions (Chan et al., 2018). The primary causes of impaired compaction at CFS remain unknown: although our data argues against delayed replication as a cause, it is possible that replication or repair intermediates, or aberrant chromatin structures resulting from them, impair condensin recruitment. This is supported by the observation that extending G2, potentially allowing time for the repair of such intermediates, results in reduction of both cytogenetic abnormalities and mitotic misfolding.

Chromosome priming for mitosis is characterised as a cell cycle coupled structural and compositional change in chromatin that facilitates mitotic condensation and sister chromatin separation. This idea is not

without precedent: the Kleckner lab have suggested that through the cell cycle chromatin is continuously remodelled using the energy of topological stress to drive chromatin compaction (Kleckner et al., 2004; Liang et al., 2015). Mechanistic steps for chromatin folding from interphase to mitosis are poorly defined, but key events include condensin loading, histone H3 phosphorylation and catenane resolution, by topoisomerases. Consistently, we find that the H3S10p mark is depleted at FRA1C in mitosis.

Chromosome misfolding is not restricted to CFSs and normal genomic regions show a low frequency of lesion formation in the presence of replication stress suggesting that common fragile sites do not have a unique set of chromatin features. Instead, CFSs are at the extreme end of a spectrum of aberrant chromatin structures that have a propensity to exhibit replication stress and inefficient priming leading to misfolding in mitosis and subsequent chromosome instability.

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## **Author contributions**

L.B., R.N. and C.N. undertook experiments, N.G., L.B., R.N. and C.N. designed experiments and analysed data, K.S. and W.C.E. designed experiments and provided reagents. N.G. and L.B. wrote the manuscript with input from all authors.

## **Competing Financial Interests**

The authors declare no competing financial interests

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