The presence of extra chromosomes leads to genomic instability

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Aneuploidy is a hallmark of cancer and underlies genetic disorders characterized by severe developmental defects, yet the molecular mechanisms explaining its effects on cellular physiology remain elusive. Here we show, using a series of human cells with defined aneuploid karyotypes, that gain of a single chromosome increases genomic instability. Next-generation sequencing and SNP-array analysis reveal accumulation of chromosomal rearrangements in aneuploids, with break point junction patterns suggestive of replication defects. Trisomic and tetrasomic cells also show increased DNA damage and sensitivity to replication stress. Strikingly, we find that aneuploidy-induced genomic instability can be explained by the reduced expression of the replicative helicase MCM2-7. Accordingly, restoring near-wild-type levels of chromatin-bound MCM helicase partly rescues the genomic instability phenotypes. Thus, gain of chromosomes triggers replication stress, thereby promoting genomic instability and possibly contributing to tumorigenesis.
Most eukaryotic organisms are diploid as they contain two sets of chromosomes. A deviation from the normal chromosome number often markedly affects their physiology. Whole-chromosome gains or losses—called numerical aneuploidy—have particularly detrimental effects in Metazoans, as cells often suffer from impaired proliferation, increased sensitivity to proteotoxic stress and altered gene expression1–3. Structural aneuploidy, characterized by sub-chromosomal unbalanced rearrangements, that is, duplications or deletions of large genomic regions, also affects cellular functions. Both numerical and structural aneuploidy are found in nearly 75% of malignant tumours4, where they are often associated with ongoing whole-chromosome gains and losses, a process known as chromosomal instability (CIN), and with additional structural rearrangements due to genomic instability (GIN). Both CIN and GIN phenotypes promote cancer and contribute to tumour heterogeneity and drug resistance5,7.

Experiments in mouse models carrying mutations that impair faithful chromosome segregation revealed that the resulting CIN is sufficient to trigger tumour formation in some tissues, although it should be noted that CIN and aneuploidy also exert tumour-suppressive effects (reviewed in ref. 8). Mounting evidence shows that GIN and replication stress occur early in tumorigenesis, and many known oncoproteins and oncogenic mutations act by triggering replication stress and subsequently genomic instability9–11. The occurrence of both structural and numerical aneuploidy in early stages of cancer provokes the question of whether there is a link between these two features. It was recently shown that lagging chromosomes suffer from breakage during cytokinesis12. Missegregated chromosomes are often contained in micronuclei in daughter cells, where they experience DNA damage likely due to aberrant replication13. Chromosomally unstable colorectal cancer cell lines show elevated DNA replication stress that was proposed to further promote chromosome missegregation5. Aneuploidy also correlates with chromosomal and genomic instability in transformed Chinese hamster embryo cells15. Yet, delineating the functional relationship between numerical aneuploidy and genomic instability, and the molecular mechanisms involved has remained challenging.

To understand the relationship between CIN and GIN, it is crucial to determine whether and how numerical aneuploidy by itself affects genome stability. Recently established model cells with defined aneuploid karyotypes have facilitated the analysis of the immediate consequences of aneuploidy per se14,16–19. Direct comparison of cognate euploid and aneuploid cells revealed that aneuploidy triggers distinct and conserved changes in gene expression20,21. Addition of even a single extra chromosome causes profound defects in the maintenance of proteostasis as aneuploid cells are more sensitive to inhibitors of protein folding, protein translation and degradation, activate protein degradation pathways and show marked protein folding defects2,3,5,8,18,22,23. Observations in budding and fission yeasts suggested that aneuploidy impairs the fidelity of chromosome segregation, and increases mutation and recombination rates, although the underlying mechanisms remain enigmatic17,24,26. Analysis of trisomy of chromosome 7 or 13 in the p53-deficient cancer cell line DLD1 revealed increased chromosome missegregation of the extra chromosome and a frequent cytokinesis failure in trisomy 13 (ref. 27). Yet, no systematic analysis of the effects of aneuploidy on the maintenance of genome stability has been performed in higher eukaryotes so far.

Here we use a series of trisomic and tetrasomic human cell lines with defined karyotypes derived from near-diploid and chromosomally stable parental cell lines. By direct comparison with the parental cell lines, we found that addition of even a single chromosome is associated with accumulation of replication-related DNA damage and chromosomal rearrangements. Strikingly, we found profound abundance changes of proteins involved in DNA replication in response to the presence of extra chromosomes, in particular, consistently low levels of the replicative helicase MCM2-7. Our data provide the first evidence that the presence of even a single extra chromosome in human cells triggers genomic instability by impairing DNA replication, thus establishing a new link between numerical and structural aneuploidy that might play a role in cancer.

Results
Accumulation of pre-mitotic DNA errors in aneuploid cells. To determine whether the presence of one or two extra chromosomal gains induces genomic instability, we created a panel of trisomic and tetrasomic human cell lines using microinjected-mediated chromosome transfer (MMCT; see Supplementary Information for further details). We analysed five different cell lines that were derived from the chromosomally stable colorectal cell line HCT116 and five cell lines originating from the primary human cell line RPE1 (retinal pigment epithelium) immortalized by the overexpression of human telomerase. Each of these cell lines contained an extra copy of either chromosome 3, 5, 8, 12 or 21. The chromosomal content was verified by single-nucleotide polymorphism (SNP) array analysis, and the cell lines were named according to the identity of the extra chromosome and its copy number, for example, HCT116 8/3 for trisomy of chromosome 8 in the HCT116 cell line (Fig. 1a). Evaluation of microscopic images of anaphases revealed that all 10 analysed trisomic and tetrasomic cells contained anaphase bridges at higher frequency than the parental cell lines (Fig. 1b–d). This finding was further confirmed using high-resolution imaging combined with staining with a centromere-specific CREST antibody. On average, 46% of HCT116 3/3, 25% of HCT116 5/3 and 28% of HCT116 5/4 anaphase cells contained anaphase bridges and broken chromosome fragments devoid of centromeric staining that are indicative of pre-mitotic errors, whereas only 10% of HCT116 anaphase cells showed similar defects (Fig. 1e,f). A similar trend was observed in RPE1-derived cell lines, with an increase from 2.5% in control to 12.7% in RPE1 5/3 and 9% in RPE1 21/3 (Fig. 1f). In contrast, the analysed cell lines showed a mild, but insignificant increase in the frequency of lagging chromosomes in comparison with the parental HCT116 or RPE1 cells (Supplementary Fig. 1a–e).

Next, we analysed the occurrence of ultrafine anaphase DNA bridges (UBFs), thread-like DNA structures that associate with the Bloom’s syndrome helicase (BLM) protein and link the dividing DNA masses28. UBFs originate either from DNA catenanes or from replication or recombination intermediates, and their occurrence is suggestive of un-replicated or abnormal DNA structures29. Staining with an antibody against Bloom helicase (BLM) revealed increased frequency of UBFs by up to 60% in the trisomic and tetrasomic cells compared with controls (Fig. 1g,h). Collectively, these findings suggest that the presence of even a single extra chromosome increases the frequency of pre-mitotic errors, while chromosome segregation is not significantly impaired.

DNA damage and chromosome breaks increase with aneuploidy. In order to determine whether trisomy and tetrasomy lead to increased levels of DNA damage, we determined the occurrence of 53BP1-containing nuclear bodies in cyclin A-negative G1 cells. Such 53BP1 foci have been suggested to mark unrepairred replication-induced DNA lesions that persist through mitosis29. We determined the number of 53BP1 foci in G1 cells using automatized image acquisition and analysis (Fig. 2a).

The
Figure 1 | Trisomy and tetrasomy elevates the frequency of pre-mitotic errors. (a) Chromosome copy number changes in the parental HCT116, RPE1 and the respective trisomic and tetrasomic cell lines. Chromosome gains are marked in red and chromosome losses in grey. Note that both HCT116 and RPE1 contain several previously documented structural copy number variations that remained largely unchanged in the trisomic and tetrasomic derivatives. The identity of the extra chromosome and the number of copies were used for identification of each cell line, for example, HCT116 3/3 contain three copies of chromosome 3. Two cell lines with identical trisomies, but originating from different single cells, were selected for HCT116 3/3: clone 11 and clone 13 (c11 and c13) and for RPE 5/3 12/3 (c3 and c7) to determine the effect of clonal differences. (b) Representative images of a HCT116 5/3 anaphase cell with anaphase bridges and fragments. (e) Representative images of an HCT116 5/3 anaphase cell stained with DAPI, anti-centromere antibody CREST and anti-α-tubulin. Arrowhead marks an acentric chromosome fragment. (f) Quantification of acentric chromosomal fragments and anaphase bridges. Bridges extend fully between DNA masses; acentic fragments were distinguished from whole-lagging chromosomes by absence of the CREST signal. (g) Examples of HCT116 5/3 anaphase cells stained with DAPI and antibodies against BLM (yellow arrowheads), which bind to UFBs. White arrowhead marks an anaphase bridge. (h) Quantification of UFBs. Plots (c,d,f and h) show mean ± s.e.m. of three independent experiments. At least 100 anaphases were scored in each experiment in c,d,f and h, in RPE1 21/3, only 68 (d), 51 (f) and 54 (h) anaphase cells were scored in each experiment. Nonparametric t-test; *P<0.05, **P<0.01 and ***P<0.001.
Figure 2 | Trisomy and tetrasomy elevates DNA damage. (a) Example of control and aneuploid cells stained with DAPI and antibody against 53BP1 and cyclin A2 to distinguish G1 cells (cyclin A2 negative). (b) Average number of 53BP1 foci per G1-phase cell counted in untreated HCT116 (control), upon treatment with 0.25 μM aphidicolin (APH) and in the untreated aneuploid derivatives. (c) Average number of 53BP1 foci per G1-phase cell counted in untreated RPE1 (control), upon exposure to 0.25 μM aphidicolin (APH) and in the untreated aneuploid derivatives. All plots show mean ± s.e.m. of three independent experiments, at least 500 cyclin A-negative cells were scored in each experiment. Nonparametric t-test; ***P < 0.0001. (d) Structural aberrations, such as gaps and constrictions, on metaphase chromosomes from RPE1 21/3 cells grown under replication stress conditions (0.1 μM aphidicolin and 0.73 mM caffeine). Representative metaphase spread; aberrations are indicated by red arrowheads. (e) Quantification of chromosomal aberrations detected in metaphase spreads of HCT116 cells exposed to 0.3 μM aphidicolin for 24 h. Fisher’s exact test, *P < 0.05, n = 94 (HCT116), 93 (5/3) and 95 (5/4) metaphases analysed in two independent experiments. (f) Distribution of metaphases according to the number of gaps and constrictions in RPE1, and its trisomic derivative under treatment with 0.1 μM aphidicolin and 0.73 mM caffeine for 24 h. Fisher’s exact test, **P < 0.01, n = 94 (RPE1) and 119 (21/3) metaphases analysed in two independent experiments.

The presence of extra chromosomes caused a significant twofold increase in the average number of 53BP1 foci per cell as well as a higher percentage of cells with >3 53BP1 foci in all trisomic and tetrasomic cell lines (Fig. 2b,c and Supplementary Fig. 2a,b). Of note, the numbers of 53BP1 foci tend to correlate with the amount of excessive DNA (Supplementary Fig. 2c). Treatment with the replication inhibitor aphidicolin further increased the number of 53BP1 foci in the tested cell lines (Supplementary Fig. 2d,e).

The higher levels of DNA damage may indicate perturbed DNA replication and/or repair at genomic regions sensitive to replication stress defined as fragile sites that can be observed as structural aberrations on metaphase chromosomes. Our analysis of metaphase spreads from cells grown under normal conditions showed a small, but insignificant trend indicative of higher fragility in response to extra chromosomes (Supplementary Fig. 2f,g). Aphidicolin treatment evoked significantly higher numbers of structural aberrations in trisomic and tetrasomic cells than in control HCT116 and RPE1 cells (Fig. 2d–f). While ~50% of the control HCT116 cells contained >5 breaks, gaps and constrictions, a similar level of aberrations was observed in almost 70% of the metaphases in the trisomic HCT116 5/3 (Fig. 2e). In HCT116, the effect scales with aneuploidy as 25% of metaphases showed very high fragility (>16 gaps and breaks) in tetrasomic cells compared with only 7% of the control diploid cells (Fig. 2e). Increased levels of structural aberrations were also found in trisomic RPE1 21/3 cells compared with the control cells (Fig. 2f). Taken together, the addition of an extra chromosome leads to increased DNA damage and significantly higher levels of chromosome fragility under replication stress.

Extra chromosomes increase sensitivity to replication stress. Replication stress conditions alter the cell cycle profile by slowing the progression of the cells through S-phase and/or by arresting the cells at the S-phase or G2/M checkpoints. Cell cycle analysis demonstrated that whereas under normal conditions both aneuploid HCT116 5/3 and 5/4, and their cognate controls showed nearly identical cell cycle profiles, there was a marked difference upon replication stress (Fig. 3a). Treatment with a low aphidicolin concentration led to an accumulation in late S-phase and G2/M in control cells, and this accumulation was further...
break point junctions revealed microhomologies in all cases, one tail-to-tail inversion (Fig. 4b,c). Sanger sequencing of the head-to-tail tandem duplications, one tail-to-head deletion and cells (Supplementary Fig. 4a). The rearrangements involved three of the six rearrangements have occurred designed to amplify the break point junctions confirmed that five chromosomes (Fig. 4a). Verification by PCR using primers trisomic line and four in the tetrasomic line on five different cell (HCT116 5/3 and HCT116 5/4). Comparison with the control parental cell line HCT116 and cognate trisomic and tetrasomic performed mate-pair next-generation sequencing (NGS) of the chromosomes might induce stably inherited genomic rearrangements that the replication stress caused by the presence of extra chromosomes.Aneuploidy causes genomic rearrangements. We hypothesized that the replication stress caused by the presence of extra chromosomes might induce stably inherited genomic rearrangements in human cells. To determine whether this is the case, we performed mate-pair next-generation sequencing (NGS) of the parental cell line HCT116 and cognate trisomic and tetrasomic cell (HCT116 5/3 and HCT116 5/4). Comparison with the control cell line revealed two unique chromosomal rearrangements in the trisomic line and four in the tetrasomic line on five different chromosomes (Fig. 4a). Verification by PCR using primers designed to amplify the break point junctions confirmed that five of the six rearrangements have occurred de novo in the aneuploid cells (Supplementary Fig. 4a). The rearrangements involved three head-to-tail tandem duplications, one tail-to-head deletion and one tail-to-tail inversion (Fig. 4b,c). Sanger sequencing of the break point junctions revealed microhomologies in all cases, indicative of replication-mediated rearrangement formation (Fig. 4c and Supplementary Fig. 4b).

To determine the rate of de novo chromosomal rearrangements in aneuploids and in parental cells, we generated two sets of 12 clonal cell lines, each originating either from a single HCT116 5/3 cell or from a single HCT116 cell. After 30 cell doublings, DNA was isolated from each clonal cell line and subjected to SNP-array analysis (Supplementary Fig. 4c). Eight out of the 24 trisomic clonal lines contained de novo copy number aberrations (CNAs), whereas none were detected in the 24 tetrasomic cell lines derived from the parental HCT116 cells (Fig. 4d). In total, we identified 12 CNAs on 7 different chromosomes that include a gain of chromosome 7, four duplications and seven deletions ranging from 105 kb to several megabases (Fig. 4e and Supplementary Table 2). Permutation testing confirmed that the accumulation of de novo CNAs among HCT116 5/3 as compared with HCT116 cells was unlikely to have occurred by chance (P value = 0.0021). By comparison of the identified break point junctions with previously documented common fragile sites, we found that one site overlapped with the fragile sites mapped in the HCT116 cell line after treatment with the replication inhibitor aphidicolin, and 8 out of the 12 CNAs were mapped to fragile sites identified in lymphocytes (Fig. 4e and Supplementary Table 2). Taken together, the presence of extra chromosomes significantly increases the occurrence of CNAs in human cells. The break point sequences, the types of identified chromosomal rearrangements and their frequent overlap with common fragile sites indicate that they arose due to defects during DNA replication.

Reduced MCM2-7 levels contribute to genomic instability. The presence of extra chromosomes triggers global and highly conserved expression changes that affect a large number of cellular pathways. We asked whether these expression changes might explain the phenotypes observed in aneuploid cells. To this end, we analysed protein expression data previously generated using quantitative mass spectrometry. Unsupervised hierarchical clustering revealed downregulation of several replication factors in aneuploid cells (Fig. 5a). Strikingly,
expression of all six subunits of the replicative helicase MCM2-7 (hereafter MCM) was consistently and significantly decreased in all analysed cells. MCM is a heterohexameric DNA helicase required for licensing of replication origins and for replication progression. Insufficient licensing owing to low levels of MCM helicase impairs the activation of dormant origins under replication stress conditions. Immunoblotting confirmed a general 20–50% decrease in all subunits of MCM helicase in HCT116 and RPE1 compared with control cells (Fig. 5b). Strikingly, a decrease of MCM2, MCM3 and MCM7 abundance was observed in 9 out of 10 HCT116- and RPE1-derived aneuploidies, regardless of the specific karyotype (Fig. 5c,d). Only chromatin-bound MCM contributes to the activation of replication origins; immunoblotting of MCM2, MCM3 and MCM7 from the chromatin fraction in both asynchronous and synchronized cells confirmed that trisomic and tetrasomic cells load significantly less helicase on DNA than cognate controls (Fig. 5e,f and Supplementary Fig. 6). It should be noted that immunoblotting confirmed downregulation of additional replication proteins, but none of them as consistently as the subunits of the helicase MCM (Fig. 5c,d).

We hypothesized that the low levels of MCM2-7 contribute to the replication defects observed in aneuploid cells. To test this hypothesis, we partially depleted MCM2 by short interfering RNA (siRNA) in parental HCT116 and RPE1. The abundance of another subunit, MCM7, conjointly decreased, indicating a...
Figure 5 | The abundance of replication factors is decreased in response to extra chromosomes. (a) Unsupervised hierarchical clustering of the protein abundance fold changes (calculated log2 aneuploid-to-diploid ratio) of factors assigned to KEGG (Kyoto Encyclopedia of Genes and Genomes)-defined term replication; manually curated. HCT116 5/4 and HCT116 3/3 marked in the figure in bold are cell lines previously constructed by Minoru Koi; these cell lines were used only for the global proteome analysis. See Methods for more details. (b) Immunoblotting of subunits of the replicative helicase MCM2-7 in whole-cell extracts from trisomic cell lines and their respective controls. (c–d) Levels of chromatin-bound replication proteins in the parental cell lines and the trisomic and tetrasomic cell lines. Note that MCM2, 3 and 7 are downregulated in all cell lines with extra chromosomes except for RPE1 5/3 12/3 c3. Ponceau staining was used as loading control. (e,f) Levels of chromatin-bound subunits of the MCM2-7 helicase in asynchronous and synchronized cells in HCT116 5/3 and RPE1 21/3, and respective controls.

The partial depletion of MCM2 was sufficient to trigger accumulation of 53BP1 foci and an increased frequency of anaphase bridges in the presence or absence of aphidicolin in HCT116 (Fig. 6b,c) as well as in RPE1 (Supplementary Fig. 7a–c). Other replication factors such as CDC6, ORC2 and RPA1 were also partially downregulated in some cell lines with extra chromosomes; however, downregulation of CDC6 and ORC2 in control cells did not trigger accumulation of 53BP1 foci and anaphase bridges, whereas partial downregulation of RPA1 caused high levels of DNA damage (Supplementary Fig. 7d–i). To further test which of the downregulated replicative factors are limiting in cells with extra chromosomes, we transfected both diploid cells and respective trisomic and tetrasomic cell lines with plasmids carrying either functional or mutant alleles of MCM2, ORC1 and RPA1. The mutant protein MCM2-457 A cannot be phosphorylated by Dbf4-dependent kinase (DDK) kinase, thereby rendering replication firing inefficient\( ^3 \). ORC1ΔBAH shows decreased binding to DNA\(^2 \), and RPA1 L221P impairs DNA replication and leads to accumulation of DNA damage\(^3 \). None of the mutant alleles exert detrimental effects in the presence of the endogenous wild-type allele; however, they are toxic for a cell in the absence of the wild-type protein. We found that overexpression of the MCM2-457 A allele was highly toxic in trisomic and tetrasomic cell lines, but showed no effect in the control cell lines (Fig. 6d). In contrast, overexpression of ORC1ΔBAH and RPA1 L221P mutants did not impair the proliferation of any of the aneuploid cell lines significantly more than overexpression of the wild-type allele (Fig. 6e,f). We conclude that the MCM helicase is the limiting factor for replication in cells with extra chromosomes.

Finally, we asked whether increasing the levels of MCM2-7 can rescue the accumulation of DNA damage in aneuploid cells. To this end, we transiently transfected HCT116 5/3 and 5/4 with either empty pcDNA vector or with a version carrying pCMV-MCM7. The levels of the chromatin-bound MCM7 as well as MCM2 increased in cells transfected with pCMV-MCM7...
in comparison with control cells (Fig. 6g). Importantly, the increase in the MCM2-7 abundance resulted in a significant decrease in 53BP1 foci formation as well as lower occurrence of anaphase bridges in four different cell lines with extra chromosomes (HCT116 3/3, 5/3, 5/4 and RPE1 21/3), but did not affect the control HCT116 or RPE1 (Fig. 6h,i and Supplementary Fig. 8a,b). Overexpression of other replication factors (PolD1,4, RPA1,2,3 and CDC6) or control renilla luciferase did not affect the accumulation of 53BP1 foci (Supplementary Fig. 8c,d). Supplementing the medium with a high concentration of DNA synthesis precursors showed a mild rescue of the 53BP1 foci formation in only one cell line and no rescue of the EdU incorporation (Supplementary Fig. 8e–g). Altogether, our findings strongly suggest that the presence of extra chromosomes causes replication stress and genomic instability in human cells owing to downregulation of components of the MCM2-7 helicase.

**Discussion**

Here we demonstrate for the first time that the addition of even a single chromosome to human cells promotes genomic instability by increasing DNA damage and sensitivity to replication stress. Replication stress is emerging as one of the primary causes of...
genomic instability during early stages of tumorigenesis9–11. Our findings provide a rationale for how random missegregation of a single chromosome might contribute to genomic instability and potentially to early events in tumorigenesis.

To systematically analyse the effect of trisomy and tetrasmoy on genomic instability in human cells, we analysed five different trisomies and tetrasomies derived from HCT116 and five RPE1-derived cell lines. Remarkably, we found strikingly similar phenotypes regardless of the identity of extra chromosomes. Indeed, the presence of extra chromosomes leads to (1) accumulation of DNA damage likely originating from replication errors (Figs 1 and 2); (2) increased sensitivity to additional replication stress (Fig. 3a,b); and (3) abnormal DNA replication (Supplementary Fig. 3 and Fig. 3c). The uniformity of the phenotypes and the fact that the degree of some defects tends to scale with the amount of the added DNA suggest that the presence of an additional chromosome alone is the culprit, which causes the genetic instability in these cells.

The altered DNA replication and increased DNA damage affects genomic instability in trisomic and tetrasomic cell lines, as documented by the increased occurrence of de novo CNAs. These structural rearrangements were caused by the presence of extra chromosomes, as parental HCT116 did not show any CNAs after the same number of generations (Fig. 4a,d). The CNAs affected all chromosomes evenly, and no enrichment on the extra chromosome was observed. The pattern of identified CNAs, showing a bias towards deletions and tandem duplications, and the occurrence of microhomology at the break point junctions further support the idea that the addition of extra chromosomes specifically impairs DNA replication, thus inducing replication-induced breaks at stalled/arrested replication forks31.

What causes the genetic instability in response to extra chromosomes? It has been shown that trisomy and tetrasomy cause genome-wide gene expression changes; one of the most prominent features is a strong downregulation of factors related to DNA replication20,21. In particular, the subunits of the replicative helicase MCM-2-7 are significantly downregulated in all aneuploid cell lines that we analysed (Fig. 5). Loading of the MCM helicase together with ORC2-5 proteins, CDT1 and CDC6, is essential for licensing of origins of replication, and MCM is also required for fork progression. Replication dynamics are unaffected by partial depletion of MCM under normal growth conditions as the number of MCM complexes loaded onto the DNA is greater than the number of the actual active origins34,35. Under replication stress, however, a reduction in the level of MCM proteins leads to a decreased licensing of dormant origins that are needed to allow completion of the DNA replication. Accordingly, cells are markedly sensitive to MCM dosage changes. Several lines of evidence suggest that the observed decrease of 20–50% of MCM2-7 abundance indeed contributes to the genomic instability observed in cells with extra chromosomes. First, depletion of MCM in control cells to levels equivalent to those observed in tri- and tetrasmoyes results in comparable increases in DNA damage foci, whereas partial depletion of ORC2, RPA1 and CDC6 did not cause similar phenotypes (Fig. 6a–c and Supplementary Fig. 7a–i). Second, depletion of MCM3 was previously shown to lead to an increase in fragile site instability39, similarly to cells with extra chromosomes that show increased levels of fragile sites on metaphase spreads under replication stress conditions. Moreover, fragile site instability may originate from origin paucity and/or from a failure in dormant origin activation39,40. Third, mouse models homozygous for an MCM4 mutation that impairs the stability of the MCM4 subunit, or an MCM2RES-CreERT2 mutation that reduces the levels of MCM2, suffer from impaired proliferation, slow replication rates and genomic instability41–43, comparable to trisomic and tetrasmoy cell lines in this study. Fourth, overexpression of mutant MCM2 protein is toxic in cells with extra chromosomes but not in control cell lines, whereas mutant RPA1 and ORC1 do not affect the proliferation of aneuploid cell lines more than overexpression of the wild-type allele, suggesting that indeed the MCM complex is the limiting DNA replication factor in trisomic and tetrasmoy cell lines. Finally, we show that elevating the levels of MCM2-7 by exogenous overexpression alleviates the replication-induced defects and DNA damage in aneuploid cells (Fig. 6g,h). The effect of MCM overexpression was stronger in trisomies and tetrasmoyes with a relatively high levels of genomic instability, such as HCT116 5/4 or RPE1 21/3, than in HCT116 5/3 with a rather mild defect. This is in agreement with the idea that high levels of MCM2-7 helicase become critical in cells with elevated replication stress, and also suggest that other factors play a role in genomic instability of aneuploids as well.

Alternative explanations for the observed phenotypes are possible. For example, overexpression of a specific gene located on a supernumerary chromosome might impair maintenance of genome stability. However, the fact that the trisomies and tetrasmoyes of five different chromosomes (3, 5, 8, 12 and 21) lead to strikingly similar phenotypes does not support this explanation. Another possibility is that the extra DNA simply titrates away a limiting factor, thus slowing down replication. This hypothesis implies that additional DNA and its replication alone should trigger the same phenotypes. However, transcriptional silencing of chromosome 21 using XIST expression in cells with trisomy 21 results in improved proliferation44, similarly as the full removal of chromosome 21 by counter-selection against TKNNeo45. Moreover, haploid yeast with extra chromosomes show genetic instability, but the phenotype is not observed in cells that contain a yeast artificial chromosome that can be replicated, but not transcribed by these cells46. Thus, we propose that it is not the need to replicate additional DNA, but rather the pathway deregulation and stoichiometric imbalances caused by aneuploidy that promote the observed genetic instability.

Why the replication factors are downregulated in response to aneuploidy remains enigmatic. This question goes together with the observation that aneuploidy causes global expression changes, with DNA replication being among the most consistently downregulated pathways across several analysed cell lines and species20,23. One possibility is that some pathways are deregulated due to the proteotoxic stress and protein folding defect in trisomic and tetrasmoy human cells23. Accordingly, several factors involved in DNA repair and replication are well-characterized clients of molecular chaperones46. Alternatively, activity of E2F transcription factors that control expression of MCM subunits or DNA polymerases47 might be altered in response to trisomy and tetrasmoy. Interestingly, recent results revealed that ageing haematopoietic stem cells in mice suffer from replication stress, cell cycle defects and chromosomal aberrations owing to a decreased expression of MCM helicase48. The reasons for the downregulation of MCM in ageing haematopoietic stem cells remain unclear. Together with our observations in trisomic and tetrasmoy cells, we suggest that the expression levels of MCM helicase are a highly sensitive measure of cellular stress.

In summary, our results show that addition of even a single chromosome renders human cells sensitive to replication stress and elevates the occurrence of CNAs in human cells, thereby suggesting a novel mechanism for how chromosome segregation errors may fuel genomic instability. The observed phenotypes may provide new insights into the causes of developmental defects associated with congenital trisomies and might help address the question of why patients with Down’s syndrome show altered spectra of malignant diseases with an increase in haematological malignancies and a decrease in solid tumours49.
Markedly, cancer cells often express DNA replication factors including the MCM helicase at high levels, and MCM2 and MCM7 are considered as biomarkers and potential therapeutic targets for cervical, colorectal and other tumours (for example, refs 50–52). Identification of the mechanisms that allow cancer cells to adapt to aneuploidy by elevating the expression of replication factors might illuminate novel opportunities for cancer therapy.

**Methods**

**Cell lines.** The cell line RPE1 IKTERT (referred to as RPE1) and RPE1 IKTERT H2B-GFP were a kind gift from Stephen Taylor (University of Manchester, UK). HCT116 H2B-GFP was generated by lipofection (FugeneHD, Roche) of HCT116 (American Type Culture Collection no. CCL-247) with pBOS-H2B-GFP (BD Pharmingen) according to manufacturer’s protocols. Trisomic and tetrasomic cell lines were generated by microcell-mediated chromosome transfer as described below. The donor mouse cell lines A9(Neo3), A9(Neo5), A9(Neo8) and A9(Neo21) were purchased from the Health Science Research Resources Bank (HSRRB), Osaka, Japan. All cell lines were maintained at 37°C with 5% CO2 atmosphere in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% foetal bovine serum, 100 U penicillin and 100 U streptomycin. The cell lines HCT116 3/3, HCT116 H2B-GFP 5/3, HCT116 H2B-GFP 5/4, RPE1 5/3 12/3, RPE1 H2B-GFP 21/3 and A9(Neo5) were grown supplemented with 400 µg ml−1 G418. The cell line HCT116 5/4 as well as the cell lines stably transfected with H2B-GFP were grown in media supplemented with 6 µg ml−1 blasticidin S. Before each experiment, aneuploid cells were grown one passage in medium without the antibiotic selection and were then tested for mycoplasma contamination. The cell lines HCT116, HCT116 5/4 and HCT116 3/3 were kindly provided by Minoru Koi, Baylor University Medical Centre, Dallas, TX, USA. These cell lines were used only for the global proteome analysis (Fig. 5a). For nucleic acid purification, the culture medium was supplemented with adenosine (Sigma A9251), cytidine (Sigma C6454), uridine (Sigma U3750) and guanosine (Sigma G6752) to a final concentration of 50 µM for 48 h. In order to synchronize cells in S-phase, HCT116- and RPE1-derived cells were cultured for 30 h in 2 mM thymidine, washed three times in PBS and were released into the standard DMEM medium.

**Microcell-mediated chromosome transfer.** To generate aneuploid HCT116 and RPE1 containing an additional chromosome 3, 5, 8 or 21, microcell fusion (Supplementary Methods) was used. Briefly, murine donor cells containing additional human chromosome with a resistance gene were treated for 48 h with colchicine (final concentration 60 ng ml−1). Donor cells were trypsinized and seeded on plastic dishes. After the cells were attached to the surface, bullets were centrifuged at 27,000g for 30 min at 30–34°C in DMEM supplemented with 10 µg ml−1 cytochalasin B. Cell pellets were resuspended in serum-free DMEM and filtered to clear suspension from mouse cells. Filtered microcells were mixed with phytohaemagglutinin (PHA-P) and added to the recipient cell line HCT116 H2B-GFP, RPE1 or RPE1 H2B-GFP. Fusion of microcells with the recipient cells and filters were incubated for 10–15 min at 37°C. After fusion, the cell-bullets were filtered to clear suspension from mouse cells and washed several times in 3:1 methanol/acetic acid, spread on a wet glass slide and air dried at 42°C for 5 min.

**Chromosome painting.** Chromosome spreads were prepared as described above. Each sample was labelled with probes for two different chromosomes: a transferred chromosome and a control chromosome. Probes (Chromobios GmbH, Raubling, Germany) for chromosomes 2, 3, 5 and 21 were tagged with TAMRA, FITC, Cy-5 and TAMRA, respectively. The chromosomes were labelled according to the manufacturer’s instructions and counterstained with 4’,6-diamidino-2-phenylindole (DAPI). Images were obtained by a fully automated Zeiss inverted microscope.

**Array comparative genomic hybridization.** Genomic DNA for aCGH analysis was extracted using the Qiagen Gentra Puregene Kit according to manufacturers’ instructions. The aCGH analysis was performed by IMGM Laboratories, Martinsried, Germany. Commercially available human genomic DNA (Promega) was used as a reference sample for all four 4 × 4K array-based analyses (HCT116, HCT116 5/4, HCT116 3/3, HCT116 H2B-GFP 5/4, RPE1 and RPE1 5/3 12/3). RPE1 H2B-GFP 21/3 was analysed by SurePrint G 4 × 180K G3 Human CGH Microarray. Genomic DNA extracted from HCT116 was used as a reference for the high-density CGH analysis of HCT116 5/4 by the 2 × 400K array. gDNA concentration and DNA absorbance ratio (260 nm/280 nm) were measured by NanoDrop ND-1000 ultra-low (260 nm/280 nm) was measured by NanoDrop ND-1000 ultraviolet–visible spectrophotometer (PeqLab). An amount of 1 µg of gDNA was used for each reaction. gDNA integrity was tested on an 1.0% agarose gel stained with ethidium bromide. A measure of 1.0 µg of DNA was subjected to restriction digestion with a combination of Alu I and Rsa I restriction enzymes. The digested gDNA samples were directly labelled with each restriction enzyme primer fragments and radiolabelled using Oligo-a-GTP (dUTP = 2’-deoxyuridine 5’-triphosphate) for the experimental samples and Cy-3 dUTP (dUTP = 2’-deoxyuridine 5’-triphosphate) for the reference samples (Genomic DNA Enzymatic Labeling Kit, Agilent Technologies). After purification, each experimental sample was combined with its respective reference sample and hybridized to respective arrays. All microarrays had been washed with increasing stringency using Oligo-a-GTP (dUTP) and Cy-3 dUTP (dUTP) (Agilent Technologies) followed by drying with acetone (Sigma-Aldrich). Fluorescent signal intensities for both dyes were detected with Scan Control 8.4.1 Software (Agilent Technologies) on the Agilent DNA Microarray Scanner and extracted from the images using Feature Extraction 10.5.1.1 Software (Agilent Technologies). The software Feature Extraction 10.5.1.1 as well as the software Genomic Workbench 5.0.14 was used for quality control, statistical data analysis and visualization. Raw microarray data were normalized. ADM-2 aberration algorithm was applied together with centralization algorithm. Aberrations for all samples were filtered from the whole-genome data and analysed based on a threshold of log2 ≥ 0.39 for amplifications and log2 ≤ −0.30 for deletions with at least five consecutive aberrant probes.

**MATE-pair NGS.** Whole-genome mate-pair sequencing of HCT116 5/3 and HCT116 5/4, and the respective parental control was performed as described in 44. Briefly, DNA was sheared to 3 kb fragments, followed by mate-pair library preparation according to the SOLiD 5500 Long Mate-Pair procedure (Life Technologies). Each library was sequenced in 2 × 50 bp configuration on a single lane of a SOLiD 5500 instrument. Colour space reads were mapped to the human reference genome (GRCh37) using BWA version 0.5.0. Discordant mate pairs were detected and clustered for all samples together as described 44. Unique clusters of discordant mate-pair reads were identified by filtering out clusters supported by read pairs in >1 sample. Based on discordant mate-pair clusters, we designed primers for break point junction amplifications using Primer3 software. PCR amplifications were performed using AmpliTag Gold polymerase (Life Technologies) under standard conditions. Sanger sequencing was performed to identify break point junction sequences at nucleotide resolution.

**SNP-array profiling.** Human CytoSNP-12 bead chip arrays (Illumina) for detecting CNAs were used in clonal aneuploid and control cell lines. Array hybridization was performed according to the manufacturer’s recommendations. CNAs were called using Nexus software (version 7.5.1) with standard settings. To identify unique CNAs in clonal cell lines, we used the Nexus call coordinates and removed all calls of the same type with a reciprocal overlap of at least 60%. Unique CNA calls were manually curated based on copy number and allele frequency profiles. Permutation testing was performed to calculate the significance of the differences in the distribution of unique CNAs among HCT116 (controls) and HCT116 5/3 (cases) clonal lines. We generated 1,000,000 permutations of the case/ control labels per set and determined the P value as the proportion of randomizations where all 12 unique CNAs were found in HCT116 5/3 clonal lines.

**Mapping the fragile sites.** Genomic positions of common and rare fragile sites were obtained from refs 32,33. The coordinates matching the chromosomal bands with fragile sites were overlapped with CNA break points using BEDTools 45. The genomic coordinates were obtained from ref 32,33. The coordinates matching the chromosomal bands with fragile sites were overlapped with CNA break points using BEDTools 45. The positions matching the genome coordinates were obtained from ref 32,33.
cells were fixed with 3.7% buffered formaldehyde (12 min at room temperature), permeabilized in 0.2% Triton X-100 in PBS (5 min) and co-immunostained with 33B1 (1:1,000; Santa Cruz sc-22760) and cyclin A2 (1:200; Abcam ab61726) overnight at 4°C, followed by a secondary anti-rabbit antibody (Alexa Fluor 647 1:1,000; Jackson ImmunoResearch 711-605-152) and anti-mouse antibody (Alexa Fluor 594 1:1,000; Jackson ImmunoResearch 715-858-150) for 1 h at room temperature.

Electroporation. Cells were electroporated using the Amaxa Nucleofector II apparatus according to the manufacturer’s instructions for HCT116 and RPE1 cells, respectively. In brief, 1 million cells were resuspended in Cell Line Nucleofector Solution V containing 2 μg of respective plasmids and transferred to cuvettes. For siRNA, cells were resuspended in Cell Line Nucleofector Solution V containing the indicated concentration of siRNA. The previously sequenced siRNAs were acquired from Eurofins Genomics and are as follows: MCM2 (5'-GGGACACAAUGUAGGUCAUGGAG-3'), GL2 (5'-CGAUGCGGAUAUCUUGATT-3'), ORC2 (5'-GAUACGCUAGACUGGUAAG-3'), CDC6 (5'-ACAUAGACACAAUAAGGC-3') and RPA2 (5'-AAACUGGGUGAACGG-3'). HCT116 cells were electroporated using the D-032 program, and for RPE1 cells the program was U-017. Co-transfection with a GFP overexpressing vector revealed that ~50–70% of the cells are transfected in each experiment. All cells were scored for 33B1 foci and anaphase bridges quantification after transfection.

Statistical analysis. Statistical analyses were performed using two-tailed t-test, two-tailed Fisher’s exact test or χ²-test as indicated in the corresponding figure legend (t-test; P<0.05, **P<0.01, and ***P<0.001). Values are shown as the mean ± s.e.m. of multiple independent experiments. The quantifications were software based (CellProfiler); in other cases, the investigators were blinded to allocation during outcome assessment.

**Microscopy and image analysis.** Confocal microscopy was performed using a fully automated Zeiss inverted microscope (AxioObserver Z1) equipped with a Plan Neofluar 40x/0.75 (Carl Zeiss) objective. Acquired images were processed using the Slidebook software (version 5.0; Intelligent Imaging Innovations). The numbers of 53BP1 foci per nucleus were determined by an automated pipeline using the public domain, free software CellProfiler (http://www.cellprofiler.org/). Image processing was performed for the whole data set using standard CellProfiler modules for illumination correction, segmentation, thresholding and manual counting. For 53BP1 foci, if two foci were detected in the same channel, the distance between foci was calculated in ImageJ.

Metaphase preparation for chromosomal fragility analysis. Cells were harvested after a 40-min treatment with 100 μM/ml colchicine followed by a 30-min incubation in 0.4% KCl at 37°C for multiple changes of 3:1 methanol:acetic acid fixative. Cells were then dropped onto slides and stained with propidium iodide. Gaps and constrictions on metaphase chromosomes were analysed using a Nikon fluorescent microscope. At least 50 metaphases for each condition were analysed. Gaps and constrictions on metaphase chromosomes were analysed using a Nikon fluorescent microscope. At least 50 metaphases for each condition were analysed. Gaps and constrictions on metaphase chromosomes were analysed using a Nikon fluorescent microscope. At least 50 metaphases for each condition were analysed.
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