Infection with retroviral vectors leads to perturbed DNA replication increasing vector integrations into fragile sites

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Genome instability is a hallmark of cancer. Common fragile sites (CFSs) are specific regions in the human genome that are sensitive to replication stress and are prone to genomic instability in different cancer types. Here we molecularly cloned a new CFS, FRA11H, in 11q13. The genomic region of FRA11H harbors a hotspot of chromosomal breakpoints found in different types of cancer, indicating that this region is unstable during cancer development. We further found that FRA11H is a hotspot for integrations of Murine Leukemia Virus (MLV)-based vectors, following CD34+ infections in vitro as well as ex-vivo during gene therapy trials. Importantly, we found that the MLV-based vector infection in-vitro leads to replication perturbation, DNA damage and increased CFS expression. This suggests that infection by MLV-based vectors leads to replication-induced genome instability, raising further concerns regarding the use of retroviral vectors in gene therapy trials.

Common fragile sites (CFSs) are specific regions in the human genome that appear as gaps and constrictions in metaphase chromosomes from cells grown under mild replication stress conditions1. These sites are present in all individuals and are considered to be part of the normal chromosomal structure. In vitro, most CFSs are induced using low concentrations of aphidicolin, an inhibitor of polymerase α, δ and ε2,3. Under these conditions, CFSs are hotspots for sister chromatid exchanges, translocations and deletions4–6. At present, 87 common fragile sites have been characterized at low resolution by their cytogenetic appearance on metaphase chromosomes (http://www.ncbi.nlm.nih.gov/gene), but the exact repertoire of common fragile sites depends on the inducer, cell type and method of analysis. Using fluorescence in situ hybridization (FISH) 39 of the CFSs have been molecularly cloned, and their exact genomic localization determined7.

In vivo, CFSs correlate with chromosomal breakpoints in tumors8,9. Recently, several studies have analyzed genomic deletions in cancer cells and demonstrated that CFSs are preferentially unstable in the early stages of cancer development and precede instability in non-fragile regions10–16. A large scale analysis of 750 cancer cell lines revealed an instability signature in CFSs that differed significantly from that found in cancer genes, indicating that there is a unique mechanism leading to genomic instability in fragile sites8. CFSs were also found to be involved in the occurrence of duplications and amplifications in cancer12–14. The breakpoints setting the amplicon boundaries are within the CFSs and they drive breakage-fusion-bridge (BFB) cycles.

Altogether, the results of these studies suggest that DSBs at fragile sites may themselves contribute to oncogenesis by triggering genomic instability that can lead to disruption of tumor suppressor genes or oncogenes harbored within these regions.

Another type of instability at CFSs involves the integration of foreign DNA into the genome. In vitro studies have shown that under replication stress conditions, plasmid DNA integrates preferentially into CFSs17. In vivo, CFSs were also found to be preferential sites for integration of viral DNA. Human papillomavirus (HPV), the most important cancer related virus, was found to integrate preferentially into CFSs in the genome of most cervical cancer cells18. Another oncogenic retrovirus found to preferentially integrate into CFSs is the Xenotropic Murine Leukemia related virus (XMRV)19, a relatively new type of retrovirus associated with a subset of prostate tumors20.

We have previously found preferential integrations of retroviral-derived vectors, based on Moloney Murine Leukemia virus (MLV), into CFSs21 both in vitro and in gene therapy trials. Unfortunately, in two gene therapy trials on patients with X-linked severe combined immunodeficiency (SCID-X1), five of the 20 treated patients developed monoclonal acute lymphoblastic leukemia-like lymphoproliferation. The leukemia in four of these patients involved activation of the LOM2 proto oncogene22,23, which resides within the CFS FRA11E21.
Here we studied a region in 11q13 known to be a hotspot for chromosomal instability in many cancer types. Recurrent amplifications in this region are found in 15% of breast carcinomas, 13% of lung cancers, 21% of bladder tumors, 50% of esophageal cancers and 45% of oral squamous cell carcinomas. Recently, a large scale analysis of 50 gastric tumors revealed that the 11q13.3 region is among the most common genomic deleted regions.

The 11q13 band encompasses 13.7 Mb, in which a CFS FRA11H was characterized by low resolution cytogenetic mapping (http://www.ncbi.nlm.nih.gov/gene/?term=FRA11H). However, whether this CFS contributes to the instability in 11q13 remains unclear.

Here we show that the recurrent instability leading to amplifications in 11q13 is within the common fragile site FRA11H. We further reveal that FRA11H is a preferential site for retroviral integrations. For this we first analyzed 1238 retroviral integration sites (RISs), mapped in cells of patients with X-linked SCID (Supplementary Table 1). The results revealed that both BAC clones show FISH signals proximal, distal or on both sides of the chromatin constrictions and gaps in the 11q13 region (Figure 1 and Table 1).

These results indicate that a genomic region of 7 Mb flanked by the analyzed probes is part of the CFS, FRA11H, and that this fragile site is probably involved in chromosome instability in different cancer types.

We further studied whether FRA11H is a preferential site for retroviral integrations. For this we first analyzed 1238 retroviral integration sites (RISs), mapped in cells of patients with X-linked SCID patients.

Table 1 | Summary of FISH results. The position of the FISH probes relative to the gaps and constrictions in 11q13. The results of the adjacent BACs RP11-456I5, RP11-707G14 and RP11-809N8 were pooled.

| BAC name     | Telomeric | On | Split | Centromeric |
|--------------|-----------|----|-------|-------------|
| RP11-15L8    | 19        | 3  | -     | 3           |
| RP11-149G19  | 16        | -  | 1     | 3           |
| RP11-456I15  | 8         | 3  | -     | 9           |
| RP11-707G14  |           |    |       |             |
| RP11-809N8   |           |    |       |             |
SCID (SCID-X1) treated with a retrovirus-based gene therapy vector carrying the γ chain (γc) gene
26,27. Our analysis revealed 10 retroviral integrations into the ~7 Mb of FRA11H (Figure 1), a significantly
higher frequency than the frequency in the entire non-fragile regions in the genome (binomial test p < 0.001). We then analyzed more
than 40,000 MLV integrations into human CD34+ cells recently mapped by Cottoglio et al.28. Of these, 2,197 unique integrations were
mapped on chromosome 11. This massive integration mapping allowed us to study the distribution of viral integrations into CFSSs
at a higher resolution and statistical power. First we analyzed viral integrations into FRA11H and compared this to the frequency of
integrations along the entire chromosome 11 (excluding centromeric, telomeric sequences and other mapped CFSSs: FRA11E,
FRA11F and FRA11G (Supplementary Table 2)). Our analysis revealed that the 7 Mb FRA11H region harbors 170/2197 MLV inte-
grations, which is significantly higher than the frequency in the 110 Mb non-fragile regions in chromosome 11 (p = 1.63*10^{-5}).

Previously, we analyzed a limited number of MLV integrations into another fragile site on human chromosome 11, FRA11E29. Here
we analyzed the viral integration into FRA11E in the large integration database mapped by Cottoglio et al.28. The analysis revealed
an extremely high MLV integration frequency, with 67 integrations into the 2 Mb of FRA11E, significantly different from the integration
frequency into the entire chromosome 11 (p = 2.12*10^{-5}). These results are consistent with our previous report on a limited number of
integration sites mapped in SCID patients, which indicated that FRA11H is a hotspot for MLV integrations in human CD34+ cells21.

We further aimed to investigate the basis for the preferential integrations of retroviral MLV-based vectors into CFSSs. CFSSs are sen-
titive to replication stress conditions and under these conditions their chromatin is uncondensed even in mitosis. Hence, we raised the
possibility that MLV retroviral infection leads to replication stress resulting in open chromatin conformation in fragile sites which
might predispose these regions to the MLV integration protein complex29. To test this possibility we infected HeLa cells with an
MLV-based vector (based on pTG-nls-LacZ plasmid), with high infection efficiency (~96%) (Figure 2). Using the high resolution
DNA combing method, which enables replication analysis of single DNA molecules (an example is shown in Figure 3a), we analyzed the
DNA replication rate 24 hours following infection, and compared it
to the rate in control cells, grown for 24 hours in a medium from
packaging cells that does not produce virions. Our results revealed a
significant reduction (~35%) in the replication rate following viral
infection. The mean replication rate for the control HeLa cells was
1.32 ± 0.04 Kb/min (n = 111), whereas the MLV based-vector infection significantly decreased the rate to 0.86 ± 0.02 Kb/min (n =
237) (p < 7.8*10^{-5}) (Figure 3b). Interestingly, following MLV infection the distribution of fork rates changed dramatically. In the
control cells, in 25% of the forks the rate was >1.5 Kb/min, whereas
only 3.3% of the forks progressed at this high rate following infection
with the MLV-based vector. In order to verify that MLV-induced
replication stress is a general phenomenon and is not cell-specific,
we further analyzed the replication dynamics in normal human fibroblast cells expressing hTERT (BJ). As was found for HeLa cells,
the replication rate significantly (p-value = 1.188e-07) decreased following the MLV-based vector infection, from an average of 2.05 ± 0.07 Kb/min (n = 108) in the control non-infected cells, to 1.55 ± 0.06 Kb/min (n = 94) in BJ cells infected by the MLV-based vector (Figure 3c). Furthermore, in BJ infected by the MLV-based vector a dramatic (4-fold) increase in the fraction of slow forks (<1 Kb/min) was found, from 3.7%, in non-infected control cells to 14.8% in the infected cells. The perturbed replication dynamics described in BJ cells is clearly an under-estimation of the phenomenon, since only 65 ± 7% of the cells were infected by the vector (performed in three independent experiments), as revealed by X-gal positive staining. Altogether, our results indicate that a short time after retroviral-based vector infection, the DNA replication was dramatically perturbed. This replication perturbation may lead to the collapse of replication forks, resulting in DNA damage.

In order to study whether the replication perturbation caused by retroviral infection is the result of high viral titer, leading to high multiplicity of infection (MOI), we infected HeLa cells with GFP reporter gene, by a low MOI (1 MOI) of MLV-based vectors. 24 h post infection, cells were sorted by fluorescence-activated cell sorting (FACS), to differentiate GFP positive and negative cells. Replication analysis using DNA combing revealed a significantly slow fork progression rate in the GFP cells (average of 0.6 ± 0.02 Kb/min (n = 127)), compared to the GFP negative cells (average of 1.0 ± 0.04 Kb/min (n = 71)) (p-value = 1.441e-11).

Lentiviral-based vectors are considered as good candidate vectors for gene therapy trials due to their potential to mediate a prolonged therapeutic gene transfer, both in dividing and non-dividing cells. We further analyzed the effect of lentiviral infection on the replication dynamics in HeLa cells infected with a lenti Green Fluorescent Protein (GFP) vector. Our results show that even with a high titer (10 MOI), the replication fork rate remains similar to the observed rate in the non-infected (GFP-negative) cells (average of 1.0 ± 0.03 Kb/min (n = 107)) (p-value = 0.89) (Supplementary Figure 1). This result indicates that replication stress caused by MLV-based vectors, is not a general characteristic of viral infection.

We further explored whether the replication perturbation conferred by retroviral MLV vector infection leads to fragile site expression. For this we analyzed HeLa cells 24 hours after infection with a retroviral-based vector. Our results show that retroviral infection leads to a significant (P < 0.005) level of gaps and constrictions in the infected cells (1.93 CFSs/metaphase) compared to non-infected control cells (1.19 CFSs/metaphase) (Figure 4). Importantly, half of the retroviral-infected cells showed a significant number of fragile sites (≥2). This indicates that infection with a retroviral-based vector leads to the expression of CFSs as a result of replication stress.

In order to investigate the possibility that the replication perturbation induced by the retroviral MLV-based vector infection leads to accumulation of DSBs we analyzed γH2AX foci, which are known to be formed at DSBs (Figure 5a) in HeLa cells 24 h post-infection with an MLV-based vector. Immunofluorescence analysis revealed significantly (p < 8.9*10^-4) more γH2AX foci in HeLa cells 24 h post infection by an MLV-based vector, with an average of 2.25 ± 0.4 foci/cell (n = 60) compared to 0.87 ± 0.2 foci/cell (n = 61) in the control cells (Figure 5b). Next we analyzed DSB-formation in BJ cells infected with an MLV-based vector. Because the infection in BJ cells was less efficient (only 67% of the cells were positive to the x-gal staining (data not shown), or the αβ-gal antibody (Figure 2b)), we analyzed γH2AX foci only in β-gal positive cells (Figure 5c). Immunofluorescence analysis revealed a significant (p < 5*10^-14) increase in γH2AX foci in BJ cells, 24 h post- infection by the MLV-based vector, with an average of 3.5 ± 0.2 foci/cell (n = 105), compared to 0.95 ± 0.2 foci/cell (n = 103) in the control cells (Figure 5d). In order to verify that the observed effects of the MLV-based vector infection resulted from the viral infection and not from the introduction of foreign DNA, we analyzed the formation of DNA damage following transfection with a plasmid DNA which is the backbone of the retroviral-based vector. As the transfection efficiency of the retroviral plasmid was only >20%, we transfected HeLa cells with the retroviral plasmid encoding GFP (pLVC-GFP). No significant difference (p = 0.67) in the number of γH2AX foci in the GFP positive cells (2.4 ± 0.3, n = 57) was found compared to the GFP negative cells (2.5 ± 0.2, n = 320) (Figure 6), although the transfection itself is toxic, resulting in DNA damage and cell death. These results indicate that transfection of plasmid DNA by itself does not lead to replication-induced DSBs, indicating that such DSBs are specific to the infection by retroviral virons.

In summary, our results reveal that retroviral infection leads to replication perturbation and DSBs suggesting a mechanism for the significantly higher frequency of retroviral integration into CFSs.

Discussion

Genome instability is a hallmark of cancer cells. One type of instability is amplifications that increase the expression of oncogenes. The
Figure 5 | DSBs accumulated following retroviral-based vector infection. (a) Examples of HeLa cells infected with MLV-based vector following immunofluorescence with anti-β-gal (green) and γH2AX (red). The green outlines mark positive β-gal cells. Nuclei were stained with DAPI (blue). (b) Percent of nuclei with the indicated number of γH2AX foci. HeLa infected by a retroviral based vector (HeLa-Retro, n = 60), control cells (HeLa-control) (control, n = 60), (p < 8.9*10^-24). (c) Examples of BJ cells infected with MLV-based vector following immunofluorescence with anti-β-gal (green) and γH2AX (red). The green outlines mark positive β-gal cells. Nuclei were stained with DAPI (blue). (d) Percent of nuclei with the indicated number of γH2AX foci. BJ cells infected by a retroviral-based vector (BJ-Retro, n = 105), control cells (BJ-control) (control, n = 103), (p < 5*10^-14); (these results are a summary of two independent infections).
high frequency of amplifications found in 11q13 indicates that this region harbors an oncogene/s which may give a proliferative advantage to the cancer cells. Indeed FRA11H harbors the oncogene CCND1 that encodes the cyclin D protein, which is involved in different types of cancer32. However, the 11q13 amplified region in these cancers is significantly larger than the oncogene, indicating that in addition to the selection force, other mechanisms contribute to 11q13 instability. One such mechanism involves the inherent instability of CFS under replication stress conditions. Instability in CFSs was shown to drive genomic amplifications by setting the boundaries of the amplicons. This mechanism was described for the amplification of different oncogenes including MET (FRA7G)12,14,33, PIP (FRA7I)34, SMAD5 (FRA5C)35 among others36,37. The preferential breaks promoting genomic amplifications within the replication-sensitive CFSs indicate that the instability in these sites involves replication stress conditions. Several studies have indicated that there is replication stress at early stages of cancer development16,38 which is a result of aberrant activation of oncogenes10,15,39. Our results revealed a large fragile site (at least 7 Mb long), FRA11H, in 11q13, which may explain the large amplifications at this site in many cancers.

A different type of genome instability is the integration of foreign DNA into the human genome. Retroviral integrations are mediated by viral and cellular proteins, and are distributed along the genome with a preference for transcription start sites (TSS) and regulatory elements28,40,41 and DNase I hypersensitive sites42. Large scale analyses of retroviral based vectors have found preferential integration of viral DNA into CFSs29,31. Here we showed that the CFS FRA11H is a preferential site for MLV-based vectors. Many fragile sites are involved in genomic instability in cancer43. It is important to note that 11q13, the chromosomal band harboring FRA11H, is a known hotspot for genomic instability in different types of cancer (Supplementary Table 1), although further studies are required to shed light on the importance of FRA11H sequences as a hotspot for genomic instability in cancer.

We further showed that in vitro infection by a retroviral-based vector leads to perturbed DNA replication of the host cell, as indicated by the decrease in replication rate and the increase in DNA damage (Figures 2 and 4). The infection also leads to CFS expression (Figure 4), which appears as uncondensed open chromatin at metaphase chromosomes.

The uncondensed chromatin at CFSs compared to the non-fragile DNA might lead to higher accessibility of these regions to the viral integration complex, which may account for the high number of MLV integrations into FRA11H and FRA11E. Integration to the open chromatin of CFSs is consistent with previous results showing preferential MLV integration into other open chromatin regions in the genome, such as TSS40, regulatory elements28 and DNase I hypersensitive sites42.

Altogether, our findings showing that in vitro infection by retrovirus vectors leads to replication perturbations raises new concerns regarding the safety of these vectors. This replication perturbation may increase genome instability and mutation rates that could lead to tumorigenicity. This risk is greater especially in patients with challenged immune systems, such as those with SCID. Hence, future studies should explore the molecular basis of this replication stress,

Figure 6 | DSBs following transfection with a plasmid DNA encoding for GFP. (a) Examples of HeLa cells infected with MLV-based vector following immunofluorescence with anti-GFP (green) and γH2AX (red). The nuclei were stained with DAPI (blue). (b) Percent of nuclei with the indicated number of γH2AX foci. HeLa cells expressing GFP following transfection (HeLa-GFP, n = 57), transfected cells that do not express GFP (HeLa-Negative) (control, n = 320), (p = 0.67); (these results are a summary of two independent transfections).
and the ways in which it affects specific risks associated with gene therapy trials and treatments.

**Methods**

**Cells and growth conditions.** The simian virus 40-transformed human fibroblast cell line GM05847 (Cordell Cell Repository, Camden, N.J.) was grown in Eagle minimal essential medium supplemented with 10% fetal calf serum 100,000 U penicillin and 100 mg/l streptomycin. Hela cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100,000 U penicillin and 100 mg/l streptomycin.

**Retroviral-based vector infection and transfection.** For the MLV-based vector infection, we used packaging cells stably expressing a pseudo-type gibbon ape leukemia virus (GALV) envelope (TGA), and the pE8-Nla-Lacz plasmid which is a MLV-based vector (a gift from Prof. Moshe Y. Flugelman, Technion, Israel), and from the infection, we used packaging cells stably expressing a pseudo-type gibbon ape leukemia virus (GALV) envelope (TGA), and the pE8-Nla-Lacz plasmid which is a MLV-based vector (a gift from Prof. Moshe Y. Flugelman, Technion, Israel). Cells were then harvested, and genomic DNA was extracted, combed, and analyzed as previously described. The primary antibody for fluorescence detection of IdU was mouse anti-Brdu (Becton Dickinson), and the secondary antibody was goat anti-mouse Alexa Fluor 488 (Invitrogen). The length of the replication signals was measured in micrometers and converted to kilo bases according to a constant and sequence-independent stretching factor (1 mm = 2 Kb), as previously reported.

**Statistical analysis.** A one-tailed t-test was performed for the replication rate analysis, \( \gamma \text{H2AX} \) foci and chromosomal CFS expression. A binomial test was used to examine the frequency of MLV integrations into CFSs, compared to non-fragile regions. Statistical analysis was performed on at least two biological repeats.

**Immunofluorescence for detection of \( \gamma \text{H2AX} \) foci and \( \text{pH}2 \) gal.** Cells were fixed in 3.7% formaldehyde/PBS for 10 min, permeabilized with 0.5% Triton/PBS, and blocked with 5% BSA/PBS. The primary \( \gamma \text{H2AX} \) antibodies used were mouse anti- \( \gamma \text{H2AX} \) (Upstate Biotechnology). Appropriate secondary antibodies were added, Cy3 conjugated (Jackson ImmunoResearch Laboratories) or Alexa Fluor 594 (Invitrogen). For \( \text{pH}2 \) gal analysis we used anti- \( \text{pH}2 \) Galactosidase (Promega) as a primary antibody, and Alexa Fluor 488 (Invitrogen) as a secondary antibodies. Images were taken with a Bio-Rad confocal microscope. For focus information analysis at least 50 nuclei for each condition were analyzed.

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Author contributions
A.C.B. and B.K. designed the experiments and wrote the manuscript. A.C.B. performed all the experiments presented in the manuscript. M.K. assisted in the cloning of the FRA11H region. K.M. assisted in the DNA combing experiments and analyses.

Additional information
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