The FANC pathway is activated by adenovirus infection and promotes viral replication-dependent recombination

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ABSTRACT
Deciphering the crosstalk between a host cell and a virus during infection is important not only to better define viral biology but also to improve our understanding of cellular processes. We identified the FANC pathway as a helper of viral replication and recombination by searching for cellular targets that are modified by adenovirus (Ad) infection and are involved in its outcome. This pathway, which is involved in the DNA damage response and checkpoint control, is altered in Fanconi anaemia, a rare cancer predisposition syndrome. We show here that Ad5 infection activates the FANC pathway independent of the classical DNA damage response. Infection with a non-replicating Ad shows that the presence of viral DNA is not sufficient to induce the monoubiquitination of FANCD2 but still activates the DNA damage response coordinated by phospho-NBS1 and phospho-CHK1. E1A expression alone fails to induce FANCD2 monoubiquitination, indicating that a productive viral infection and/or replication is required for FANC pathway activation. Our data indicate that Ad5 infection induces FANCD2 activation to promote its own replication. Specifically, we show that FANCD2 is involved in the recombination process that accompanies viral DNA replication. This study provides evidence of a DNA damage-independent function of the FANC pathway and identifies a cellular system involved in Ad5 recombination.

INTRODUCTION
Cellular infection by DNA viruses can be seen as a dynamic interplay: on the one side, the virus tries to manipulate host cell activities to allow its own replication, while on the other, the cell activates defence responses to counteract viral expansion. Thanks to the analysis of their interactions with the host, viruses have proved to be a useful tool in understanding cellular processes, such as replication, apoptosis and the immune response.

Adenovirus (Ad) is a double-stranded DNA virus that promotes cell-cycle progression and unprogrammed DNA replication and generates a huge amount of exogenous DNA for the host cell to deal with (1). The Ad genome is unique among animal viruses with double-stranded DNA genomes because of its linear nature; following infection, double-stranded DNA termini are sensed as double-stranded breaks (DSBs) by the host cell and are able to activate the DSB repair machinery. However, this response is only detected in the absence of the E4 region of Ad (2,3). Infection with a ΔE4 virus results in the...
formation of long concatemers of viral DNA, which does not occur during the wild-type (wt) Ad5 infection (4); this demonstrates that the viral genome is a substrate for DSB repair and that proteins from the E4 region are necessary to block the DNA repair response. The E4-derived proteins target the MRE11:RAD50:NBS1 (MRN) complex, a sensor and central player in the response to DSBs (5,6). This targeting is accomplished through two mechanisms: the redistribution of the MRN complex to nuclear speckles and its targeting for proteasome-mediated degradation (3). The MRN complex is necessary to fully activate both ataxia-telangiectasia mutated (ATM)- and ATM-Rad3-related (ATR)-dependent signalling, which orchestrate the DNA damage response (DDR) (7). Consequently, through the dismantling of the MRN complex, Ad abrogates a major host DNA damage defence (2,8). However, many other viruses, including SV40, HIV and EBV, exploit the DDR to their own advantage (9).

In addition to several other targets, ATR signalling is involved in the activation of the FANC pathway (10–12), which is thought to coordinate the stabilization and recovery of spontaneous and DNA damage-induced replication fork stalling with checkpoint functions (13–15). The FANC pathway consists of a network of proteins, 13 of which are encoded by genes mutated in the rare genetic disease Fanconi anaemia (FA) (15), which is characterized by bone marrow failure, genomic instability and a predisposition to cancer. FA cells are hypersensitive to DNA interstrand crosslink (ICL)-inducing agents, such as the anticancer drugs cisplatin and mitomycin C (MMC) (15–17). Genomic instability and hypersensitivity to ICLs strongly suggest a role for the FANC pathway in the DDR. During S phase or in response to DNA damage, eight of the FANC proteins (FANCA, -B, -C, -E, -F, -G, -L and -M) that form the FANC core complex mediate the monoubiquitination of both FANCD2 (18) and FANCI (19).

Following monoubiquitination, the FANCD2/FANCI complex recruits FAN1 (20) and relocalizes to nuclear foci to activate the DDR coordinated by proteasome-mediated degradation (3). The MRN complex is necessary to fully activate both ataxia-telangiectasia mutated (ATM)- and ATM-Rad3-related (ATR)-dependent signalling, which orchestrate the DNA damage response (DDR) (7). Consequently, through the dismantling of the MRN complex, Ad abrogates a major host DNA damage defence (2,8). However, many other viruses, including SV40, HIV and EBV, exploit the DDR to their own advantage (9).

In this study, we examined whether Ad5 infection targets the FANC pathway, and we show here that Ad5 infection induces FANCD2 monoubiquitination and recruitment to viral replication centres. We demonstrate that FANCD2 monoubiquitination requires Ad5 replication, but that this event is independent of the classical cellular DDR. Accordingly, infection with a non-replicating virus fails to induce FANCD2 mono- ubiquitination while still inducing the DDR coordinated by phospho-NBS1 and phospho-CHK1. Finally, our experiments indicate that the absence of FANCD2 impacts the kinetics of viral replication and affects viral recombination. Our data provide evidence of a DNA lesion-independent function of the FANC pathway and identify a cellular system involved in Ad5 recombination.

**MATERIALS AND METHODS**

**Cell lines and culture conditions**

Human umbilical vein endothelial cells (HUVECs) obtained from healthy donors, as previously described (29), were cultured in EBM-2 (Lonza) supplemented according to the manufacturer’s instructions. The FANC pathway-proficient HeLa and MRC5 cell lines and the FA cell lines PD20 (FANCD2

**Viruses, probe preparation and drugs**

The Ad5 (provided by M. Crescenzi, ISS, Rome, Italy) and Ad2 (provided by P. Boulanger, Université Lyon, Lyon, France) viruses were propagated in HeLa cells, AdGFP (a AElE3 Ad whose E1 region has been replaced with GFP) and mutE1A13s (dl520, Ad5-derived; M. Crescenzi, ISS, Rome, Italy) were propagated in 293 cells (31) and ΔE4 (dl1004, Ad5-derived; provided by G. Kettnner, John Hopkins University, Baltimore, MD, USA) was propagated in W162 medium (32). Two successive rounds of caesium chloride equilibrium centrifugation were performed to purify the viral preparations, and the stocks were then titrated according to optical units (OPU), high-performance liquid chromatography (HPLC) and plaque-forming units (PFU) as...
previously described (33–35). All viral stocks exhibited a viral particle to infectious unit ratio (OPU/PFU) between 10 and 40. Quantitative polymerase chain reaction (q-PCR) was used to determine the purity of mutant viral stocks as previously described (36), and the stocks were virtually pure, with <0.003% contamination. To prepare the probe used in the Southern blot analysis, viral DNA from purified Ad2 stock was extracted with the Nucleospin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions. The purified DNA was then used to prepare the probe using PCR with the DIG labelling system (Roche) according to the manufacturer’s instructions, and oligonucleotides were used to amplify the region between nucleotides 13 441 and 14 961 (NCBI Reference Sequence: AC_000007.1). Hydroxyurea (HU) was purchased from Sigma and resuspended in water.

Infections and transient transfections
For all infections, cells were infected in a low volume of DMEM supplemented with 2% FCS 24 h after plating. For all infections, cells were infected in a low volume of DMEM supplemented with 2% FCS 24 h after plating. After incubation for 1 h at 37°C, the virus was removed by aspiration and multiple washes, and fresh medium was added to the cells. In HUVECs, infections were performed in the same media used for culture because it already contained 2% FCS. For transfections, cells were grown in six-well plates. For E1A transfection, pCMVE1A12S and pCMVE1A13S plasmids (kindly provided by M. Crescenzi, ISS, Rome, Italy) were transfected alone or in combination using Fugene (Roche) according to the manufacturer’s instructions. FANCD2 expression was knocked down using small interfering RNA (siRNA) transfection using duplexed siRNA (Eurogentec) directed against the sequence 5’GGAGAUGAGUGU CUACUA3’. As a negative control, luciferase siRNA with the sequence 5’CGUACCGGGAUACUUCGA3’ was used. The siRNAs were resuspended in RNase-free water at 20 µM, and cells were transfected using Oligofectamine (Invitrogen) according to the manufacturer’s protocol for adherent cells.

RNA and DNA extraction and preparation
Total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer’s instructions, and cDNA retrotranscription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Total DNA was extracted with the Nucleospin Tissue kit (Macherey-Nagel) according to the manufacturer’s instructions. RNA and DNA quantification was performed using an ND-1000 spectrophotometer (NanoDrop Technologies).

Cell lysates and immunoblot analysis
At the indicated time points, infected cells were lysed in RIPA buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), complete mini EDTA-free protease inhibitor cocktail tablets (Roche) and PhosStop phosphatase inhibitor cocktail tablets (Roche)] for 30 min on ice. The soluble protein concentration was then determined using the Bradford assay (Bio-Rad), and the lysates were sonicated after the addition of Laemmli buffer. A total of 20 µg of total protein was separated using SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Whatman). Membranes were blocked in 5% low-fat milk in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.1% Tween 20) followed by primary antibody incubation overnight (O/N) at 4°C. The following proteins were then probed using their respective antibodies at the indicated dilution: FANCD2 (1:2000, ab2187, rabbit), pS343/NBS1 (1:2000, ab75778, rabbit) and vinculin (1:400, ab18058, mouse) (Abcam); γH2AX (1:1000, 05-636, mouse) and NBS1 (1:1000, 07-317, rabbit) (Millipore); actin (1:1000, sc-1615, goat), ATR (1:500, sc-28901, rabbit), CHK1 (1:400, sc-7898, rabbit) and β-tub (1:500, sc-101527, mouse) (Santa Cruz Biotechnology); E1A (1:600, MS-587 - P1, mouse; Labvision Corporation); pS345CHK1 (1:1000, 2348, rabbit; Cell Signaling); ATM (1:500, 2C1, mouse; GeneTex); and Ad5 (L5 1:2000, rabbit). Immunoreactive proteins were labelled using horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology), revealed using the enhanced chemiluminescence system (ECL plus, Pierce) and visualized with a Gene Gnome gel documentation system (Syngene).

BrdU incorporation for flow cytometry assay
At the indicated time points, media was aspirated and a low amount of fresh media with 10 µM BrdU was added to the cells. After 1 h at 37°C, cells were collected by trypsinization, washed in phosphate-buffered saline (PBS) and fixed in cold EtOH 70%. At least 24 h after fixation, cells were washed in PBS and denatured in HCl 2N for 15 min at room temperature (RT). Cells were then washed in PBS and PBS/0.1% bovine serum albumin (BSA)/0.2% Tween and incubated with anti-BrdU antibody (1:50, mouse monoclonal, Becton-Dickinson) for 30 min. After washes in PBS/0.1% BSA/0.2% Tween, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (1:50, DAKO) for 30 min in the dark. Finally, the cells were washed in PBS/0.1% BSA/0.2% Tween, then PBS and resuspended in PBS containing RNAse (100 µg/ml, SIGMA) and Propidium Iodide (PI, 50 µg/ml, Invitrogen). Samples were analysed with a FACScalibur instrument (Becton Dickinson). Twenty thousand events were acquired and populations were gated to exclude cell debris and doublets. Acquired samples were then analysed using the FlowJo software 8.8.6 (Tree Star Inc.)

Immunofluorescence
Cells were grown on coverslips in six-well plates for 24 h before infection. At the indicated time points post-infection, the cells were fixed with 4% paraformaldehyde for 10 min at RT, rinsed with PBS and permeabilized with Triton buffer (0.5% Triton X-100, 20 mM HEPES, pH 7.9, 50 mM NaCl, 3 mM MgCl2 and 300 mM sucrose) for 10 min on a rocking platform. Cells were then blocked in a solution of PBS/3% BSA/0.05%
Tween and incubated O/N at 4°C with the primary antibody. The following antibodies were used: FANCD2 (1:1000, ab2187, rabbit) and γH2AX (1:500, ab2893, rabbit) (Abcam), DBP (1:1000; 37.3, mouse; Dr D.F. Klessig) and CPD (1:200, mouse; Cosmo Bio). The cells were then washed with PBS/3% BSA/0.05% Tween and incubated for 1 h at RT with anti-mouse Alexa Fluor 488- and anti-rabbit Alexa Fluor 594-conjugated secondary antibodies (Invitrogen) followed by sequential washing in PBS/3% BSA/0.05% Tween, PBS and dH2O. After drying the preparations, the coverslips were mounted on glass slides with DAPI Vectashield (Vector Laboratories). All preparations were analysed using an Olympus BX-51 fluorescence microscope equipped with an Orca-Er camera (Hamamatsu). Grey-scale digital images were collected separately using the SimplePCI software and converted for Photoshop (Adobe Systems).

q-PCR
To quantify viral DNA and gene expression levels, gene-specific primers were designed with Primer Express software (Applied Biosystems) for the amplification of E1A (Ad5 nt 697–778) and L1 (Ad5 nt 12 322–12 423). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard in all reactions, using the following oligonucleotides for amplification: forward (5'-TGGGCTACACTGAGCACCAG-3') and reverse (5'-GGGTTGCCTGGTTGAAGTCA-3'). All reactions were performed and analysed as previously described (36).

DNA analysis using Southern blot
Analysis of the production of Ad recombinants was performed as described by Young and Silverstein (37). Briefly, 42 h post-infection (h.p.i.), the viral DNA was extracted from infected cells with a modified HIRT extraction procedure (38), digested with BamH1 (New England Biolabs) for 90 min at 37°C and then separated using electrophoresis on a 0.6% agarose gel at 50 V for 5 h. All successive passages were performed according to the DIG manual (Roche). Briefly, the gel was depurinated and denatured, and after neutralisation of the denaturation reaction, the gel was equilibrated in 20× SSC. The fragmented DNA was transferred onto a nylon membrane (Amersham) using capillary transfer O/N in 20× SSC. Transferred DNA was fixed to the membrane for 2 h at 80°C, and the membrane was then incubated O/N with the DIG-labelled probe. After stringent washings, the membrane was labelled with an anti-digoxigenin alkaline phosphatase (AP)-conjugated antibody (Roche) and revealed using a chemiluminescent reaction using CDP-Star (Roche). The signal was then detected with the Gene Gnome apparatus, and the signal intensities were quantified using Genetools software (Syngene).

Statistical analysis
The results are reported as the mean of at least three independent experiments, with error bars showing the standard error. Differences were considered significant when \( P < 0.05 \), as calculated using the paired sample \( t \)-test.

RESULTS
Ad5 infection induces FANC core-dependent FANCD2 monoubiquitination
One of the strategies used by Ad to reprogram cellular activities to optimize its own replication is to dismantle part of the cellular DDR via MRE11, NBS1 and RAD50 degradation/mislocalization. We therefore investigated the behaviour of the FANC pathway, another key component of the DDR known to play a role in both DNA repair and S phase checkpoint (11,13–15) following Ad5 infection. To evaluate the activation of the FANC pathway, we performed immunoblot analysis to investigate the appearance of the lower mobility band of FANC2 (FANC2-L), which corresponds to its monoubiquitinated form (18). We therefore infected both primary (HUVECs, Figure 1A) and transformed (HeLa, Figure 1B) cells with Ad5 and observed that while NBS1 and MRE11 were degraded as expected, FANC2 became monoubiquitinated upon infection (Figure 1A and B). Because infection with AdGFP (a non-replicating Ad5 whose E1 region has been replaced with GFP) induced neither FANC2 monoubiquitination nor NBS1 or MRE11 degradation (Figure 1A and B), we concluded that FANC2 monoubiquitination was not a consequence of the early steps of viral infection, i.e. attachment and internalization, but still required viral gene expression. We also observed that the extent of FANC2 monoubiquitination increased with time post-infection (Figure 1A and B) and that following an infection with different quantities of Ad5, the levels of monoubiquitinated FANC2 were proportional to the initial multiplicity of infection (m.o.i.) (Figure 1C). Viral infection is thought to push cells into S phase, a situation that could activate the FANC pathway. However, the level of FANC2 monoubiquitination correlated with the initial m.o.i. rather than with the percentage of BrdU-positive cells following infection (Figure 1D). It seems, therefore, that FANC2 monoubiquitination is linked more to Ad infection and replication rather than entry and accumulation into S phase of the infected cell population. Finally, it is known that FANC2 is monoubiquitinated by a fully proficient FANC core complex in response to genotoxic stress (15). Accordingly, FANC2 monoubiquitination was absent in FANC core-deficient cells (Figure 1E).

These results indicate that the Ad5-induced monoubiquitination of FANC2 is FANC core-dependent and that viral expression is required for this phenomenon to occur.

Ad5 infection induces a FANC core-dependent relocalization of FANC2 at viral replication centres
Monoubiquitinated FANC2 is targeted to subnuclear foci in response to DNA damage or replication fork stalling (28). Because we observed FANC2 monoubiquitination in response to Ad5 infection, we examined the subcellular localization of FANC2 in infected cells. Indirect immunofluorescence analysis of Ad5-infected cells demonstrated that FANC2 localizes at the sites of viral replication (viral replication centres), as shown by the
co-localization of FANCD2 with the Ad DNA-binding protein (DBP), a key protein in Ad replication (39) (Figure 2A). To exclude the possibility that FANCD2 staining of viral replication centres was an artefact due to cross-reactivity of the antibody with a viral protein at this site, we performed the same analysis in the isogenic cell lines PD20 (FANCD2⁻/⁻/⁻/⁻) and PD20 3.15 (rescued wt FANCD2 expression) (Figure 2B). Despite the formation of viral replication centres in both cell lines, we detected the co-localization of FANCD2 and DBP only in FANCD2-expressing cells. Importantly, FANCD2 re-localization to viral replication centres was also absent in FANCD2⁻/⁻ cells expressing the K561R non-monoubiquitatable form of FANCD2 (Figure 2B, third row from the top) and in cells defective for a component of the FANC core complex (Figure 2C).

Taken together, these results indicate that Ad5 infection induces a FANC core-dependent monoubiquitination of
FANCD2 that is required for its relocalization to viral replication centres and that FANCD2 is not essential for the formation of the latter.

**E1A expression is not sufficient to induce FANCD2 monoubiquitination**

It has previously been shown that the sole expression of proteins that are functionally analogous to Ad E1A, including E7 from human papilloma virus-16 (HPV-16) and LT from simian virus 40 (SV40), induces FANCD2 monoubiquitination and foci formation (40,41). Consequently, we investigated the role of E1A expression in FANCD2 monoubiquitination and nuclear relocalization.

We transfected the primary E1A splice variants 12S and 13S into HeLa cells and observed that the expression of the E1A oncoproteins was not sufficient to induce

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**Figure 2.** Ad5 induces FANC core-dependent localization of FANCD2 at viral replication centres. (A) HeLa cells were infected with Ad5 at an m.o.i. of 500 OPU. (B) FANCD2−/− cells (PD20), FANCD2-rescued cells (PD20 3.15, PD20 cells expressing wild-type FANCD2) and FANCD2−/−+K561R (PD20 cells transfected with a non-monoubiquitinable form of FANCD2) cells were infected with Ad5 at an m.o.i. of 1000 OPU. (C) FANCC-rescued (PD331 cells expressing a wild-type FANCC) and FANCC−/− (PD331) cells were infected with Ad5 at an m.o.i. of 1000 OPU. Representative images from cells infected with Ad5 that were processed at 18 h.p.i. and analysed using immunofluorescence using antibodies directed against DBP and FANCD2.
either monoubiquitination or a significant assembly of FANCD2 in subnuclear foci, as observed in cells treated with hydroxyurea (Figure 3A and B). It, therefore, seems that contrary to what has been observed for HPV-16 E7 and SV40 LT, expression of the viral oncoprotein E1A alone is not sufficient to activate the FANC pathway. In addition, we infected HeLa cells with an Ad5 mutant that was defective in the expression of the E1A13S splice variant (mutE1A13S) and only expressed E1A12S, which can interact with pRb but lacks a domain (CR3) responsible for the transactivation of other viral genes. Therefore, the mutE1A13S mutant virus is highly defective in viral expression and replication (42,43). Following infection with mutE1A13S, we observed that this mutant was unable to induce FANCD2 monoubiquitination (Figure 3C).

Altogether, these findings indicate that E1A expression alone is not sufficient to induce FANCD2 mono-ubiquitination and suggest that this event requires either the expression of other viral proteins or active viral replication.

**FANCD2 monoubiquitination is independent of the activation of the DDR in the presence of exogenous DNA**

It has previously been reported that following DNA damage, ATR is involved in optimal FANCD2 monoubiquitination and its relocalization to nuclear foci (10,12). In addition, FANCD2 monoubiquitination requires CHK1 phosphorylation (44,45), FANC D2 interacts with the MRN complex (46) and the loss or inactivation of MRN components affects FANCD2 stability (46). Because viral infection dismantles the MRN complex and inactivates the ATM/ATR signalling pathways (Figures 1A and B and 4C) (47), the observed monoubiquitination and relocalization of FANCD2 in response to Ad5 infection (Figures 1 and 2) appears uncoupled from the classical cellular DDR. To better understand the interaction between the FANC pathway and Ad5 infection, we compared FANCD2 activation in HeLa cells infected with either Ad5 or an E4-deficient Ad5 (ΔE4) (Figures 3C and 4A). The ΔE4 virus has been shown to be competent at initiating replication but unable to inactivate MRN (3). Consequently, replicated viral genomes are ligated into concatemers that cannot be packaged into the capsid (2,3). Infection with ΔE4 was accompanied by phosphorylation of NBS1 and CHK1, indicative of the activation of the cellular DDR. It has previously been reported that following DNA repair, ATM/ATR signalling is significantly affected by Ad5 infection. To further exclude the requirement of ATM/ATR in Ad-induced FANCD2 activation, we infected HeLa cells in which ATM or ATR expression was downregulated by the stable expression of sequence-specific short hairpin RNAs (Figure 4B). In addition, we infected HeLa cells that were transiently transfected with specific siRNAs targeting ATR (Supplementary Figure S1A and B). FANCD2 monoubiquitination and relocalization to viral replication centres were observed in cells in which ATM or ATR signalling was impaired by both Ad infection and sh/siRNA expression. Indeed, the siRNA-mediated ATR knockdown impaired FANCD2 monoubiquitination and relocalization to nuclear repair foci in response to HU, but it did not affect the Ad5-induced monoubiquitination or FANCD2 relocalization to viral replication centres (Supplementary Figure S1A and B). Altogether, these data indicate that FANCD2 monoubiquitination in

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**Figure 3.** E1A expression is not sufficient to induce FANCD2 monoubiquitination. (A) HeLa cells were transfected with one or a combination of two plasmids encoding E1A12S and E1A13S. Total cell extracts were isolated at 48 h.p.i. and analysed using western blot with the indicated antibodies. (B) HeLa cells transfected with E1A12S and E1A13S or treated with 1 mM hydroxyurea (HU) for 24 h were analysed using immunofluorescence. pSKT indicates the empty vector used as the control for transfection. (C) HeLa cells were infected with Ad5 and two viral mutants at an m.o.i. of 500 OPU. Total cell extracts were isolated at 48 h.p.i. and analysed using western blot with the indicated antibodies. The vertical lines indicate areas in which the original blots were cut to eliminate intervening samples.
Figure 4. Ad-induced FANCD2 monoubiquitination is independent of the activation of the DNA damage response. (A) HeLa cells were infected with the ∆E4 virus and analysed using immunofluorescence at 18 h.p.i. with the indicated antibodies. (B) HeLa cells that stably expressed ATM or ATR shRNAs, showing a reduction of 45% and 75% in protein level, respectively, were infected with Ad5 at an m.o.i. of 500 OPU, and total cell extracts were collected at different h.p.i. and analysed using western blot. n.t.: not transfected; shScr: scrambled shRNA, used as negative control, M: mock-infected cells. (C) HeLa cells were infected with Ad5 or AdGFP at the indicated m.o.i., and total cell extracts were collected at 24 h.p.i. and analysed using western blot with the indicated antibodies. (D) HeLa cells infected at an m.o.i. of 500 (second row from the top) or 5000 (third row from the top) OPU of AdGFP or mock-treated cells (first row from the top) were analysed using immunofluorescence at 18 h.p.i. for FANCD2 localization. (E) HeLa cells were infected with Ad5 at an m.o.i. of 500 OPU, and total cellular extracts were analysed using the indicated antibodies using western blot. (F) HeLa cells infected with Ad5 at an m.o.i. of 500 OPU were analysed using immunofluorescence at 22 h.p.i. Upper two rows: co-staining of DBP/FANCD2; bottom two rows: co-staining of DBP/γH2AX. (G) Cells infected as in (F) were processed for immunofluorescence assays at the indicated h.p.i., analysed for DBP distribution and assigned to four arbitrary stages of infection (I: early stage - IV: late stage). At the same time, infected cells were scored positive for H2AX phosphorylation (γH2AX). Histograms are the result of the mean of three experiments using ~250 infected cells/slide. Errors bars represent the standard error. (H) HeLa cells were infected with Ad5 at an m.o.i. of 500 OPU, irradiated at 14 h.p.i. with UVC (80 J/m²) through a 5-μm-pore filter and analysed using immunofluorescence 6 h post-irradiation. A representative image is shown from three independent experiments scoring at least 50 optical fields. CPD: cyclobutane pyrimidine dimers, one of the major lesions induced by UVC irradiation, detected using a specific antibody.
response to Ad5 infection is independent of the DDR and that the reduction in FANCD2 monoubiquitination in ΔE4-infected cells could be due to either the reduced amount of exogenous DNA (consequent to a minor viral amplification) or the impaired replication of the mutant viruses.

To determine whether FANCD2 activation in infected cells correlated with the amount of exogenous DNA, we infected HeLa cells with AdGFP at two m.o.i.s: one that leads to FANCD2 activation in response to Ad5 (500, see Figure 1B) and another that was 10-times higher. As shown in Figure 4C and D, although the lower m.o.i. did not lead to FANCD2 monoubiquitination and localization, it was sufficient to induce CHK1 and NBS1 phosphorylation, indicating activation of the DDR by the exogenous double-stranded DNA termini. Even at the higher m.o.i., AdGFP did not induce FANCD2 activation, despite the toxicity of this amount of viral DNA on the cells, as indicated through the global degradation of proteins (Figure 4C) and the altered shape of the nucleus of the infected cells (Figure 4D).

It has been described that following treatment with DNA-damaging agents, the subnuclear relocalization of monoubiquitinated FANCD2 requires the phosphorylated form of the histone variant H2AX (γH2AX) (21). As observed using western blot (Figure 4E), Ad5 infection induced the accumulation of γH2AX starting from 18 h.p.i. Because the cellular distribution of DBP changes during the course of infection and can be used to identify the different stages of infection (48), we used indirect immunofluorescence to analyse H2AX phosphorylation and FANCD2 activation in relation to DBP distribution. While FANCD2 was localized at viral replication centres early after infection (DBP in discrete nuclear foci, II; Figure 4F and G), γH2AX was detected only at later stages of infection (DBP was mostly diffused throughout the nucleus and the shape of the nucleus was altered, IV; Figure 4F and G). In fact, the percentage of cells positive for γH2AX was almost equal to the percentage of cells at the latest stage of infection (IV, Figure 4G). A similar behaviour has previously been reported for RAD51, a key protein in the strand exchange reaction of homologous recombination (49). These results suggest that FANCD2 activation is an early virus-mediated response, while γH2AX and RAD51 activation seems to be part of a cell-dependent response, possibly induced by the accumulation of damaged cellular DNA at later stages of infection (50).

It has been shown that in ultraviolet (UV)-irradiated cells, monoubiquitinated FANCD2 accumulates at the sites of damage (21). To determine whether FANCD2 relocalized to UV-induced lesions after Ad5 infection, we locally irradiated infected HeLa cells with UV light and analysed the localisation of FANCD2 and UV-induced cyclobutane pyrimidine dimers (CPDs) using immunofluorescence. In the cells in which FANCD2 presented a pattern similar to that found in Ad-infected cells (i.e. FANCD2 in viral replication centres), we observed no FANCD2 relocalization to CPD-positive sites (Figure 4H). In contrast, nuclei that showed co-localization of CPDs and FANCD2 showed no signs of infection, which implies that either viral replication centres sequester FANCD2 or that dismantling of the DDR impairs FANCD2 relocalization to the chromatin.

Taken together, these results indicate that FANCD2 monoubiquitination in response to Ad infection is not a function of the amount of viral DNA. In fact, while a high amount of viral DNA presenting DSBs is sufficient to induce the cellular DDR, the presence of free double-stranded termini is not sufficient per se to induce FANCD2 monoubiquitination. Furthermore, the localization of FANCD2 to viral replication centres following infection appears to be independent of the classic DDR.

The lack of FANCD2 affects the kinetics of viral replication

To determine whether the FANC pathway played an active role in Ad5 infection, we compared Ad5 production in isogenic FANC2-expressing and -deficient cells. As shown in Figure 5A and B, FANC2-deficient cells, either obtained by transient transfection of FANC2-specific siRNA in HeLa cells or derived from an FA patient, showed a lower production of Ad5 capsid proteins when compared to their FANC2-expressing counterparts. The observed difference could not be ascribed to a different permissiveness because microscopic analysis showed the same percentage of infected cells (~80% for HeLa cells transfected with both siFANC2 and siLuc and ~20% for both FANC2-expressing and -deficient cells). The accumulation of E1A seemed similarly independent of the presence of FANC2 (Figure 5A and B), suggesting that the expression of Ad5 early proteins is not affected by the lack of FANC2.

The pattern observed at the protein level was confirmed using quantitative analysis of viral mRNAs using q-PCR (Figure 5C). Indeed, we observed a similar rate of transcription for the E1A early gene when FANC2-expressing cells were compared with FANC2-deficient cells (solid lines). However, L1 late gene transcription showed significant differences (Figure 5C, at least 10 times, between 8 and 24 h.p.i., dashed lines). Because the transcription of Ad5 late genes is strictly dependent upon the onset of viral DNA replication (51,52), this result suggests that FANC2 plays a role in Ad5 replication. We also assessed viral genome production following the infection of FANC2-deficient and -expressing cells using q-PCR. At early times post-infection, we observed a 10-fold lower quantity of Ad5 in FANC2-deficient cells (Figure 5D), consistent with the differences observed in L1 transcription (Figure 5C). This difference was gradually lost as the infection progressed. Eventually, intracellular viral DNA reached a plateau at 28 h.p.i. in FANC2-expressing cells, and a similar level was observed 16 h later in FANC2-/- cells, demonstrating that viral production is delayed in FANC2-deficient cells.

The viral genome production was also assessed in cells defective for FANC2 monoubiquitination (FANC2-/- + K561RFANC2 and FANC2-/-) and compared to their rescued counterpart (Figure 5E). In all cases, we
observed impaired Ad5 production in FANC-deficient cells.

Taken together, our data support the hypothesis of a positive role for monoubiquitinated FANCD2 in Ad5 infection, potentially improving the viral DNA replication process.

**FANCD2 helps Ad5 replication–dependent recombination**

Previously published data have implicated the FANC pathway in replication, homologous recombination and cell-cycle checkpoints (11,13–15). To shed light on the function of FANCD2 during Ad5 replication, we analysed viral DNA replication fidelity and recombination in Ad5-infected cells.

Because FA cells are characterized by a high degree of genomic instability, we hypothesized a role for the FANC pathway in the control of the accuracy of Ad5 replication. Consequently, the lack of FANCD2 would affect the viral replication process, leading to mutated and thereby less infective viral particles. To verify this hypothesis, we
infected FANCD2-deficient and -expressing cells and collected the produced viral particles at 72 h.p.i. The collected virus samples were quantified using RT-PCR and HPLC and used to reinfec FANCD2-deficient and -expressing cells at an equal m.o.i. This procedure was repeated for three cycles (see scheme in Figure 6A). The viruses collected and quantified from each cycle were then used to infect HeLa cells at the same m.o.i. to determine if the same amount of viruses produced from cells that expressed or lacked FANCD2 retained the same infectivity. We consistently recovered a lower amount of viral particles from FANCD2-deficient cells (Figure 5 and data not shown). However, when the same amount of particle was used for HeLa cell infection, we observed no differences in the quantity of Ad5 produced from viral particles isolated from cells with or without FANCD2 (Figure 6B). The same result was observed independent of time, m.o.i. or the number of cycles of reinfection in both FANCD2-deficient and -expressing cells (Figure 6B and data not shown). This result indicates that the virus amplified in cells without FANCD2 is as infective as that amplified in cells with FANCD2. Thus, we can reasonably exclude the possibility that FANCD2 recruitment to viral replication centres is necessary to control the quality of Ad5 replication.

Ad5 undergoes extensive recombination during infection of cultured somatic cells (37,53), and although the virus is thought to use the enzymes from the host cell, the exact host recombination pathways used during Ad5 infection are still undefined. To analyse the possible involvement of FANCD2 in virus recombination, we used a recombination assay to analyse recombination during a mixed infection with Ad2 and Ad5 (54). These viruses are closely related, but the Ad2 genome has two BamH1 sites on the left side of the genome, while the Ad5 genome only has one. Recombination that occurs between these sites leads to the formation of recombinant fragments that can be detected using Southern blot hybridisation (see scheme in Figure 6C and the expected pattern in a representative experiment in Figure 6D). Despite loading the same amount of total DNA from the FANCD2+/− and FANCD2-rescued samples (IV and V, 300 ng), we consistently observed less DNA in the FANCD2+/− lane (sum of the P1, R1, R2 and P2 bands). One possible reason for this difference could be that part of the viral DNA recovered from FANC-deficient cells was degraded, and the fragments were therefore too short to be visible on the gel. To avoid the effect that this would have on the interpretation of the data, we compared the frequency of recombination between different cell lines, calculated as the percentage of recombinant fragments relative to the total amount of fragments [100 × (R1 + R2)/(P1 + P2 + R1 + R2)]. This quantification showed that the frequency of recombination in FANCD2-expressing cells was more than 15%. Conversely, a significant reduction in the recombination frequency (P < 0.05) was observed in all experiments with FANCD2+/− and FANCC+/− cells (Figure 6D and E and data not shown). In addition, FANCD2+/− cells consistently showed 25–30% less recombinants than their corrected counterpart independent of the m.o.i. used (Figure 6F).

These data suggest that FANCD2 is involved in the production of recombinant Ad molecules that originate during viral replication, which could indirectly affect the accumulation of replicated viral particles. However, because viral recombination still occurs without FANCD2, other cellular proteins are most likely involved. Further research is required to fully characterize the cellular machinery involved in Ad5 recombination.

DISCUSSION

Deciphering the crosstalk between a host cell and a virus during infection is important not only to obtain a better understanding of viral biology but also to improve our understanding of cellular processes. In this study, we analysed the interaction of Ad5 with its infected cell as an alternative means to characterize the activation and function of the FANC pathway in DNA replication and recombination. We demonstrate that Ad infection specifically induces FANCD2 monoubiquitination and relocalization to viral replication centres, where it helps viral recombination and replication.

It has been reported that expression of the HPV-16 E7 oncoprotein or the LT region from SV40, both functional analogues of E1A, alone leads to the monoubiquitination of FANCD2 and the formation of FANCD2 foci on cellular chromatin (40,41). However, our work shows that the expression of E1A is necessary but not sufficient to induce the FANC pathway: intact replication-competent virus is also required. It is important to note that our findings are not in opposition with the work of Spardy et al. (41), who observed FANCD2 monoubiquitination and focus formation using only the high-risk HPV-16 E7 oncoprotein, while a low-risk HPV-6 E7 failed to induce these events. Because Ad5 is not oncogenic in humans, this could mean that the low-risk HPV-6 E7 is a closer functional analogue of Ad5 E1A.

Our data show that the activation of the FANC pathway can be uncoupled from the activation of the DDR. It has been suggested that the Ad-induced DDR is dependent on the presence of double-stranded termini sensed as DSBs by the cellular machinery. Our data confirm this possibility because infection with a non-replicative double-stranded virus, AdGFP, activates the DDR. Notwithstanding the possible activation of the DDR, FANCD2 is not activated, indicating that the presence of DSBs is not sufficient to activate the FANC pathway. Interestingly, a recent report from Boichuk et al. (40) showed that FANCD2 was monoubiquitinated and relocalized to SV40 viral replication centres in a DDR-dependent manner (41), contrary to our findings. This is not completely unexpected because, contrary to Ad5, SV40 activates the cellular DDR process to foster its own replication. Considering that HPV, SV40 and Ad5 were shown to induce FANCD2 monoubiquitination, FANCD2 can be added to the class of common targets of viral infections, including p53, pRb and the MRN complex.

Several of the presented findings are consistent with a requirement of activated FANCD2 for optimal viral
Figure 6. FANCD2 does not affect the outcome of replication but is required for Ad replication-dependent recombination. (A) Schematic representation of the experiment in (B). FANCD2-expressing and -deficient cells were infected at the same m.o.i., and supernatants were collected at 72 h.p.i., titrated using q-PCR and HPLC and used to reinfect FANCD2-expressing and -deficient cells at the same m.o.i. This was repeated for three successive cycles. HeLa cells were infected with the collected and quantified virus from each cycle using the same m.o.i. (B) HeLa cells were infected with the collected and quantified Ad5 virus from the third cycle using an m.o.i. of 20 OPU, and viral production was quantified using q-PCR at the indicated time points using oligonucleotides to amplify the E1A region. Results are shown as the mean of at least three independent experiments, and error bars represent the standard error. (C) Schematic representation of the experiments in (D–F). Recombination (cross) within the region containing the two BamH1 sites (circles) specific for Ad2 generates two recombinant products, each with only one BamH1 site on the left side of the Ad genome. The probe (red), directed against the region between the Ad2-specific BamH1 sites, recognizes the parental fragments P1 and P2 and the recombinant fragments R1 and R2. (D) Southern blot hybridisation analysis of intracellular DNA from the Ad2 X Ad5 cross that was prepared as described in ‘Materials and Methods’ section; the data are representative of three different experiments. (Lines I–III) MRC5-infected human SV40 transformed fibroblasts, total m.o.i. 500; line I: Ad5, 60 ng; line II: Ad2, 60 ng; line III: Ad2+Ad5, 120 ng; line IV: Ad2+Ad5 from FANCD2-rescued infected cells, total m.o.i. 1000, 300 ng; and M: DIG-labelled marker. (E) Quantitative analysis of the recombination from three independent experiments as in (D), calculated as the percentage of the recombinant fragments relative to the total amount of fragments. The error bars represent the standard error of the mean. A significant decrease in recombination was observed in FANCD2-deficient cells compared to FANCD2-rescued cells (P < 0.05). No difference was observed between FANCD2-rescued and wt (MRC5) cells. (F) Quantitative analysis of the recombination in FANCD2−/− and FANCD2-rescued cells infected with Ad2 and Ad5 at different m.o.i., treated as indicated in ‘Materials and Methods’ section and analysed using Southern blot as in (E). Results are shown as the mean of three independent experiments, and error bars represent the standard error.
replication, including the recruitment of FANCD2 to sites of active viral replication and delayed viral replication in the absence of FANCD2. However, FANCD2 is not essential for a productive viral cycle because viral replication centres can form in the absence of FANCD2 and viral particles produced in FANCD2-deficient cells are as infective as those produced in FANCD2-expressing cells. These data are compatible with the hypothesis that FANCD2 plays a role in Ad5 replication-dependent recombination. Ad5 recombination proceeds through both the Holliday and the Meselson and Radding models for cellular recombination (55) (Figure 7A). However, the proteins involved in the viral recombination steps remain elusive. During the recombination of cellular DNA, the strand exchange step is mediated by RAD51 (56); however, RAD51 involvement in Ad5 recombination has been excluded (57), and it has been suggested that viral DBP is able to perform the strand exchange step (58). We speculated that FANCD2 could interact with DBP to aid in recombination. However, we observed normal DBP expression and sub-nuclear localization in FANC-deficient cells. In addition, DBP was not present in FANC2 immunoprecipitates (Supplementary Figure S1C). Therefore, it is possible that FANCD2 interacts with other viral proteins or directly binds to viral DNA. Consistent with the latter hypothesis, data from Sobeck et al. (59) have shown the capacity of FANCD2 to bind directly naked DNA. Our data demonstrate that the frequency of recombinant viral particles in mixed infections is significantly reduced (30%, P < 0.05) in the absence of the FANC pathway. We therefore propose that FANCD2 facilitates the completion of recombination (Figure 7A); its absence could either delay or prevent this process, limiting the accessibility of double-stranded viral genomes to replication machinery. In this scenario, FANCD2 seems to play an opposite role to that of BLM. Indeed, the helicase mutated in Bloom syndrome has been proposed to limit recombination, and in its absence, Ad5 recombination has been reported to be increased (60).

The adenoviral system provides an alternative means to analyse the mechanism and the consequences of FANC pathway activation.

Previous studies (10,12,45,59,61,62) suggest that FANCD2 monoubiquitination follows three pathways (Figure 7B): one that is ATR-dependent but CHK1-independent, which is probably activated by endogenous replication stress associated with CHK1 deficiency, and another pathway that is ATR- and CHK1-dependent and that is activated in response to replication problems induced by exogenous DNA damage. A third mechanism, induced by either cellular (59,61,62) or viral (our data) DNA replication, leads to FANCD2 monoubiquitination independent of both ATR and CHK1 (Figure 7B). This pathway could be exclusively involved in dealing with intrinsic problems that arise during normal replication.

In conclusion, we believe that our results have important implications for the use of oncolytic viruses in cancer therapy, in which Ad5 is one of the most-used viruses. In fact, numerous studies have reported synergy between oncolytic Ad5 and chemotherapeutic drugs, such as cisplatin, a well-known activator of the FANC pathway (63–65). No molecular mechanism has been elucidated to explain these results, but there is evidence suggesting that the interaction of the virus with the DNA repair machinery could be involved. Our finding that FANCD2 recruited to Ad5 replication centres cannot relocalize to sites of cellular damage, possibly interfering with FANCD2 function, should be taken into account when deciphering the observed synergy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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