SUPPLEMENTAL FIGURES

Figure S1

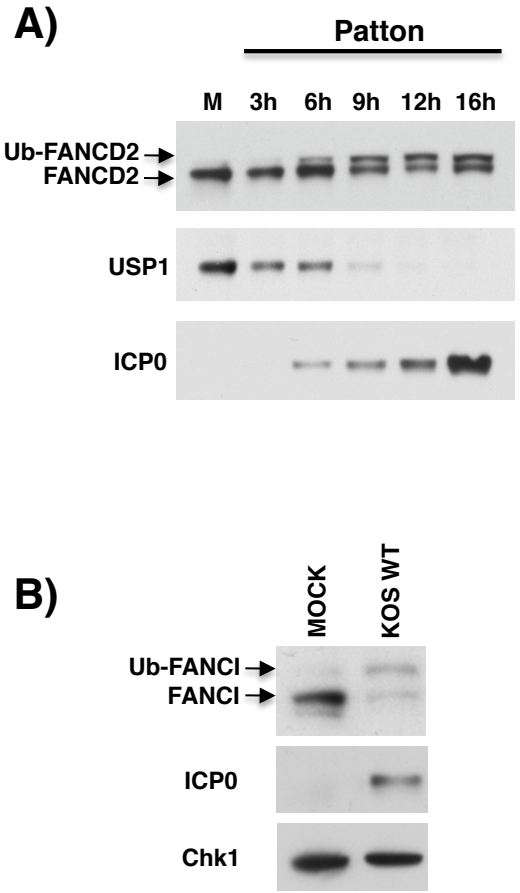


Figure S2

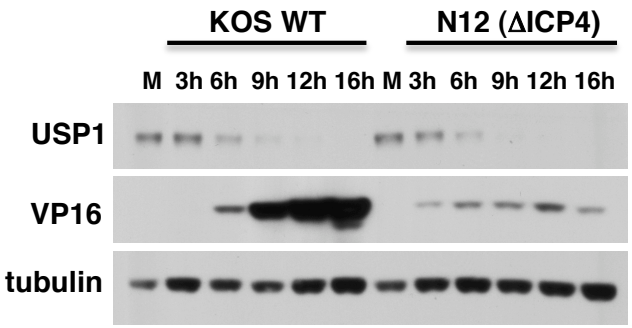

KOS WT
N12 (ΔICP4)
M 3h 6h 9h 12h 16h M 3h 6h 9h 12h 16h
USP1
VP16
tubulin

Figure S3

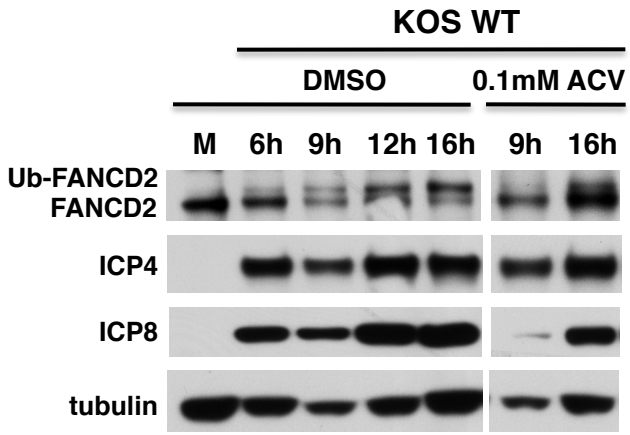
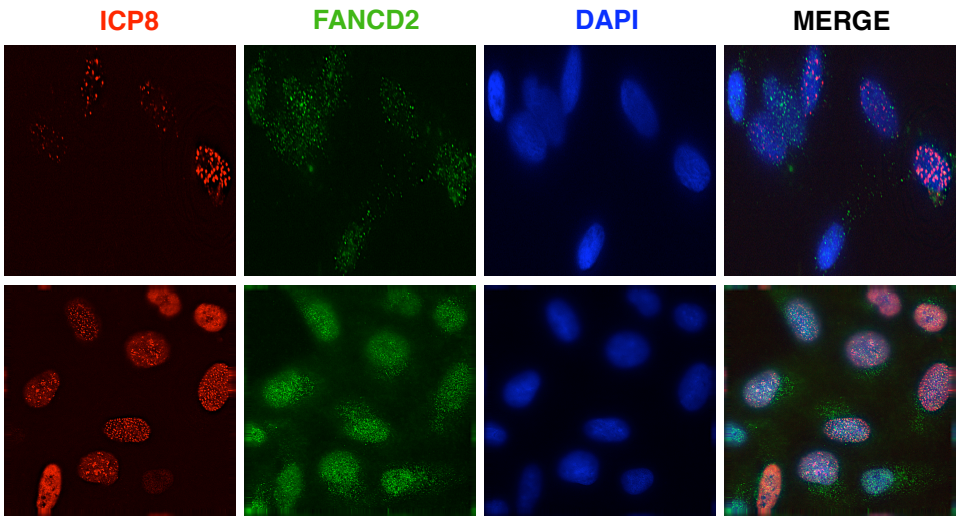
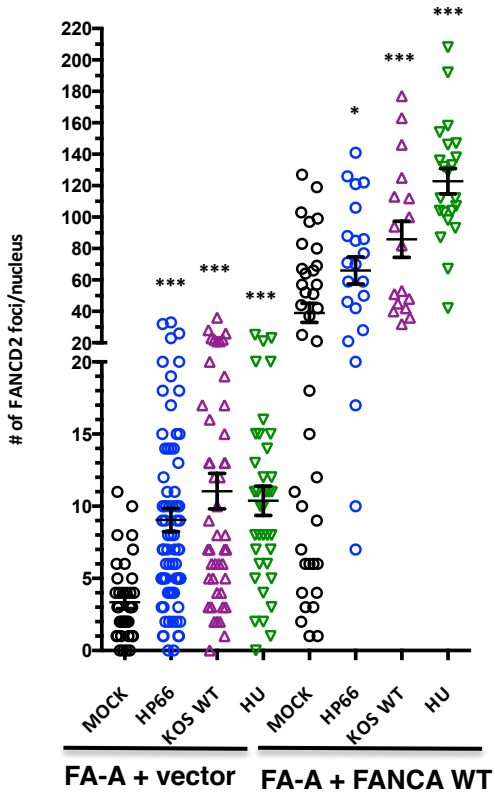

KOS WT
DMSO
0.1mM ACV
M
6h
9h
12h
16h
9h
16h
Ub-FANCD2
FANCD2
ICP4
ICP8
tubulin

Figure S4

A)

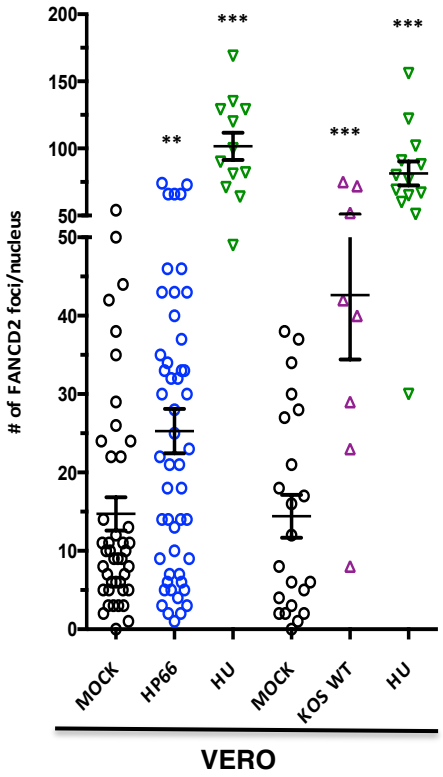


B)



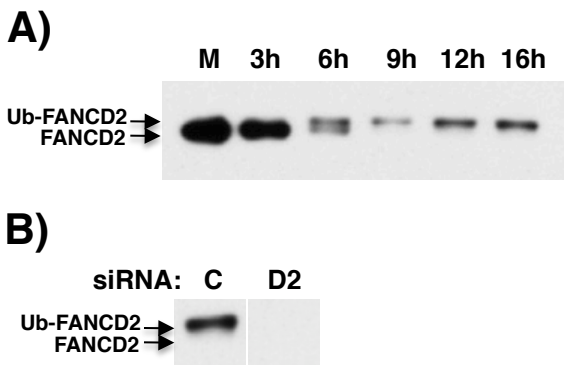
n=	38	74	46	37	38	22	17	22
Mean=	3.3	9.0	11	10	39	66	86	123
Fold difference over MOCK:	1.0	2.7	3.3	3.1	1.0	1.7	2.2	3.1

C)



n=	43	50	12	22	8	13
Mean=	15	25	102	14	43	81
Fold difference over MOCK:	1.0	1.7	6.9	1.0	3.0	5.7

Figure S5


A)
M
3h
6h
9h
12h
16h
Ub-FANCD2
FANCD2
B)
siRNA:
C
D2
Ub-FANCD2
FANCD2

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Activation of the FA pathway in HSV1-infected cells is not strain specific, related to Figure 1. A) Vero cells were mock-infected (M) or infected with WT HSV1 Patton strain (MOI=5). Total protein was harvested at different times post infection (hPI), fractionated by SDS-PAGE and analyzed by immunoblotting using the indicated antibodies. Ub-FANCD2 denotes the slower-migrating, monoubiquitinated form of FANCD2. B) As in A except the KOS strain was used (MOI=1) and cultures harvested at 24 hpi.

Figure S2. Reduction of USP1 abundance induced by an ICP4-deficient HSV1, related to Figure 2. Vero cells were mock-infected (M) or infected (MOI = 5) with an ICP4-deficient virus (N12) or wild-type HSV1 (strain KOS WT). Total protein was isolated at the indicated time post-infection and analyzed by immunoblotting. In addition to USP1, accumulation of a delayed early viral protein (VP16) and tubulin (loading control) were monitored. VP16 levels are reduced in N12-infected cells because the viral lifecycle is blocked early in infection (see Figure 2A schematic).

Figure S3. Prevention of FA pathway activation in HSV1-infected cells by acyclovir, related to Figure 2. Vero cells treated with DMSO or 0.1 mM acyclovir (ACV) were infected (MOI = 5) with WT HSV1 strain KOS. Total protein was isolated at the indicated time post-infection and analyzed by immunoblotting.

In addition to FANCD2 and tubulin (loading control), accumulation of representative viral IE (ICP4) and early (ICP8) proteins were monitored.

Figure S4. Quantifying FANCD2 foci in HSV1-infected cells, related to Figure 4. A) Extraction of ICP8 under pre-permeabilization conditions utilized to visualize FANCD2 nuclear foci. Vero cells infected with the HSV1 replication defective HP66 mutant were either pre-permeabilized with 0.5% Triton X-100-containing Scully buffer and subsequently fixed with PFA (*top row*); or (B) first fixed with PFA and then permeabilized (*bottom row*) prior to processing for indirect immunofluorescence to detect HSV1 ICP8 (red) or FANCD2 (green) as described in the supplemental experimental procedures. Nuclei stained with DAPI (blue) are also shown. Note that pre-permeabilization using Scully buffer removes diffusely staining nuclear FANCD2 and allows clear staining of FANCD2 puncta. However, it also extracts ICP8 from many cells. In the absence of the pre-permeabilization step, 100% of the nuclei are positive for ICP8 (*see bottom row ICP8 panel*) under these conditions (MOI = 5; 8 hpi). B) FA-A cells stably transduced with either empty expression vector only (FA-A + Vector) or functionally complemented with a WT FANCA cDNA (FA-A + FANCA WT) were mock-infected or infected (MOI=5) with HP66 or WT KOS. At 10 hpi, cultures were processed as described in the experimental procedures and FANCD2 nuclear foci were visualized by indirect immunofluorescence. C) As in A except Vero cells were infected and cultures processed at 8 hpi. As a positive control to detect FANCD2 nuclear foci after DNA damage, cells were treated with 2 mM

hydroxyurea (HU) for 8 or 10 h. Fluorescent images were collected with Applied Precision PersonalDV live-cell imaging system using Olympus IX-71 inverted microscope and subsequently deconvoluted. FANCD2 foci were counted using a customized ImageJ macro, and the foci number in per nucleus plotted. The number of nuclei counted (n), the mean number of nuclear foci, and the fold difference over mock-infected cells are shown beneath each graph. *p=0.0117; **p<0.005; ***p≤0.0003.

Figure S5. Monoubiquitination of FANCD2 in HSV1-infected primary cells and FANCD2-depletion by RNAi, related to Figure 5. A). Primary NHDFs were mock-infected (M) or infected with HSV1 (MOI=5). At the indicated hpi, total protein was harvested, fractionated by SDS-PAGE and analyzed by immunoblotting using anti-FANCD2 antibody. Ub-FANCD2 denotes the slower-migrating, mono-ubiquitinated form of FANCD2. B) Primary human fibroblasts transfected with non-silencing, control siRNA or FANCD2 siRNA were infected with GFP-HSV1 (MOI = 10^{-3}). After two days, total protein was isolated, fractionated by SDS-PAGE and analyzed by immunoblotting with anti-FANCD2 antibody.

SUPPLEMENTAL TABLES

Supplemental Table 1. HSV1-encoded proteins detected in anti-FANCI immune complexes, related to Figure 3. Anti-FANCI immune complexes isolated from HSV1-infected (WT vs. HP66) or mock-infected cells were analyzed by tandem MS. The virus-encoded proteins identified, spectral counts, peptide number, and % amino acid coverage are listed.

Supplemental Table 2. HSV1-encoded proteins detected in anti-FANCD2 immune complexes, related to Figure 3. Anti-FANCD2 immune complexes isolated from HSV1-infected (WT vs. HP66) or mock-infected cells were analyzed by tandem MS. The virus-encoded proteins identified, spectral counts, peptide number, and % amino acid coverage are listed.

Table S1

Accession for candidate interactors	KOS WT			HP66			MOCK			Common name for human herpesvirus candidate interactors of FANCI
	Spec#	*Pep#*	*%AA*	*Spec#*	*Pep#*	*%AA*	*Spec#*	*Pep#*	*%AA*	
sp|P06487|GI	115	9	21.3	50	8	17.2	0	0	0.0	Envelope glycoprotein I (gene name=gI)
sp|P08543|RIR1	91	46	36.0	86	33	29.1	0	0	0.0	Ribonucleoside-diphosphate reductase large subunit (gene name=UL39)
sp|P04488|GE	88	20	34.9	67	15	31.6	0	0	0.0	Envelope glycoprotein E (gene name=gE)
sp|Q9NVI1|FANCI	51	37	26.7	45	27	20.3	119	32	23.2	Fanconi anemia group I protein (gene name=FANCI)
sp|P10211|GB	50	26	36.1	79	29	35.0	0	0	0.0	Envelope glycoprotein B (gene name=gB)
sp|P06491|MCP	18	15	21.1	5	5	6.1	0	0	0.0	Major capsid protein (gene name=UL19)
sp|P04296|ICP8	16	15	16.2	8	6	5.6	0	0	0.0	Single-stranded DNA-binding protein, SSB, ICP8 (gene name=UL29)
sp|P10215|UL31	16	6	27.1	2	2	12.7	0	0	0.0	Virion egress protein UL31 (gene name=UL31)
sp|P10231|TG47	16	13	23.7	0	0	0.0	0	0	0.0	Tegument protein UL47 (gene name=UL47)
sp|P10218|UL34	9	6	33.5	2	2	9.1	0	0	0.0	Virion egress protein UL34 (gene name=UL34)
sp|P04294|AN	8	7	19.8	4	4	10.8	0	0	0.0	Alkaline nuclease (gene name=UL12)
sp|P04294-2|AN	8	7	19.8	4	4	10.8	0	0	0.0	Isoform UL12.5 of Alkaline nuclease (gene name=UL12)
sp|P10233|VP22	6	6	24.6	4	3	17.3	0	0	0.0	Tegument protein VP22 (gene name=UL49)
sp|Q69091|GD	6	5	21.1	2	2	10.4	0	0	0.0	Envelope glycoprotein D (gene name=gD)
sp|P06492|VP16	5	4	10.2	3	2	9.8	0	0	0.0	Tegument protein VP16 (gene name=UL48)
sp|P10226|PAP	5	4	11.3	2	2	4.1	0	0	0.0	DNA polymerase processivity factor (gene name=UL42)
sp|P06477|GH	4	3	3.8	6	4	7.8	0	0	0.0	Envelope glycoprotein H (gene name=gH)
sp|P10190|PORTL	4	4	14.2	5	4	10.8	0	0	0.0	Portal protein UL6 (gene name=UL6)
sp|P08392|ICP4	4	4	5.5	3	3	4.3	0	0	0.0	Trans-acting transcriptional protein ICP4 (gene name=ICP4)
sp|P06485|US02	4	3	14.4	0	0	0.0	0	0	0.0	Protein US2 (gene name=US2)
sp|P10220|DEN	4	4	2.1	0	0	0.0	0	0	0.0	Deneddylase UL36 (gene name=UL36)
sp|P10228|GC	3	3	7.4	7	4	11.9	0	0	0.0	Glycoprotein C (gene name=GC)
sp|P10228-2|GC	3	3	7.8	7	4	12.5	0	0	0.0	Isoform gCsec of Glycoprotein C (gene name=GC)
sp|P10224|RIR2	3	2	12.9	3	2	3.8	0	0	0.0	Ribonucleoside-diphosphate reductase small chain (gene name=UL40)
sp|P10210|PPR	3	3	28.0	0	0	0.0	0	0	0.0	Protease precursor (gene name=UL26)
sp|P10210-2|PPR	3	3	28.0	0	0	0.0	0	0	0.0	Isoform pAP of Protease precursor (gene name= UL26)
sp|P10230|ATI2	3	3	28.0	0	0	0.0	0	0	0.0	Alpha trans-inducing factor 78 kDa protein (gene name=UL46)
sp|P32888|VP19	3	3	14.0	0	0	0.0	0	0	0.0	Triplex capsid protein VP19C (gene name=UL38)
sp|P03176|KITH	2	2	9.0	7	4	15.4	0	0	0.0	Thymidine kinase (gene name=TK)
sp|P10234|DUT	2	2	8.6	4	2	9.2	0	0	0.0	Deoxyuridine 5'-triphosphate nucleotidohydrolase (gene name=UL50)
sp|P10221|V120	2	2	3.7	1	1	1.6	0	0	0.0	Capsid assembly protein UL37 (gene name=UL37)
sp|P10202|VP23	2	2	11.6	0	0	0.0	0	0	0.0	Triplex capsid protein VP23 (gene name=UL18)
sp|P08393|ICP0	1	1	2.5	4	2	4.4	0	0	0.0	E3 ubiquitin-protein ligase ICP0 (gene name=ICP0)
sp|P10238|ICP27	1	1	2.1	2	1	2.5	0	0	0.0	Transcriptional regulator ICP27 (gene name=UL54)
sp|P04293|DPOL	1	1	1.3	0	0	0.0	0	0	0.0	DNA polymerase catalytic subunit (gene name=UL30)
sp|P10216|UL32	1	1	2.5	0	0	0.0	0	0	0.0	Packaging protein UL32 (gene name=UL32)
sp|P10188|NP04	1	1	11.1	0	0	0.0	0	0	0.0	Nuclear protein UL4 (gene name=UL4)
sp|P10191|UL07	1	1	5.4	0	0	0.0	0	0	0.0	Protein UL7 (gene name=UL7)
sp|P10208|UL24	1	1	5.2	0	0	0.0	0	0	0.0	Protein UL24 (gene name=UL24)
sp|P04288|GM	1	1	1.9	0	0	0.0	0	0	0.0	Envelope glycoprotein M (gene name=gM)
sp|P04487|RNB	1	1	10.6	0	0	0.0	0	0	0.0	RNA-binding protein (gene name=US11)
sp|P04291|TG14	1	1	4.1	0	0	0.0	0	0	0.0	Tegument protein UL14 (gene name=UL14)
sp|P10235|UL51	0	0	0.0	2	1	5.7	0	0	0.0	Tegument protein UL51 (gene name=UL51)
sp|P04485|ICP22	0	0	0.0	1	1	2.4	0	0	0.0	Transcriptional regulator ICP22 (gene name=US1)
sp|P04485-2|ICP22	0	0	0.0	1	1	3.6	0	0	0.0	Isoform US1.5 of Transcriptional regulator ICP22 (gene name=US1)
sp|P10240|UL56	0	0	0.0	1	1	7.6	0	0	0.0	Protein UL56 (gene name=UL56)
sp|P10229|EV45	0	0	0.0	1	1	7.6	0	0	0.0	Envelope protein UL45 (gene name=UL45)

Table S2

Accession for candidate interactors	KOS WT			HP66			MOCK			Common name for human herpesvirus candidate interactors of FANCD2
	Spec#	*Pep#*	*%AA*	*Spec#*	*Pep#*	*%AA*	*Spec#*	*Pep#*	*%AA*	
splQ9BXW9IFANCD2	10	9	6.4	23	14	13.1	22	11	9.3	Fanconi anemia group D2 protein (gene name=FANCD2)
splP04488IGE	10	7	13.8	5	4	12.0	0	0	0.0	Envelope glycoprotein E (gene name=gE)
splP08543IRIR1	9	8	8.3	18	12	12.6	0	0	0.0	Ribonucleoside-diphosphate reductase large subunit (gene name=UL39)
splP06487IGI	7	2	2.3	4	4	14.9	0	0	0.0	Envelope glycoprotein I (gene name=gI)
splP10211IGB	6	5	10.8	0	0	0.0	0	0	0.0	Envelope glycoprotein B (gene name=gB)
splP06485IUS02	3	3	19.2	0	0	0.0	0	0	0.0	Protein US2 (gene name=US2)
splP06491IMCP	2	2	2.6	3	3	2.8	0	0	0.0	Major capsid protein UL19 (gene name=UL19)
splP10218IUL34	2	2	12.4	0	0	0.0	0	0	0.0	Virion egress protein UL34 (gene name=UL34)
splP32888IVP19	2	2	5.4	0	0	0.0	0	0	0.0	Triplex capsid protein VP19C (gene name=UL38)
splP10210-2IPPR	1	1	4.3	0	0	0.0	0	0	0.0	Isoform pAP of Protease precursor (gene name=UL26)
splP10210IPPR	1	1	2.2	0	0	0.0	0	0	0.0	Protease precursor (gene name=UL26)
splP10224IRIR2	1	1	2.9	0	0	0.0	0	0	0.0	Ribonucleoside-diphosphate reductase small chain (gene name=UL40)
splP10188INP04	1	1	3.5	0	0	0.0	0	0	0.0	Nuclear protein UL4 (gene name=UL4)
splP10235IUL51	1	1	4.5	0	0	0.0	0	0	0.0	Tegument protein UL51 (gene name=UL51)
splP10189IHELI	1	1	1.6	0	0	0.0	0	0	0.0	Probable ATP-dependent helicase UL5 (gene name=UL5)
splP10228-2IGC	1	1	3.1	0	0	0.0	0	0	0.0	Isoform gCsec of Glycoprotein C (gene name=GC)
splP04288IGM	1	1	5.1	0	0	0.0	0	0	0.0	Envelope glycoprotein M (gene name=gM)
splP10231ITG47	1	1	2.9	0	0	0.0	0	0	0.0	Tegument protein UL47 (gene name=UL47)
splP10228IGC	1	1	2.9	0	0	0.0	0	0	0.0	Glycoprotein C (gene name=GC)
splP10233IVP22	0	0	0.0	1	1	5.0	0	0	0.0	Tegument protein VP22 (gene name=UL49)
splP06492IVP16	0	0	0.0	1	1	4.1	0	0	0.0	Tegument protein VP16 (gene name=UL48)
splP03176IKITH	0	0	0.0	3	2	6.1	0	0	0.0	Thymidine kinase (gene name=TK)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cells and viruses

All Fanconi Anemia (FA) patient-derived cell lines [FANCA-deficient (FA-A), fibroblast line GM6914 functionally complemented with a WT FANCA cDNA (FA-A + FANCA WT) or transduced with an empty expression vector (FA-A + vector); FANCG-deficient (FA-G) fibroblasts 326SV functionally complemented with a WT FANCG cDNA (FA-G +FANCG WT) or transduced with an empty expression vector (FA-G + vector); FANCD1 (BRCA2)-deficient VU423 fibroblasts functionally reconstituted with chromosome 13, which contains a WT FANCD1 (BRCA2) locus or an empty vector) were propagated in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal bovine serum (FBS) as described (*Garcia-Higuera et al., 2001*). Vero cells (from ATCC) were maintained in DMEM containing 5% calf serum (FCS). Primary normal human diploid fibroblasts (NHDFs; Clonetics, Walkersville, MD) were cultured in DMEM containing 5% (FBS).

Wild-type HSV1 (strains Patton and KOS) stocks were prepared and titered in Vero cells. The DNA polymerase-deficient HSV1 strain HP66 (a kind gift from Don Coen, Harvard Medical School) was propagated and titered using a complementing Vero cell line that expresses the HSV-1 UL30 DNA Polymerase (*Marcy et al. 1990*). The N12 ICP4 mutant (a generous gift from Neal Deluca, Univ. Pittsburgh) was propagated on a Vero cell line engineered to express ICP4 as described (*DeLuca & Schaffer 1988*). The ICP0 null mutant 7134 and the corresponding repaired variant 7134R in which the WT ICP0 gene was restored

(kindly provided by Dr. P Schaffer, Harvard Medical School) were propagated using U20S cells (*Cai & Schaffer 1989*). UV-inactivated virus was prepared as described (*Walsh & Mohr, 2004*). WT HSV1 expressing an EGFP-Us11 fusion protein was described (*Benboudjema et al., 2003).*

Antibodies and chemicals

Anti-FANCD2 was purchased from the following commercial suppliers and used as indicated: Novus Biologicals (clone FI-17, #NB100-316) for immunoblotting; Bethyl Laboratories *(#A302-174A*) for immunoprecipitation; Novus Biologicals *(#NB 100-182*) for immunofluorescence. Anti-FANCI was purchased from Bethyl Laboratories *(#A301-254A*). ICP8 and UL42 antisera was purchased form Abcam (*#ab20194. Ab19298*), ICP0 antisera (mouse) was purchased from the Goodwin Institute, anti-VP16 was purchased from Sigma-Aldrich (#V4388), and anti-ICP4 (mouse) was purchased from either the Goodwin Institute or Abcam (*#ab6514*). Anti-4E-BP1 was purchased from Bethyl Laboratories *(#A300-501A*) and anti-raptor purchased from Millipore (# 05-1470). Polyclonal antisera directed against the USP1 C-terminus was generously provided by A. D'Andrea (Dana Farber Cancer Inst.), and polyclonal UL12 antisera (pUL12N127) was a generous gift from S. Weller (U. Connecticut Health Center). NU7441 was purchased from TOCRIS Bioscience (*#3712*), dissolved in DMSO at a concentration of 5 mM and used at the indicated concentration.

Immunoblotting

Total cellular protein was solubilized in 80 µL of sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 0.7 M β-mercaptoethanol), boiled for 3 min, fractionated by Nupage 3-8% Tris-acetate or 4-12% Bis-Tris gels (Life Technologies), and transferred to a PVDF membrane. Immunoblots were processed and developed using the ECL reagent according to the manufacturer's instructions.

Multidimensional Protein Identification Technology (MudPIT) and LTQ Orbitrap Mass Spectrometry.

The protein digest was pressure-loaded onto a 250 µm i.d capillary packed with 2.5 cm of 10-µm Jupiter C18 resin (*Phenomenex, Torrance, CA*) followed by an additional 2.5 cm of 5-µm Partisphere strong cation exchanger (*Whatman, Clifton, NJ*). The column was washed with buffer containing 95% water, 5% acetonitrile, and 0.1% formic acid. After washing, a 100-µm i.d capillary with a 5-µm pulled tip packed with 15 cm 4-µm Jupiter C18 resin (*Phenomenex, Torrance, CA*) was attached to the filter union and the entire split-column (desalting column–filter union–analytical column) was placed in line with an Agilent 1100 quaternary HPLC (*Palo Alto, CA*) and analyzed using a modified 5-step separation described previously (*Washburn et al., 2001*). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Step 1 consisted of a 75 min gradient from 0–100% buffer

B. Steps 2–5 had a similar profile except 3 min of 100% buffer A, 5 min of X% buffer C, a 10 min gradient from 0–15% buffer B, and a 105 min gradient from 10–55% buffer B (except for step 5 which %B was increased from 10% to 100%). The 5 min buffer C percentages (X) were 10, 40, 60, 100% respectively for the 5-step analysis. As peptides eluted from the microcapillary column, they were electrosprayed directly into an LTQ mass spectrometer (*ThermoFinnigan, Palo Alto, CA*). For LTQ analysis: As peptides eluted from the microcapillary column, they were electrosprayed directly into an LTQ 2-dimensional ion trap mass spectrometer (*ThermoFinnigan, Palo Alto, CA*) with the application of a distal 2.4 kV spray voltage. A cycle of one full-scan mass spectrum (400–2000 m/z) followed by 5 data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur datasystem.

Analysis of Tandem Mass Spectra.

Protein identification and quantification analysis were done with Integrated Proteomics Pipeline (*IP2, Integrated Proteomics Applications, Inc. San Diego, CA*) using ProLuCID, DTASelect2 and Census. Tandem mass spectra were extracted into ms1 and ms2 files (*McDonald et al., 2004*). MS1, MS2, and SQT-three unified, compact, and easily parsed file formats for the storage of shotgun proteomic spectra and identifications. Rapid communications in mass spectrometry) from raw files using RawExtract 1.9.9 (http://fields.scripps.edu/

downloads.php) and were searched against UniProt_Human plus Human herpesvirus 1 (strain 17) (HHV-1) (Human herpes simplex virus 1) protein database from Uniprot (both downloaded on 05/16/2012); plus sequences of known contaminants such as keratin and porcine trypsin concatenated to a decoy database in which the sequence for each entry in the original database was reversed (*Peng et al., 2003*) using ProLuCID/Sequest (*Eng et al., 1994*). LTQ data was searched with 3000.0 milli-amu precursor tolerance and the fragment ions were restricted to a 600.0 ppm tolerance. All searches were parallelized (*Sadygov et al., 2002*) and performed on The Scripps Research Institute's garibaldi 64-bit LINUX cluster with 2848 cores. Search space included all fully- and half-tryptic peptide candidates with no missed cleavage restrictions. Carbamidomethylation (+57.02146) of cysteine was considered a static modification and we require 2 peptides per protein and at least one trypitic terminus for each peptide identification. The ProLuCID search results were assembled and filtered using the DTASelect program, version 2.0 (Cociorva et al., 2007) with false discovery rate (FDR) of 0.05; under such filtering conditions, the estimated false discovery rate was below ~5% at the protein level in all analysis.

Indirect immunofluorescence

To detect FANCD2 nuclear foci, cells were pre-permeabilized with Scully buffer (0.5% Triton X-100, 20 mM HEPES-KOH, pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) for 1 min. and fixed with 4% (vol/vol) paraformaldehyde

in PBS for 10 min. at room temperature (RT). Cells were subsequently permeabilized and blocked with 10% goat serum, 0.1% NP-40 in PBS for 30 min. at RT. To detect FANCD2 and ICP8, fixed cells were incubated with anti-FANCD2 (1:500) and anti-ICP-8 (1:500) antibodies diluted in 5% goat serum in PBS and incubated for 2 hours. Cells were washed 3 times with TBS-Tween and incubated with Alexa Fluor 488-conjugated goat anti–rabbit immunoglobulin G (IgG) (Invitrogen, *#A110034*) and Alexa Fluor 594-conjugated goat anti–mouse IgG (Invitrogen, *#A11005*) at a dilution of 1:1000 in 5% goat serum in PBS for 1 hour. Cells were washed 3 times with TBS-Tween. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Cells were mounted in Dako Fluorescent Mounting Medium (DAKO, *S3023*). Fluorescent images were collected with Applied Precision PersonalDV live-cell imaging system using Olympus IX-71 inverted microscope. Images were deconvoluted. FANCD2 foci were counted with a customized ImageJ macro, and the foci number per nucleus plotted.

Staining of ICP8 was less efficient if infected cells were pre-permeabilized prior to fixation (*see supplemental figure 4A*). To ensure that all of the infected cells stained for ICP8, viral infection efficiency was tested for each IF experiment on a separate slide without a pre-permeabilization step, using 4% (vol/vol) paraformaldehyde in PBS for 10 minutes at RT and then permeabilized with Scully buffer for 1 minute at RT (*Supplemental figure 4A*). As a positive control to detect FANCD2 foci, cells were treated with 2 mM hydroxyurea (HU).

RNA interference

The siRNA duplex targeting FANCD2 (sense target 5′- AACAGCCATG GATACACTTGA -3’) was synthesized as a 21-nt complementary RNA with 2-nt overhangs (Qiagen). The All Star* Negative Control siRNA (Qiagen) was used as a non-targeting duplex. To quantify viral replication, NHDFs (40–50% confluent in DMEM + 5% FBS) were transfected on a 6 well dish with 2 µL RNAi Max + 20 nM siRNA per well according to the manufacturer's instructions (Invitrogen). After 48 h, cells were infected (MOI = 0.001) with a WT HSV1, EGFP-expressing reporter virus. Cells were evaluated and photographed using phase contrast and epifluorescence microscopy. After 2 days post-infection, cultures were lysed by freeze-thawing and the virus produced was quantified by plaque assay in Vero cells.

Lentivirus plasmid constructs expressing shRNA targeting Ku80 were purchased from Sigma-Aldrich (shKu80 (1) 5′- CCGGAATCTAAGAGAGCT GCCATCGCTCGAGCGATGGCAGCTCTCTTAGATTTTTTTG-3’) or was a kind gift from S. Weller (U. Connecticut Health Center) (Ku80 (2) 5’-AAGAGCTAAT CCTCAAGTCGG-3’). Lentivirus particles were produced by transient transfection of HEK293 LTV cells with d8.9, pMDG.2, shKu80 using Lipofectamine 2000. Lentivirus-containing supernatants were harvested after 48 h, filtered (0.45 µm), and frozen at -80°C until use. After the first transduction of FA-A cells with the indicated lentivirus, the transduction procedure was repeated 24 h later. Four days later, FA-A + vector cells transduced twice with lentivirus-expressing shRNA were subsequently infected with WT GFP-HSV1 (MOI = 0.05). After 6 d post-

infection with HSV1, cells were evaluated using phase contrast and epi-fluorescence microscopy and photographed, cultures were lysed by freeze-thawing and the virus produced was quantified by plaque assay in Vero cells.

Multicycle growth experiments

GM6914 FA-A + vector and FA-A + FANCA WT fibroblasts (1×10^5 cells/well) were seeded in a well of 6-well dish and allowed to attach overnight. The next day, cells were infected (MOI = 5, 0.5, 0.05, 0.005) with a WT HSV1, EGFP-expressing reporter virus. After 3 days post infection, live cells were evaluated and photographed using phase contrast and epifluorescence microscopy. Cultures were subsequently lysed by freeze-thawing and the virus produced was quantified by plaque assay in Vero cells. For experiments using NU7441 to inhibit DNA-PKcs, cells were pretreated with 1 mM NU7441 or DMSO for 1 h prior to infection. Cells were infected with WT HSV1, EGFP-expressing reporter virus (MOI=0.05) in the continuous presence of NU7441 (1mM) or vehicle. Fresh NU7441 or DMSO was added daily to the cultures.

FA-G and FA-D1 (BRCA2) patient-derived fibroblasts (326SV FA-G + vector and FA-G + FANCG WT; VU423 FA-D1 + vector (BRAC2 vector) and FA-D1 + Chr13 (BRAC2 WT) were seeded in a well of 6-well dish (1×10^5 cells / well) and allowed to attach overnight. The next day, cells were infected (MOI = 0.5×10^{-3} or 5×10^{-3}) with a WT GFP-HSV1. After 2 d, live cells were evaluated and photographed using phase contrast and epifluorescence microscopy. Cultures

were subsequently lysed by freeze-thawing and the virus produced was quantified by plaque assay in Vero cells.

Acyclovir treatment of Vero cells

Vero cells seeded in a 6-well dish (10^5 cells / well) were allowed to attach overnight. The next day, cells were pretreated with 0.1mM acyclovir or DMSO for 1 h and subsequently infected with WT HSV1 KOS (MOI =5) in the presence of 0.1mM acyclovir or DMSO. Total protein was collected for analysis at indicated times post-infection.

Analysis of viral DNA replication by qPCR

GM6914 FA-A + vector and FA-A + FANCA WT fibroblasts (1×10^4 cells/well) were seeded in a 24-well dish and allowed to attach overnight. The next day, cells were infected with KOS WT or HP66 viruses (MOI=5) for 5.5h and 10h. The 0 h point represents the conclusion of the 90 min absorption period. Soluble lysates prepared in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS were treated with 100 µg/ml Protease K overnight at 55°C, extracted twice with phenol, once with chloroform, and precipitated with ethanol. DNA was re-suspended in water and the relative viral genomic DNA levels were determined by quantitative PCR (qPCR) using primers specific for the HSV1 ICP27 locus (ICP27 FW 5'-TTTCTCCAGTGCTACCTG AAGG-3' and ICP27 RV 5'-

TCAACTCGCAGACACGACTCG-3'). Input DNA was normalized by amplification of the cellular RPL19 gene (RPL19 FW 5′-ATGTAT CACAGCCTGTACCTG-3′ and RPL19 RV 5′-TTCTTGGTCTCTTCCTCCTTG-3′). Relative amount of DNA was measured with the following equation: dCt (threshold cycle) = Target gene Ct- RPL19 control gene Ct. Real-time qPCR analysis was performed using FastStart Universal SYBR Green Master-ROX (Roche). Relative changes in DNA levels were calculated using the ΔΔCt method. Data plots were made using Prism 6.0 software (GraphPad).

SUPPLEMENTAL REFERENCES

Benboudjema, L., Mulvey, M., Gao, Y., Pimplikar, S.W., and Mohr, I. (2003). Association of the herpes simplex virus type 1 Us11 gene product with the cellular kinesin light-chain-related protein PAT1 results in the redistribution of both polypeptides. *J. Virol.* 77, 9192-9203.

Cai, W.Z. and Schaffer, P.A. (1989). Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. *J. Virol*. 63, 4579-4589.

Cociorva, D., Tabb, D.L., and Yates, J.R. 3rd. (2007). Validation of tandem mass spectrometry database search results using DTASelect. *Curr. Protoc. Bioinformatics.* Chapter 13:Unit 13.4. doi: 10.1002/0471250953.bi1304s16.

DeLuca, N. and Schaffer, P. (1988). Physical and functional domains of the herpes simplex virus. *J. Virol*. 62, 732-743.

Eng, J., McCormack, A.L., and Yates, J.R. 3rd. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Amer. Soc. Mass Spec*. 5, 976-989.

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M., and D'Andrea, A.D. (2001). Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell* 7, 249-262.

Marcy, A.I., Yager, D.R., and Coen, D.M. (1990). Isolation and characterization of herpes simplex virus mutants containing engineered mutations at the DNA polymerase locus. *J. Virol.* 64, 2208-2216.

McDonald, W.H., Tabb, D.L., Sadygov, R.G., MacCoss, M.J., Venable, J., Graumann, J., Johnson, J.R., Cociorva, D., and Yates, J.R. 3rd. (2004). MS1, MS2, and SQT-three unified, compact, and easily parsed file formats for the storage of shotgun proteomic spectra and identifications. *Rapid Commun. Mass Spectrom*. 18, 2162-2168.

Peng, J., Elias, J.E., Thoreen, C.C., Licklider, .LJ., and Gygi, S.P. (2003). Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. *J. Proteome Res*. 2, 43-50.

Sadygov, R.G., Eng, J., Durr, E., Saraf, A., McDonald, H., MacCoss, M.J., and Yates, J.R. 3rd. (2002). Code developments to improve the efficiency of automated MS/MS spectra interpretation. *J. Proteome Re*s. 1, 211-215.

Tabb, D.L., McDonald, W.H., and Yates, J.R. 3rd. (2002). DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J. Proteome Res*. 1, 21-26.

Washburn, M.P., Wolters, D., and Yates, J.R. 3rd. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* 19, 242-247.