SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse strains

Fancc-/- mice have been previously described (Haneline et al., 1998).

Genomic PCR for *Fancc*

Genomic DNA from mice or cells was prepared as described (Orvedahl et al., 2011) and PCR was performed using 3 primers in each reaction. Primer sequences were as follows: Murine *Fancc* (MFAC)-21: 5'-CCTGCCATCTTCAGAATTGT-3'; MFAC-22: 5'-GAGCAACACAAATGGTAAGG-3'; MFAC-23: 5-TTGAATGGAAGGATTGGAGC-3'. PCR conditions were 5 min at 95^0C, 30 sec at 95^0C, 2min at 55^0C, and 90 sec at 72^0C, for a total of 31 cycles followed by 10 min at 72^0C. $Fancc^{+/+}$ genomic DNA yields an 800 bp band; $Fancc^{-/-}$ genomic DNA yields a 600 bp band; and $Fancc^{-/-}$ genomic DNA yields both an 800 and a 600 bp band.

Autophagy Assays

Autophagosome numbers were measured in MEFs from $Fancc^{+/+}$/GFP-LC3 or $Fancc^{-/-}$ /GFP-LC3 mice by counting the number of GFP punctae per cell (in the presence or absence of Baf A1, an inhibitor of lysosomal acidification) by an observer blinded to experimental condition. Autophagic flux was also measured by p62 western blot analysis and the ratio of LC3-II/LC3-I in the presence or absence of Baf A1.

Animal Studies

All infections were performed by intracerebral (i.c.) inoculation into the right cerebral hemisphere of the designated number of plaque-forming units (PFUs) of virus diluted in 30 μl Hanks balanced salt solution (HBSS). One week-old litters derived from *Fancc*$^{+/-}$ breeding pairs were infected with 1000 PFUs of SIN dsTE12Q. Six-eight week-old *Fancc*$^{+/+}$ and *Fancc*$^{-/-}$ littermate control mice were infected i.c. with $5x10^4$ PFUs of HSV-1 ICP34.5Δ68-87, HSV-1-TK$^-$, or HSV-1-TK$^-$R. For mortality studies, mice were monitored daily for 21 days. For measurement of CNS viral titers, freeze-thawed 10% (weight/volume) homogenates of the right hemispheres were used for plaque assay titration. For histopathology studies, the left hemispheres were fixed in 4% paraformaldehyde, embedded in paraffin, and cut sagitally from the medial surface into 5 μM adjacent sections. All animal procedures were performed in accordance with institutional guidelines and with approval from the UT Southwestern Medical Center Institutional Animal Care and Use Committee.

Western Blot Analyses

For western blot analyses, cells were lysed directly with Laemmli buffer containing 2.5% β-mercaptoethanol, boiled for 5 min, separated on SDS-PAGE denaturing gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Membranes were blocked with Fast Blocking Buffer (Pierce) and signals were visualized with Supersignal®West Pico or Femto Chemiluminescent Substrate kit (Pierce) on a digital imaging system (BioSpectrum, UVP). Loading was normalized by densitometry of actin signal using ImageJ.

Mammalian Cells

MEFs and cultured as described (Su et al., 2003). After 7 days in culture, differentiated BMDMs were harvested and cultured in DMEM supplemented with 10% FBS and 10% L929 cell-conditioned media. Cells were not exposed to antibiotics during processing or differentiation. HeLa cells (provided by V. Stollar [Li et al. 1997]) stably-expressing GFP-LC3 (Kabeya et al., 2000) were generated and cultured as described (Orvedahl et al., 2010). For HeLa FANCC^{KO} cells generated by CRISPR/Cas9 genome editing, sequences encompassing candidate gRNA sites were analyzed for common (>1%) SNPs using the UCSC genome browser and no common SNPs were detected in the two gRNAs used (hFANCC exon3.g4: TGCCAACAGTTGACCAATTGNGG and hFANCC exon3.g5: GCCAACAGTTGACCAATTGTNGG. The edited *FANCC* sequence was confirmed by deep sequencing using primers flanking the gRNA target site. No wild-type sequence was detected in 6075 reads. HeLa/Parkin cells were generated by stable transfection of a pIRES-hyg3 vector (Clontech) expressing Parkin cDNA. Primary human fibroblasts from a patient with the *FANCC* c.1897C>T stop mutation (FANCC R548X) (Murer-Orlando, et al., 1993) were immortalized by transduction with a lentiviral vector expressing the SV40 large T antigen (Virts, et al., 2015). They were then transduced with a retroviral control vector or a retrovirus vector expressing wild-type FANCC.

SIN and HSV-1 Viruses

SIN-mCherry.capsid/GFP-LC3 (Orvedahl et al., 2010), the recombinant SIN vector dsTE12Q (Liang et al., 1998), HSV-1 ICP34.5Δ68-87 (Orvedahl et al., 2007), and HSV-1-TK$^-$ and its marker rescue virus, HSV-1-TK$^-$R (Pyles and Thompson, 1994), have been

described previously. The Sindbis virus (SIN) strain SVIA (ATCC) is derived from a low-passage isolate of the wild-type AR339 SIN strain (Taylor et al., 1955). SVIA and infectious SIN produced from SIN recombinant chimeric vectors were titered by plaque assays using BHK-21 cells as described (Hardwick and Levine, 2000). Infectious HSV-1 was produced as described (Orvedahl et al., 2007) and virus stocks were titered using Vero cells. For *in vitro* virus infections, cells were rinsed 2 times with Dulbecco's phosphate buffered saline (PBS) and infections were then performed in DMEM containing reduced serum (1% FBS) for 1 hr.

Retroviruses and Lentiviruses

pMXs-IP-HA-Parkin (Yoshii et al., 2011) (Addgene #38248) was cotransfected with the helper plasmids pUMVC and pCMV-VSV-G (Stewart et al., 2003) (Addgene #8849 and #8454) into HEK293T cells. pLenti-C-Myc-DDK-IRES-Neo (Origene) was modified by substituting the Myc-DDK tag for a 3X Flag tag. Wild-type or c.67delG mutant FANCC cDNAs were then cloned into pLenti-3X Flag-Neo and the resultant lentiviral vectors were cotransfected into HEK293 cells with the helper plasmids pCMVΔR8.91 (Zufferey et al., 1997) and pMDG (Naldini et al., 1996). Retro- or lentiviral supernatant was filtered through a 0.45 μm membrane and then added to target cells in the presence of polybrene (8 μg/ml). After 3 hr, the virus-containing medium was removed and replaced with fresh medium and, after an additional 48 hr, cells were cultured in selection medium containing 0.5 μg/ml puromycin or 500 μg/ml G418. After selection was completed, cells were maintained in 0.25 μg/ml puromycin and/or 100 μg/ml G418.

Quantitative RT-PCR

Total cellular RNA was extracted with the RNEasy Plus kit (Qiagen) and 1 μg RNA was used to synthesize cDNA (iScript, BioRad). Validated PCR primer pairs were purchased from Integrated DNA Technologies and used in PCR reactions with SYBR Green Master Mix (Qiagen) on a 7500 Fast Real-Time PCR system (Applied Biosystems).

Chemicals

Antimycin A and Oligomycin (OA, Santa Cruz) were resuspended in DMSO and stored in small aliquots at -80^{o}C. Bafilomycin A1 (Baf A1, Sigma), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma), and MitoTEMPO (Santa Cruz) were resuspended in DMSO and stored at -20^{o}C. Mitomycin C (Sigma) was resuspended in water and stored at -80^{0}C. Tris-buffered ATP (Thermo) was stored at -20^{o}C. Ultra pure LPS from E. coli 0111:B4 (Invivogen) was resuspended in sterile water and stored at -20^{o}C.

siRNA Treatment

siRNAs were purchased from Dharmacon and siRNA sequences are provided in Supplementary Table 1. All siRNA experiments were performed using reverse transfection at a final concentration of 60 nM siRNA using RNAiMAX (Invitrogen) according to manufacturer's instructions. At 48-72 hr after siRNA transfection, protein knockdown was assessed by western blot analysis or qRT-PCR.

Antibodies Used for Western blot Analyses

The following antibodies were used for western blot analyses: HRP-conjugated anti-actin

(Santa Cruz sc-47778-HRP, 1:2000 dilution), rabbit anti-ATG7 (Sigma A2856, 1:500 dilution), mouse monoclonal anti-ATP5B (Millipore MAB3494, 1:1000 dilution), rabbit anti-COXIV (Millipore AB10526, 1:500 dilution), rabbit anti-FANCA (Novus NBP1-18977, 1:500 dilution), rabbit anti-FANCC (Fanconi Anemia Research Fund FANCC-C2, 1:1000 dilution), rabbit anti-GABARAP (Novus NBP1-71771, 1:100 dilution), rabbit anti-GATE-16 (Millipore ABC24, 1:100 dilution), HRP-conjugated anti-Flag (Sigma A8592, 1:2000 dilution), rabbit anti-HSV-1/2 (Fitzgerald 20-HR50; 1:500 dilution), rabbit anti-LC3 (Novus NB100-2220, 1:750 dilution), goat anti-HSP60 (Santa Cruz sc-1052, 1:1000 dilution), mouse monoclonal anti-p62 (Abcam ab91562 1:1000 dilution), mouse monoclonal anti-Parkin (Cell Signaling Technology mAb4211, 1:1000 dilution), and rabbit anti-TOMM20 (Santa Cruz sc-11415, 1:1000 dilution).

Co-Immunoprecipitations

For FANCC-Flag/SIN capsid, FANCC-Flag/mCherry-Parkin, FANCA/SIN capsid, FANCC-Flag/Atg8 family proteins and FANCA/HA-Parkin co-immunoprecipitation (coIP), cells were lysed in coIP lysis buffer (Tris-HCl [pH7.4], 150mM NaCl, 1 mM EDTA, 1% TritonX-100, 1x cOMPLETE protease inhibitor cocktail [Roche], and 1x HALT phosphatase inhibitor cocktail [Thermo Fisher]) for 30 min at 4°C. Lysates were centrifuged at 2,500 rcf for 2 min and anti-Flag M2 antibody (Sigma F1804, 1:200) or anti-FANCA antibody (Novus NBP1-189771:200) was added to supernatants for 1.5 hr followed by the addition of Dynabeads Protein G (Invitrogen) for 30 min. Beads were washed 3 times with ice-cold DPBS for 5 min at 4°C and then boiled in Laemmli buffer + β-mercaptoethanol for 5 min.

LC3 Immunoprecipitation and Proteinase K Protection Assay

Mechanical lysis of HeLa cells was performed with 10 strokes through a 25 gauge needle and subcellular fractionation was performed using the QProteome mitochondria isolation kit (Qiagen) according to manufacturer's instructions. The microsomal + mitochondrial fraction was incubated with anti-LC3 antibody (Novus NB100-2220, 1:200 dilution) for 3 hr at 4°C followed by the addition of Dynabeads Protein G for 30 min. Beads were washed 5 times with ice-cold DPBS for 5 min at 4°C. For western blot analysis, Dynabeads were boiled in Laemmli buffer + β mercaptoethanol for 5 min. For the Proteinase K protection assay, Dynabeads were incubated in DPBS + Proteinase K (Sigma, final concentration 2.5 ng/mL) with or without 0.5% Triton X-100 on ice for 30 min followed by addition of preheated Laemmli buffer + β-mercaptoethanol and boiling for 5 min.

Immunoprecipitation of Intact Mitochondria

Mechanical lysis of HeLa cells and subcellular fractionation was performed as in the preceding section. The purified mitochondrial fraction was incubated with anti-TOMM20 antibody (Santa Cruz sc-17764, 1:200 dilution) for 3 hr at 4°C followed by the addition of Dynabeads Protein G for 30 min. Beads were washed 3 times with ice-cold DPBS for 5 min at 4°C. For western blot analysis, Dynabeads were boiled in Laemmli buffer + β-mercaptoethanol for 5 min.

Preparation of Dynabeads for Electron Microscopy

Dynabead samples were fixed in 2.5% glutaraldehyde in 0.1 M (pH 7.4) sodium cacodylate buffer for 2 hr and centrifuged for 2 min at 3,000 rpm. The supernatant was removed and Dynabeads were resuspended in 0.1M sodium cacodylate buffer and centrifuged for 2 min at 3,000 rpm. This process was repeated 4 times, and then samples were embedded in 3% agarose, sliced into small blocks (1 mm^3), rinsed with 0.1M sodium cacodylate buffer three times, and post-fixed in 1% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M sodium cacodylate buffer for 1.5 hrs at room temperature. Samples were rinsed with water and en bloc stained with 4% uranyl acetate in 50% ethanol for 2 hr in the dark, then dehydrated with increasing concentrations of ethanol, transferred into propylene oxide, infiltrated with Embed-812 resin and polymerized in a 60°C oven overnight. Blocks were sectioned with a diamond knife (Diatome) on a Leica Ultracut 7 ultramicrotome (Leica Microsystems), collected onto copper grids, post stained with 2% aqueous uranyl acetate and lead citrate, and imaging was performed as described in the "Electron Microscopy" section of the Supplementary Experimental Procedures.

Electron Microscopy

Tissue samples from 12 month-old *Fancc*$^{+/+}$ and *Fancc*$^{-/-}$ mice and MEFs subjected to starvation or viral infection were prepared for electron microscope as described (Liang et al., 1999) and imaged using a JEOL 1200EX microscope equipped with a SIS Morada CCD camera.

Immunofluorescence Microscopy and Image Analysis

For immunofluorescence analysis of cultured cells, samples were prepared as described (Shoji-Kawata et al., 2013). Slides were incubated for 1 hr with primary antibody and 30 min with secondary antibody and mounted with Prolong Diamond (Invitrogen). Primary antibodies were rabbit anti-ASC (Novus NBP1-78977), mouse monoclonal anti-ATP5B (Millipore MAB3494, 1:1000 dilution), and/or rabbit anti-TOMM20 (Santa Cruz sc-11415, 1:1000 dilution). Secondary antibodies were highly cross-adsorbed goat anti-mouse or anti-rabbit AlexaFluor 647 and/or goat-anti-rabbit AlexaFluor 488 secondary antibodies (Invitrogen, 1:750 dilution) with the addition of Phalloidin AlexaFluor 594 (Invitrogen, 1:100 dilution) for experiments involving cell segmentation. Images were acquired with a Zeiss AxioImager Z2 microscope equipped with a Photometrics CoolSnap HQ2 camera using a Zeiss PLAN APOCHROMAT 20X/0.8 NA air objective using the same acquisition times for samples stained with identical primary antibody pairs. For experiments using a binary (>10 or <10 mitochondria/cell) readout, single images were acquired and analysis was performed by an observed blinded to experimental condition. For automated image analysis, *Z*-stacks were acquired using the same system and deconvolved with AutoDeBlur (Bitplane). Thresholding for background secondary antibody staining was performed in all experiments. Quantitative image analysis was performed using the Cell module in Imaris version 8.0 (Bitplane). For mitophagy assays, a segmetation channel was created using the Channel Arithmetic function in Imaris XT = ((2 x phalloidin signal) + DAPI signal). Cell segmentation was then performed with this new channel using DAPI-positive, ~15 μm seeds. Additional filtering was applied to exclude all cells touching the edge of the image. ATP5B and TOMM20 puncta were modeled as ~0.75 μm "vesicles." The same creation parameters

for cell segementation and mitochondrial “vesicle” detection were then applied to all images within a given experiment using Imaris Batch. Data for numbers of cytoplasmic ATP5B and TOMM20 “vesicles” for all cells in a given experiment were then plotted in Imaris Vantage and then exported for further analysis. In the images generated for figures related to quantitative image analysis of mitophagy, nonspecific nuclear staining was masked in Imaris by creating a nuclear surface using the DAPI channel and setting non-DAPI signal within the nuclear surface to zero. For ASC speck counting, ASC specks and nuclei were modeled as surfaces in Imaris and the percentage of ASC speck-positive cells was calculated as (Number of ASC specks/Number of nuclei) x 100.

Immunohistochemistry

TUNEL staining of paraffin-embedded brain sections was performed according to manufacturers’ instructions (Apoptag® peroxidase *In Situ* Apoptosis Detection Kit; Chemicon International), using Sigma *FAST*™ 3, 3’ diaminobenzidine (DAB) tablets as the peroxidase substrate. Immunohistochemical staining of paraffin-embedded brain sections was performed using a rabbit anti-SIN capsid antibody (provided by M. MacDonald, 1:2000 dilution) or a mouse monoclonal anti-HSV ICP5 major capsid protein (Santa Cruz sc-56989, 1:500 dilution). Primary antibodies were detected with the ABC Elite kit (Vector Laboratories) according to manufacturer’s instructions. Whole-section montages were acquired with a Zeiss AxioScan.Z1 slide scanner with a Zeiss PLAN APOCHROMAT 20X/0.8 NA objective. The percentage of SIN or HSV-1 capsid-positive area per brain section was quantitated using custom macros in ImageJ. The

number of TUNEL-positive cells per area in mouse brain sagittal sections was counted by an observer blinded to experimental conditions.

Cell Death Assays

For measurement of mitomycin C-induced cell death, cells were mock-treated or treated with mitomycin C (1 μM) for 24 hr. For measurement of cytokine-induced cell death, cells were mock-treated or treated with TNF-α (50 ng/mL, R+D Systems) + IFN-γ (100 I.U./mL, R+D Systems) for 48 hr and then cell death was assessed using the Guava Nexin reagent (Millipore) according to manufacturer's instructions. Data were collected with a Guava EasyCyte 6HT-2L flow cytometer (Millipore) and analyzed with InCyte (Millipore).

Assessment of Mitochondrial ROS

To measure mitochondrial ROS, at indicated time points, cell culture medium was removed and replaced with fresh culture medium containing 5 μM MitoSOX (Invitrogen) for 30 min according to manufacturer's instructions. Cells were detached by trypsinization (patient fibroblasts) or incubation in DPBS + 1 mM EDTA (BMDMs) and MitoSOX fluorescence intensity was analyzed by flow cytometry.

Measurement of IL-1β

BMDMs were treated in triplicate as indicated and cell culture supernatants were collected and centrifuged to remove floating cells and cell debris. IL-1β ELISA (R+D Systems) was then performed on the resulting supernatants and quantitation of IL-1β

levels was performed using a PolarStar Omega plate reader (BMG Labtech) using a 4-parameter fit to a standard curve.

Supplemental Table 1, Related to Supplemental Experimental Procedures:

siRNA Target Sequences.

Gene name	siRNA #	Sequence
FANCA	1	CGCUUUGGCUGCUGGAGUA
	2	GGACAGAUCUGCACGGCUC
FANCC	1	GGUAUGCACCUAUAGAUUA
	2	GGAAUCGUCUUGGCAUUGA
	3	GAGAGAAUCAUCUUAAUGG
FANCF	1	GGUCAACGUUUGCACUAUG
	2	UAACUGCCCUGGAGACCUG
FANCL	1	GAAGUUGCCUUAAAGAAUA
	2	GAUAGGAACUCUUGGUUGG
FANCD2	1	UGGAUAAGUUGUCGUCUAU
	2	CAACAUACCUCGACUCAUU
BRCA1	1	CAACAUGCCCACAGAUCAA
	2	UGAUAAAGCUCCAGCAGGA
BRCA2	1	GAAACGGACUUGCUAUUUA
	2	GAAGAAUGCAGGUUUAAUA
ATG7	1	GGGUUAUUACUACAAUGGUGUU

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