Cell Reports

Supplemental Information

ATM Localization and Heterochromatin Repair Depend on Direct Interaction of the 53BP1-BRCT$_2$ Domain with γH2AX

Robert A. Baldock, Matthew Day, Oliver J. Wilkinson, Ross Cloney, Penelope A. Jeggo, Antony W. Oliver, Felicity Z. Watts, and Laurence H. Pearl

SUPPLEMENTARY TABLE 1. Crystallographic Data Collection and Refinement Statistics – Relates to FIGURE 1

Data Collection	
Wavelength (Å)	0.9173
Resolution range (Å)	48.06 - 3.0 (3.107 - 3.0)*
Space group	P 2_1 2_1 2_1
Cell dimensions a, b, c (Å) α, β, γ (°)	 70.26, 94.50,131.74 90, 90, 90
Total reflections	77558 (7954)
Unique reflections	18110 (1778)
Multiplicity	4.3 (4.5)
Completeness (%)	99.66 (99.83)
Mean I / σ(I)	12.57 (1.40)
Wilson B-factor	77.04
R_{merge}	0.1044 (1.057)
$CC^1/_2$	0.997 (0.563)
CC*	0.999 (0.849)
Refinement	
Reflections used for R-free	
R_{work}	0.2053 (0.3441)
R_{free}	0.2512 (0.4353)
Number of non-hydrogen atoms	6537
macromolecules	6473
ligands	9
water	56
Protein residues	854
RMS bond-lengths	0.014
RMS bond-angles	1.80
Ramachandran favored (%)	92
Ramachandran allowed (%)	6.4
Ramachandran outliers (%)	1.6
Clashscore	4.68
Average B-factor	88.6
macromolecules	88.9
ligands	85.4
solvent	63.4

* Statistics for the highest-resolution shell are shown in parentheses

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein Expression and Purification

His_6-SUMO-53BP1-$BRCT_2$ and His_6-SUMO-MDC1-$BRCT_2$ were expressed from a modified pET-15b vector (EMD-Millipore, Darmstadt, Germany) in *Escherichia coli* BL21(DE3) (Novagen). Cell pellets were re-suspended in lysis buffer containing 50mM HEPES pH 7.5, 300mM NaCl, 0.25mM TCEP, 10% w/v glycerol, then disrupted by sonication, and the resulting lysate clarified by centrifugation at 40,000 x *g* for 60 minutes at 4°C. The supernatant was applied to a 5ml HiTrap TALON crude column (GE Healthcare, Little Chalfont, UK), washed with buffer containing 50mM HEPES pH 7.5, 300mM NaCl, 0.25mM TCEP, 10mM imidazole, with any retained protein then eluted by application of the same buffer but now supplemented with 250mM imidazole.

For FP experiments, a Superdex 75 16/60 size exclusion column (GE Healthcare) was used to purify the His_6-SUMO-53BP1-$BRCT_2$ and His_6-SUMO-MDC1-$BRCT_2$ proteins to homogeneity in 25mM HEPES pH 7.5, 200mM NaCl, 1mM EDTA, 0.25mM TCEP, 0.02% (v/v) Tween-20.

For crystallographic studies, the N-terminal metal affinity and solubility tag (His_6-SUMO) was removed from the His_6-SUMO-53BP1-$BRCT_2$ by incubation with GST-SENP1c protease (in house) for 12 hours at 4°C. A Superdex 75 16/60 size exclusion column (GE Healthcare) was used to purify 53BP1-$BRCT_2$ to homogeneity in 20mM HEPES pH 7.5, 200mM NaCl, 0.25mM TCEP.

GST-$P53^{Core}$ was expressed from a modified pGEX-6P-1 vector (EMD-Millipore) in *E. coli* BL21(DE3). Cell pellets were re-suspended in lysis buffer containing 50mM HEPES pH 7.5, 300mM NaCl, 2.5mM EDTA, 0.25mM TCEP, 10% w/v glycerol, then disrupted by sonication, and the resulting lysate clarified by centrifugation at 40,000 x *g* for 60 minutes at 4°C. The supernatant was applied to a 5 ml GSTrap FF column (GE Healthcare), washed with lysis buffer and then eluted by application of the same buffer but now supplemented with 20mM Glutathione. The N-terminal GST affinity tag was removed by incubation with Human rhinovirus-3C protease (in house) for 12 hours at 4°C. A Superdex 75 16/60 size exclusion column (GE Healthcare) was used to purify $P53^{Core}$ to homogeneity in 20mM HEPES pH 7.5, 200mM NaCl, 0.25mM TCEP.

To produce the $P53^{Core}$ / 53BP1-$BRCT_2$ complex, 53BP1-$BRCT_2$ was mixed with a slight molar excess of $P53^{Core}$ and the resulting complex purified using a Superdex

75 16/60 size exclusion column (GE Healthcare) in 20mM HEPES pH 7.5, 200mM NaCl, 0.25mM TCEP.

Fluorescence Polarisation Experiments

Fluorescein-labelled peptides (Peptide Protein Research Ltd, Bishops Waltham, UK) at a concentration of 200nM, were incubated at room temperature with increasing concentrations of His_6-SUMO-53BP1-$BRCT_2$ in 25mM HEPES pH 7.5, 200mM NaCl, 1mM EDTA, 0.25mM TCEP, 0.02% (v/v) Tween-20 in a black 96-well polypropylene plate (VWR, Lutterworth, UK). Fluorescence polarisation was measured in a POLARstar Omega multimode microplate reader (BMG Labtech GmbH, Offenburg, Germany). Binding curves represent the mean of 4 independent experiments, with error bars of 1 standard deviation. All data were fitted by non-linear regression, to a one site – specific binding model in Prism 6 for Mac OS X (v 6.0d, GraphPad Software) in order to calculate the reported disassociation constants (K_d).

Crystallisation, X-ray Diffraction Data Collection, Phasing, Model Building and Refinement

Co-crystallisation trials of the $P53^{Core}$ / 53BP1-$BRCT_2$ complex were set up in MRC 2 96-well sitting-drop vapour-diffusion plates by mixing 200nl of 12mg/ml protein solution with 200nl of mother, over a well volume of 50µl. Crystals that grew from condition F1 (200mM NaF, 100mM Bis-Tris Propane pH 6.5, 20% (w/v) PEG 3,350) of the PACT *premier* HT-96 (Molecular Dimensions, Newmarket, UK) screen, were soaked in mother liquor with the addition of both 20% (v/v) PEG 400 and 20µM γH2AX-pS139 peptide (pSQEY) for 60 minutes prior to plunge-freezing in liquid nitrogen.

Diffraction data were collected at the I04-1 beamline at the Diamond Synchrotron Lightsource (Didcot, UK) and auto-processed using the Xia2 software pipeline. The structure was determined by molecular replacement using Phaser (McCoy et al., 2007) with PDB entry 1KZY as a search model. The structure was built using COOT (Emsley et al., 2010) and refined with Phenix (Zwart et al., 2008).

Tissue Culture, Cell lines and Reagents

HeLa, $H2AX^{+/+}$ and $H2AX^{-/-}$ MEF cells were cultured in DMEM supplemented with 10% (v/v) FCS, penicillin, streptomycin and L-glutamine. Caffeine (Sigma-Aldrich, Gillingham, UK) was used at a final concentration of 10mM. ATMi (KU55933, Abcam, Cambridge, UK), DNA-PKi (NU7441, Santa Cruz Biotechnology, Heidelberg,

Germany) and ATRi (ATR-kinase inhibitor II, Merck-Millipore) were all used at a concentration of 10μM, except for the CHK1 inhibitor UCN-01 (Sigma-Aldrich) that was used at a concentration of 200nM. Cells were irradiated using a Caesium^{137} gamma source.

siRNA depletion of 53BP1/KAP-1 and siRNA-resistant expression of 53BP1

HeLa cells were seeded at high confluence onto 35mm plates and reverse transfected with either 53BP1-siRNA or a negative control - Stealth RNAi (Life Technologies, Paisley, UK). Cells were transfected using Hiperfect (Qiagen, Manchester, UK) with oligonucleotides diluted into OptiMEM serum free media (Life Technologies) and to a final concentration of 20nM. 53BP1-siRNA oligonucleotides used: 5'-AGAACGAGGAGACGGUAAUAGUGGG-3' and 5' CCCACUAUUACCGUCUCCUCGUUCU 3' (Invitrogen). Cells were cultured for a further 48 hours for efficient depletion. Following this cells were washed in 1xPBS before transfection with siRNA-resistant 53BP1 constructs using NanoJuice Core Transfection Reagent (Merck-Millipore) and booster at a ratio of 3:1 (Reagent:DNA). Resistance to siRNA was conferred by 3 silent point mutations in the 53BP1 cDNA clone in pCMH6K (A231G, A234G and A237C) (Noon et al., 2010). Each plate was transfected with 1.5μg of plasmid DNA. Cells were incubated for a further 16 hours to allow cells to express the plasmid before irradiation. For dual depletion of both 53BP1 and KAP1, both oligonucleotides were transfected at a final concentration of 20nM. KAP1 siRNA oligonucleotides used: 5' GCGGAAAUGUGAGCGUGUAtt 3' and 5' UACACGCUCACAUUUCCGCtg 3' (Life Technologies).

Antibodies

Dilutions shown refer to immunofluorescence unless otherwise stated: α-HA probe mouse monoclonal (Santa Cruz Biotechnology) at 1:200, α-phosphorylated ATM (S1981) rabbit monoclonal (Abcam, ab81292) at 1:400, α-γH2AX (S139) rabbit polyclonal (Merck-Millipore, DR1017) at 1:400, α-γH2AX (S139) mouse monoclonal (Merck-Millipore, 05-636) at 1:800, α-53BP1 rabbit polyclonal (Bethyl Laboratories, Montgomery, TX, USA, A300-272A) at 1:400 for IF and 1:5000 for WB, α-BRCA1 mouse monoclonal (Santa Cruz Biotechnology, sc-642) at 1:100, α-NBS1 rabbit polyclonal (Novus, Abingdon, UK, NB100-143) at 1:500, α-ATR (Santa Cruz Biotechnology, Sc-1887) and α-KAP1 (Abcam, a10484) at 1:2500 for WB. Secondary

antibodies used were: HRP-rabbit (Cell Signaling Technology, Danvers, MA, USA) at 1:2500 for WB, FITC-mouse (Sigma-Aldrich), TritC-Rabbit (Sigma-Aldrich) and Cy5-Rabbit (Life Technologies) all used at 1:200.

Immunofluorescence

Cells cultured on coverslips were fixed using 4% (w/v) PFA in PBS for 10 minutes and permeablised using 0.1% (v/v) Triton-X100 in PBS for 30 seconds. Cells were washed 3 times with PBS before incubation for 1 hour at room temperature with the primary antibodies diluted to the indicated concentration in 4% (w/v) BSA in PBS. Cells were washed a further 3 times with PBS before incubation for 30 minutes with the secondary, fluorophore-coupled, antibodies. Secondary antibodies were diluted as before in 4% (w/v) BSA in PBS. Cells were washed a final 3 times before mounting on glass slides with ProLong Gold with DAPI mounting media (Life Technologies).

Live cell imaging and UV-laser microirradiation

Cells were seeded at a lower confluence before 53BP1 depletion by siRNA and eYFP-SV40$_{NLS}$-53BP1-BRCT$_2$ plasmid constructs were transfected into cells using NanoJuice as before. Cells were incubated for 20 minutes with 100μg/ml Hoecsht 34580 (Scientific Laboratory Supplies, Hessle, UK, 63493) prior to excitation with a 405nm laser. Protein localisation was tracked over 3 minutes using a 488nm laser.

GFP-Trap

Stable cell lines were generated by G418 antibiotic selection for HeLa cells transfected with wild-type or R1811E mutant forms of the eYFP-SV40$_{NLS}$-53BP1-BRCT$_2$ expression construct.

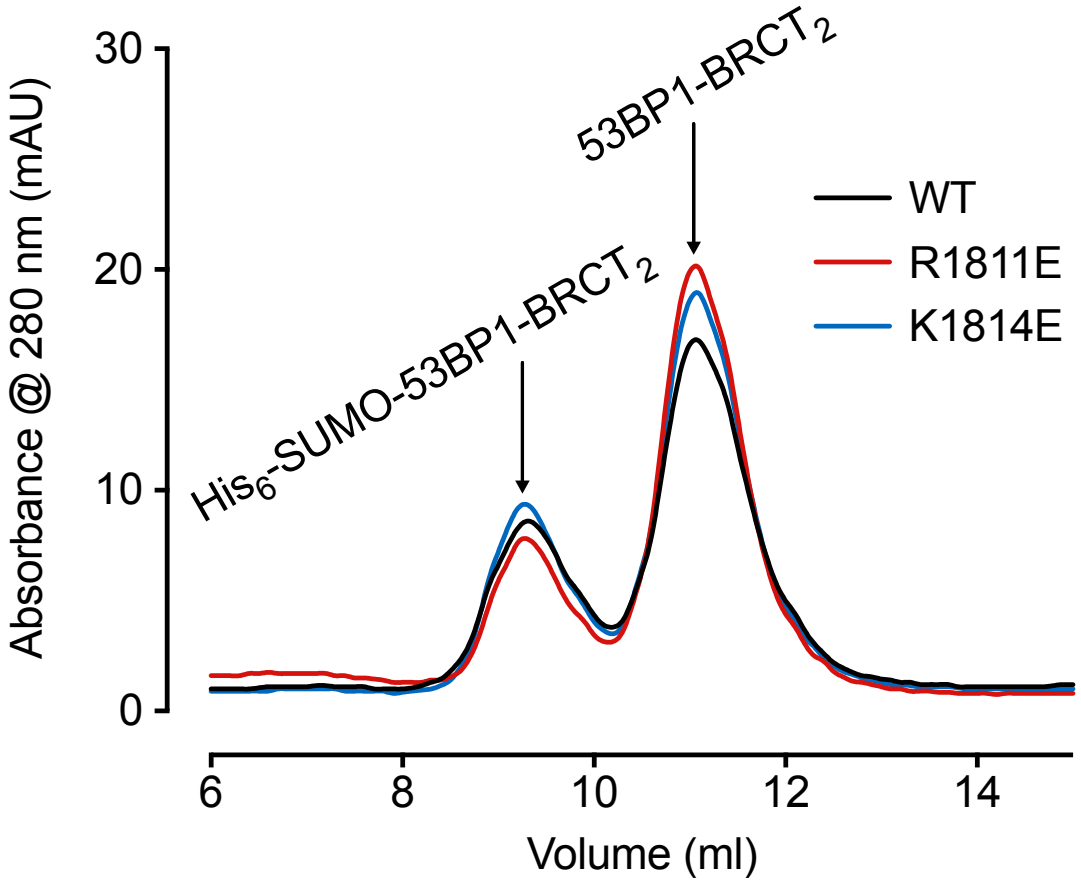
Irradiated cells were: harvested by scraping 4 x 20cm petri dishes - seeded at 1.8 x 10^5 cells / ml and grown in 30ml of DMEM medium with G418 selection (400μg/ml) for 48 hours at 37°C in a 5% CO_2 environment – into PBS buffer, pelleted by centrifugation, and then stored at -20°C.

Cellular lysates were generated by re-suspension of the frozen cell pellets in 2ml of RIPA buffer (Sigma-Aldrich) supplemented with EDTA-free protease- and PhosSTOP

phosphatase-inhibitor tablets (Roche Diagnostics, Burgess Hill, UK) and 40µl Benzonase endonuclease (25 Units/µl, Merck-Millipore), followed by disruption in a Bioruptor Pico with water cooler (Diagenode, Seraing, Belgium). Cell debris and insoluble material were removed by centrifugation at 16,000 x *g*, for 10 minutes at 4°C, followed by dilution in 10mM Tris-HCl pH 7.5, 150mM NaCl, 0.5mM EDTA, 0.5mM TCEP supplemented with protease and phosphatase inhibitor tablets as before, to a final volume of 10ml. The diluted supernatant was then incubated with ~200µl bed volume of GFP-Trap_A resin (Chromotek, Planegg-Martinsried, Germay) following the manufacturer's recommended protocol. After binding and washing of the beads with dilution buffer, any retained protein was detected by chemi-luminescent western blot, following SDS-PAGE separation and transfer to a nitrocelluose membrane. Antibodies used: rabbit polyclonal anti-GFP (2555S, Cell Signaling), donkey anti-rabbit IgG-HRP conjugate (NA934V, GE Healthcare), mouse monoclonal anti-phospho-Histone H2AX(Ser139) (JBW301, Merck-Millipore), sheep anti-mouse IgG-HRP conjugate (NXA931, GE Healthcare).

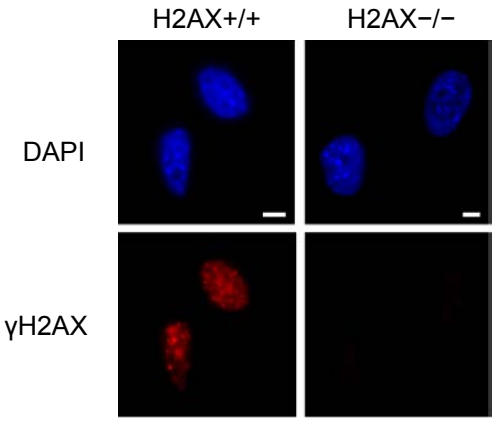
SUPPLEMENTARY FIGURE LEGENDS

FIGURE S1 - Gel Filtration analysis of wt and point-mutant 53BP1-BRCT2 constructs – relates to FIGURE 1



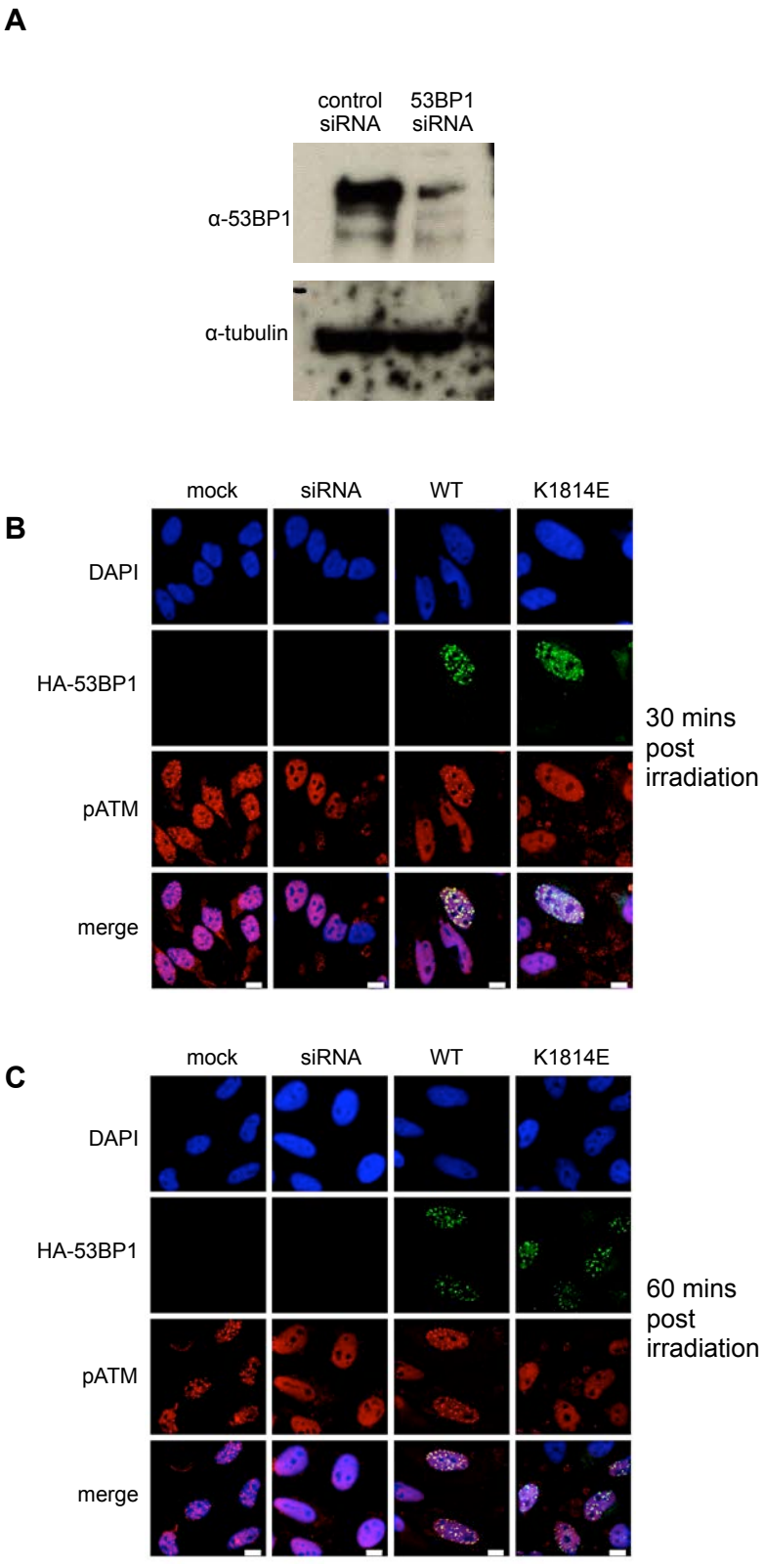
Elution profiles for 53BP1-$BRCT_2$ constructs with/without His_6-SUMO tag used for purification. Regardless of the presence of point-mutations that disrupt the ability of the $BRCT_2$ domain to bind γH2AX, the proteins elute from a calibrated gel filtration column at positions consistent with dimers of the predicted monomer molecular weight.

FIGURE S2 - γH2AX staining in MEFs - relates to FIGURE 2



Immunofluorescence staining for γH2AX in mouse embryonic fibroblast lines with. No γH2AX response is seen in H2AX -/- cells.

FIGURE S3 - 53BP1 siRNA knock down and effect of 53BP1 mutants on focus formation - relates to FIGURE 3

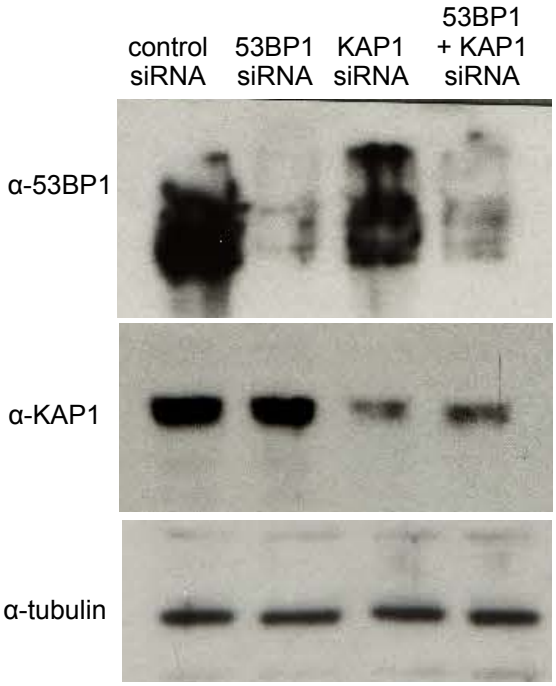


A) Western blot of HeLa cells confirming substantial knock-down of 53BP1 protein levels in siRNA treated cells.

B) Immunofluorescence staining 30 minutes post irradiation for HA-53BP1 and pATM in γ-irradiated HeLa cells that were : mock transfected, transfected with 53BP1 siRNA, siRNA treated and transfected with an siRNA-resistant wild-type HA-53BP1 or a K1814E mutant 53BP1.

C) As B) but visualized 60 minutes post-irradiation.

FIGURE S4 - 53BP1 and KAP1 siRNA knock-downs and focus γH2AX focus persistence - relates to FIGURE 4



Western blot of HeLa cells confirming substantial knock-downs of 53BP1 and KAP1 protein levels in siRNA treated cells.

SUPPLEMENTARY MOVIES

MOVIE S1 – Example time-lapse of fluorescence microscope field of HeLa cells showing of eYFP-MDC1-$BRCT_2$ recruitment to laser stripe damage; positive control for formation of DNA double-strand breaks and γH2AX by laser microirradiation. Crosshairs mark start and end of laser path(s). Relates to **FIGURE 2**

MOVIE S2 – As **S1** but for eYFP-NLS-53BP1-$BRCT_2$ showing wild-type $BRCT_2$ mediates recruitment to DNA damage. Relates to **FIGURE 2**

MOVIE S3 – As **S3** but for eYFP-NLS-53BP1-$BRCT_2$ with K1814E mutation, which abrogates interaction with γH2AX and prevents recruitment to laser stripe. Relates to **FIGURE 2**

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