Supplementary Information

Supplementary figure legends

Supplementary Figure 1: Accumulation of SUMO1, SUMO3 and Ubc9 at sites of laser-induced DNA damage becomes apparent within minutes after irradiation. a-d, Kinetics of: (**a**), GFP-53BP1,13.5 min at 1.5 min intervals (10 frames in total); (**b**), RFP-SUMO1, 19.5 min at 1.5 min intervals (14 frames in total); (**c**), RFP-SUMO3, 27 min at 3 min intervals (10 frames in total); (**d**), GFP-Ubc9, 19.5 min at 1.5 min intervals (14 frames in total). Movie speed: 2 frames/sec. **a**, U2OS cells stably expressing GFP-53BP1 were laser-micro-irradiated and imaged. 53BP1 was used as a marker for damage-response kinetics in the system. **b**, **d**, U2OS cells were co-transfected with GFP-Ubc9 and RFP-SUMO1, and 24 h following transfection, cells were laser micro-irradiated and imaged. **c**, U2OS cells were transfected with RFP-SUMO3 alone, and 24 h following transfection, cells were laser micro-irradiated and imaged. Note that all cells displayed SUMO accumulation at 20 min after laser micro-irradiation, while some but not others lost SUMO lines 1 h after irradiation.

Supplementary Figure 2: Accumulation of SUMO1, SUMO3 and Ubc9 at various times after DNA-damage induction. **a**, **b**, U2OS cells were laser micro-irradiated, allowed to recover at 37^0C for 1, 4 or 8 h, pre-extracted, fixed and co-stained for SUMO1 and 53BP1 (**a**) or SUMO2/3 and 53BP1 (**b**). **c**, Persistence of SUMO proteins at sites of laser micro-irradiation is detected in both G_1 and S/G_2 cells. U2OS cells stably expressing GFP-CtIP were laser micro-irradiated and, 2 h post-treatment, cells were fixed, permeabilized and stained as indicated. **d**, U2OS cells transfected with GFP-Ubc9 were laser micro-irradiated, fixed, permeabilized and stained for 53BP1 at 1, 2 or 4 h after damage induction.

Supplementary Figure 3: Accumulation of SUMO proteins at DNA-damage sites requires ATM activity, RNF8 and RNF168. a, ATM activity is needed for SUMO1 and SUMO2/3 accumulation at DNA-damage sites. U2OS cells were pre-treated with ATM inhibitor KU-55933 (ATMi) or DMSO, laser micro-irradiated, allowed to recover at 37^0C for 4 h, pre-extracted, fixed and co-stained for 53BP1 and either SUMO1 or SUMO2/3. Note that only cells displaying residual 53BP1 recruitment were analyzed so that we could be certain that they had been laser-treated. **b**, RNF8 is needed for SUMO1, SUMO3 and 53BP1 accumulation at DNA-damage sites. U2OS cells stably expressing GFP-SUMO1 or GFP-SUMO3 were transfected with Luciferase (Luc) or RNF8 siRNAs for 48 h, laser micro-irradiated, allowed to recover at 37^0C for 2 h, fixed, permeabilized and co-stained for γH2AX and 53BP1. **c**, RNF168 is needed for SUMO1 and SUMO2/3 accumulation at DNA-damage sites. hTERT-RIDDLE syndrome fibroblasts complemented with vector or HA-RNF168 were laser micro-irradiated, allowed to recover at 37^0C for 2 h, fixed, permeabilized and co-stained for γH2AX and SUMO1 or SUMO2/3.

Supplementary Figure 4: **Effects of depleting SUMO E3 ligases on the accumulation of SUMO proteins and 53BP1 at sites of DNA damage. a**, PIAS4 is required for accumulation of SUMO1 and 53BP1 at sites of laser micro-irradiation. U2OS cells were transfected with Luciferase (Luc), PIAS1, PIAS2, PIAS3, PIAS4 or MMS21 siRNAs for 48 h, laser micro-irradiated, allowed to recover at 37^0C for 4 h, pre-extracted, fixed and co-stained for SUMO1 and 53BP1. **b**, PIAS1 is required for accumulation of SUMO2/3 at sites of laser micro-irradiation; procedures were as in (**a**), except cells were co-stained for SUMO2/3 and 53BP1. **c**, Quantification of the percentage of cells displaying MDC1 accumulation on laser-lines that also display 53BP1 accumulation for cells transfected with Luciferase (Luc), PIAS1 or PIAS4 siRNAs as described in Fig. 2a and b. Each column represents at least 200 MDC1-positive cells accumulated over two independent experiments. **d**, PIAS4 is required for 53BP1 IRIF. U2OS

cells were transfected with Luciferase (Luc) or two independent PIAS4 siRNAs, exposed to 2 Gy of IR, allowed to recover at 37^0C for 2 h, pre-extracted, fixed and co-stained for γH2AX, 53BP1 and DAPI. For depletions, see Supplementary Fig. 10.

Supplementary Figure 5: Complementation of 53BP1 IRIF using siRNA-resistant PIAS4 and demonstration that PIAS4 recruitment to laser–lines is independent of 53BP1. a,b, U2OS cells stably expressing siRNA-resistant RFP-PIAS4 (PIAS4siR) or RFP alone (Vector) were transfected with PIAS4 or Luciferase (Luc) siRNAs, exposed to 2 Gy of IR, allowed to recover at 37°C for 2 h, pre-extracted, fixed and co-stained for γH2AX, 53BP1 and DAPI. Representative images are shown in (**a**), while in (**b**), each column represents at least 200 γH2AX-positive cells accumulated over two independent experiments. **c,** PIAS4 accumulation at sites of DNA damage is independent of 53BP1. U2OS cells were transfected with 53BP1 siRNAs, followed by transfection with RFP-PIAS4 24 h later; after a further 24 h, cells were laser micro-irradiated, allowed to recover at 37°C for 2 h, fixed, permeabilized and co-stained for γH2AX and 53BP1. **d,** Representative images used in GFP-53BP1 FRAP analyses shown in Fig. 2e. For depletions and siRNA-resistant PIAS4, see Supplementary Fig. 10.

Supplementary Figure 6: Sumoylation of 53BP1 *in vivo* and demonstration that PIAS4 is required for 53BP1 sumoylation. a, HEK293 cells were co-transfected with full length (1-1972) HA-53BP1 and GFP-SUMO1 or GFP (samples were derived from splitting samples used in Fig. 2h). 48 h later, extracts were prepared 2 h after mock-treatment (-) or treatment with 10 Gy of IR. Half of each extract was used for GFP-SUMO1 or GFP immunoprecipitations by GFP-Trap-A beads (Fig. 2h), while HA-53BP1 was immunoprecipitated from the other half using anti HA antibodies (Supplementary Fig. 6a). Samples were analyzed by 4-18% gradient SDS-PAGE and immunoblotted with the indicated antibodies. **b,** Endogenous 53BP1 is modified by endogenous SUMO1 in a manner that is impaired by PIAS4 depletion. U2OS cells were

transfected with the indicated siRNAs and, 48 h later, extracts were prepared 2 h after mock-treatment (-) or treatment with 10 Gy of IR as in (**a**), and 53BP1 was immunoprecipitated. Samples were analyzed as in (**a**) and probed with the indicated antibodies. For depletions, see Supplementary Fig. 10. **c, d,** HA-53BP1 fragments are modified by GFP-SUMO1. Procedures for GFP-SUMO1 and GFP immunoprecipitations are as in (a) except HA-NLS-53BP1-N (1-1052), HA-53BP1-C (1052-1972) (**c**), HA-53BP1-CΔBRCT (1052-1710) or HA-53BP1-BRCT (1483-1972) (**d**), were co-transfected with GFP-SUMO1 or GFP alone. **e,** Summary of the data presented in Fig. 2h and Supplementary Figs 6a-d.

Supplementary Figure 7: Accumulation of BRCA1 in IRIF requires PIAS1, and expression of recombinant wild-type PIAS1 restores BRCA1 accumulation at sites of DNA damage in cells depleted of endogenous PIAS1. a, U2OS cells were transfected with siRNAs targeting the PIAS1 coding sequence (siPIAS1) or PIAS1 3'UTR (siPIAS1-3'UTR), exposed to 2 Gy of IR, allowed to recover at 37^0C for 2 h, pre-extracted, fixed and co-stained for γH2AX, 53BP1 and DAPI. **b,** Representative images used in GFP-BRCA1 FRAP analyses shown in Fig. 3b (for depletions, see Supplementary Fig. 10). **c-e,** U2OS cells were transfected with PIAS1-3'UTR (PIAS1-3'UTR) or Luciferase (Luc) siRNAs, and 8 h later cells were transfected with GFP alone (-) (**c**), GFP-PIAS1 wild-type (WT), GFP-PIAS1 E3 ligase-dead (LD) or GFP-PIAS1 lacking its SAP domain (ΔSAP) (**d**) followed by a second round of siRNA transfection 24 h later. After another 24 h, cells were laser micro-irradiated, allowed to recover at 37^0C for 2 h, pre-extracted, fixed and co-stained as indicated. Representative images are shown in (**c**) and (**d**); in (**e**), each column represents at least 200 γH2AX-positive cells accumulated over two independent experiments.

Supplementary Figure 8: PIAS1 and PIAS4 promote accumulation of RPA32 at DNA-damage sites. a-c, U2OS cells were transfected with the indicated siRNAs and, 48 h later were

laser micro-irradiated, allowed to recover at 37^0C for 2 h, pre-extracted, fixed and co-stained as indicated. Representative images are shown in (**c**). Panel (**a**) shows the percentage of γH2AX-positive cells that were positive for RPA32 at laser-lines. Panel (**b**) shows the percentage of γH2AX-positive cells that were positive for RPA32 laser-lines after normalisation to the proportion of S/G_2 cells as measured by the FACS analysis presented in Supplementary Fig. 10e (note that PIAS4 depletion results in higher proportion of S/G_2 cells). Each column in (**a**) and (**b**) represents at least 200 γH2AX-positive cells accumulated over two independent experiments. Note that even cells displaying weak RPA32 lines were counted as positive.

Supplementary Figure 9: Ub/FK2 accumulation at sites of DNA damage requires PIAS1 and PIAS4, while accumulation of UbH2A and RNF168 requires only PIAS4. a, Percentage of RNF8 positive cells that were positive for Ub/FK2; representative images are shown in Fig 4b. PIAS4 has a greater effect on ubiquitin accumulation than does PIAS1, which may reflect the fact that PIAS4 affects both BRCA1 and RNF168 accumulation, while PIAS1 only affects BRCA1 accumulation. **b,** Percentage of γH2AX positive cells that were positive for UbH2A; representative images from a similar experiment showing GFP-BRCA1 and UbH2A are shown in Fig 4c. Each column in (**a**) and (**b**) represents at least 100 RNF8 or γH2AX-positive cells accumulated over two independent experiments. **c,** Procedures were as in Fig. 4d, except cells were transfected with two independent PIAS4 siRNAs. **d,** Representative images are shown in (**c**), while (**d**) shows the percentage of γH2AX positive cells that were positive for RNF168, each column represents at least 200 γH2AX-positive cells accumulated over two independent experiments. Note that the efficiency of the siRNA depletions, as shown in Supplementary Fig. 10c, correlate with the relative affects on RNF168 accumulation

Supplementary Figure 10. siRNA-mediated depletion of proteins, and flow cytometric analyses of PIAS1-depleted and PIAS4-depleted cells. a, siRNA-mediated depletion of

endogenous MDC1, 53BP1, BRCA1 and Ubc9. U2OS cells were transfected with Luciferase (Luc), MDC1, 53BP1, BRCA1, or Ubc9 siRNAs and, 48 h following siRNA transfection, cellular extracts were prepared and subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. **b**, siRNA-mediated depletion of recombinant MMS21, PIAS1, PIAS2, PIAS3 and PIAS4. U2OS cells were co-transfected (see Methods) with Flag-MMS21, GFP-PIAS1, GFP-PIAS2, GFP-PIAS3 or Flag-S-tag-PIAS4 together with Luciferase (Luc), MMS21, PIAS1, PIAS2, PIAS3 or PIAS4 siRNAs as indicated. Extracts were prepared and subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. **c,** siRNA-mediated depletion of recombinant RFP-PIAS4 and demonstration of siRNA resistance of RFP-PIAS4-siR. Procedures as in (**b**) except cells were transfected with RFP-PIAS4 or RFP-PIAS4 siRNA resistant. **d,** siRNA mediated depletion of RNF8. Procedures as in (**a**), except cells were transfected with 2 independent RNF8 siRNAs. **e**, Cell-cycle profiles of irradiated and non-irradiated PIAS1-depleted and PIAS4-depleted cells. U2OS cells were transfected with Luciferase (Luc), PIAS1 or PIAS4 siRNAs and, 48 h following siRNA transfection, cells were exposed to 5 Gy of IR, harvested at the indicated times and subjected to flow cytometric analysis.

Supplementary tables

Supplementary Table 1

siRNAs used in this work			
siRNA	Sense sequence	Reference/Cat. Num	Suppliers
Luc.	CGUACGCGGAAUACUUCGA	[17]	MWG Biotech
MDC1	GUCUCCCAGAAGACAGUGA	[28]	MWG Biotech
53BP1	GAAGGACGGAGUACUAAUA	This work	MWG Biotech
BRCA1	GGAACCUGUCUCCACAAAG	[32]	MWG Biotech
CtIP	GCUAAAACAGGAACGAAUC	[33]	MWG Biotech
PIAS1	GGAUCAUUCUAGAGCUUUA	SI00113974	QIAGEN
PIAS1 3'UTR	CGAAUGAACUUGGCAGAAA	This work	MWG Biotech
PIAS2	CUUGAAUAUUACAUCUUUA	SI00684390	QIAGEN
PIAS3	CCCUGAUGUCACCAUGAAA	SI00684418	QIAGEN
PIAS4-1	GGAGUAAGAGUGGACUGAA	SI00684439	QIAGEN
PIAS4-2	AGGCACUGGUCAAGGAGAA	This work	MWG Biotech
MMS21	CUCUGGUAUGGACACACAGCU	[34]	MWG Biotech
Ubc9	GUAGCUGUCCCAACAAAGA	[35]	MWG Biotech
Ligase4	AGGAAGUAUUCUCAGGAAUUA	This work	MWG Biotech
RNF8a	UGGACAAUUAUGGACAACA	This work	MWG Biotech
RNF8c	CCAAGAACAAAGAAUUAG	This work	MWG Biotech
RNF168	GGCGAAGAGCGAUGGAGGA	[7]	MWG Biotech

Supplementary Table 2

Primers used in this work	
XhoI-SUMO1	5'-GGACTCGAGATTCTGACCAGGAGGCAAAACCTTC-3’
BamHI-SUMO1	5’-CCGGGATCCTCAACCCCCCGTTTGTTCCTGATAAAC-3’
XhoI-SUMO2	5'-GGACTCGAGATGCCGACGAAAAGCCCAAGGAAG-3’
BamHI-SUMO2	5'-CCGGGATCCTCAACCTCCCGTCTGCTGTTGGAAC-3’
XhoI-SUMO3	5'-GGACTCGAGATTCCGAGGAGAAGCCCAAGGAG-3’
BamHI-SUMO3	5'-CCGGGATCCTCAACCTCCCGTCTGCTGCTGGAAC-3’
XhoI-PIAS1	5’-TATACTCGAGCTGCGGACAGTGCGGAACTAAAG-3’
EcoRI-PIAS1	5’-TATAGAATTCTCAGTCCAATGAAATAATGTCTGG-3’
XhoI-PIAS1ΔSAP	5’-TATACTCGAGCTAAGGCTGGCTGTAGTCCTGC-3’
PIAS1-C351A-F	5’-CCCTTACAGCTTCTCATCTACAATGTTTTGAC-3’
PIAS1-C351A-R	5’-GTAGATGAGAAGCTGTAAGGGCCCGACACGG-3’
XhoI-PIAS2β	5’-TATACTCGAGCTGCGGATTTCGAAGAGTTGAG-3’
EcoRI-PIAS2β	5’-TATAGAATTCTTAGTCCAATGAGATGATGTCAGG-3’
XhoI-PIAS3	5’-TATACTCGAGCTGCGGAGCTGGGCGAATTAAAGC-3’
EcoRI-PIAS3	5’-TATAGAATTCTCAGTCCAGGGAAATGATGTCTG-3’
XhoI-PIAS4	5’-TATACTCGAGCTGCGGCGGAGCTGGTGGAGGCCAAAAACATG-3’
EcoRI-PIAS4	5’-TATAGAATTCTCAGCAGGCCGGCACCAGGCCCTTCTGGAAG-3’
XhoI-PIAS4ΔSAP	5’-TATACTCGAGCTCAGTTTGACTGTAGCCCTG-3’
PIAS4-C342A/C347A-F	5’-GCAGAGACCGCCGCCCACCTGCAGGCCTTCGACGCCGTCTTCTAC-3’
PIAS4-C342A/C347A-R	5’-GGCGTCGAAGGCCTGCAGGTGGGCGGCGGTCTCTGCCCGGCAGGG-3’
PIAS4siR-F	5’-CTGGGTTTCGTGGGCCGATCCAAGTCCGGACTGAAGCACGAGCTCGTC-3’
PIAS4siR-R	5’-GACGAGCTCGTGCTTCAGTCCGGACTTGGATCGGCCCACGAAACCCAG-3’
ClaI-MMS21	5’-TGAATCGATCCAGGACGTTCCAGTTCAAATTC-3’
ClaI-MMS21	5’-TGAATCGATCTACTCGGAATGACGATGTCTTTTC-3’
BamHI-Stag-Ubc9	5’-CGCGGATCCAAAGAAACCGCTGCTGCTAAATTCGAACGC CAGCACATGGACAGCTCGGGGATCGCCCTCAGCAG-3’
XhoI-Ubc9	5’-CCGCTCGAGTTATGAGGGCGCAAACTTCTTGG-3’

Supplementary Table 3

Antibodies used in this work			
Antibody	Species	Reference /Suppliers	Used
HA.11	Mouse	Covance	IF
HA probe F7	Mouse	Santa-Cruz	IP/IB
FLAG-M2	Mouse	Sigma	IB
GFP	Mouse	Roche	IB
Stag	Rabbit	Bethyl	IB
SUMO1	Rabbit	Abcam	IF
Sentrin1/SUMO1	Mouse	Zymed	IB
SUMO2/3	Rabbit	Abcam	IF
SUMO2/3	Rabbit	Upstate	IB
MDC1	Rabbit	[36]	IB
MDC1	Mouse	Sigma	IF
53BP1	Mouse	[37]	IF
53BP1	Rabbit	Novus	IF/IB
53BP1 pS25	Rabbit	Bethyl	IB
SMC3 pS1083	Rabbit	Bethyl	IB
mNBS1 pS343	Rabbit	Bethyl	IB
NBS1 (1D3)	Mouse	Abcam	IB
53BP1(N-terminal)	Rabbit	Sigma	IP
BRCA1 (D9)	Mouse	Santa-Cruz	IF/IB
BRCA1 (C20)	Rabbit	Santa-Cruz	IP/IB
BRCA1 (D20)	Rabbit	Santa-Cruz	IP/IB
RPA32	Mouse	Abcam	IF/IB
RPA32-pS4/pS8	Rabbit	Bethyl	IB
γH2AX	Rabbit	Cell-Signaling	IF
Tubulin	Mouse	Sigma	IB
CtIP	Mouse	[17]	IB
Ubc9	Goat	Abcam	IB
Ligase4	Rabbit	Abcam	IB
Conjugated Ub (FK2)	Mouse	BIOMOL	IF
UbH2A	Mouse	Upstate	IF
RNF8	Rb	[38]	IB
RNF168/RIDDLIN	Rabbit	[7]	IF
PIAS4	Rabbit	Abgent	IB
Alexa Fluor-488 (green)	Goat anti Mouse/Rabbit	Molecular Probes	IF
Alexa Fluor-594 (red)	Goat anti Mouse/Rabbit	Molecular Probes	IF
Alexa Fluor-647 (far red)	Goat anti Mouse/Rabbit	Molecular Probes	IF

Supplementary references

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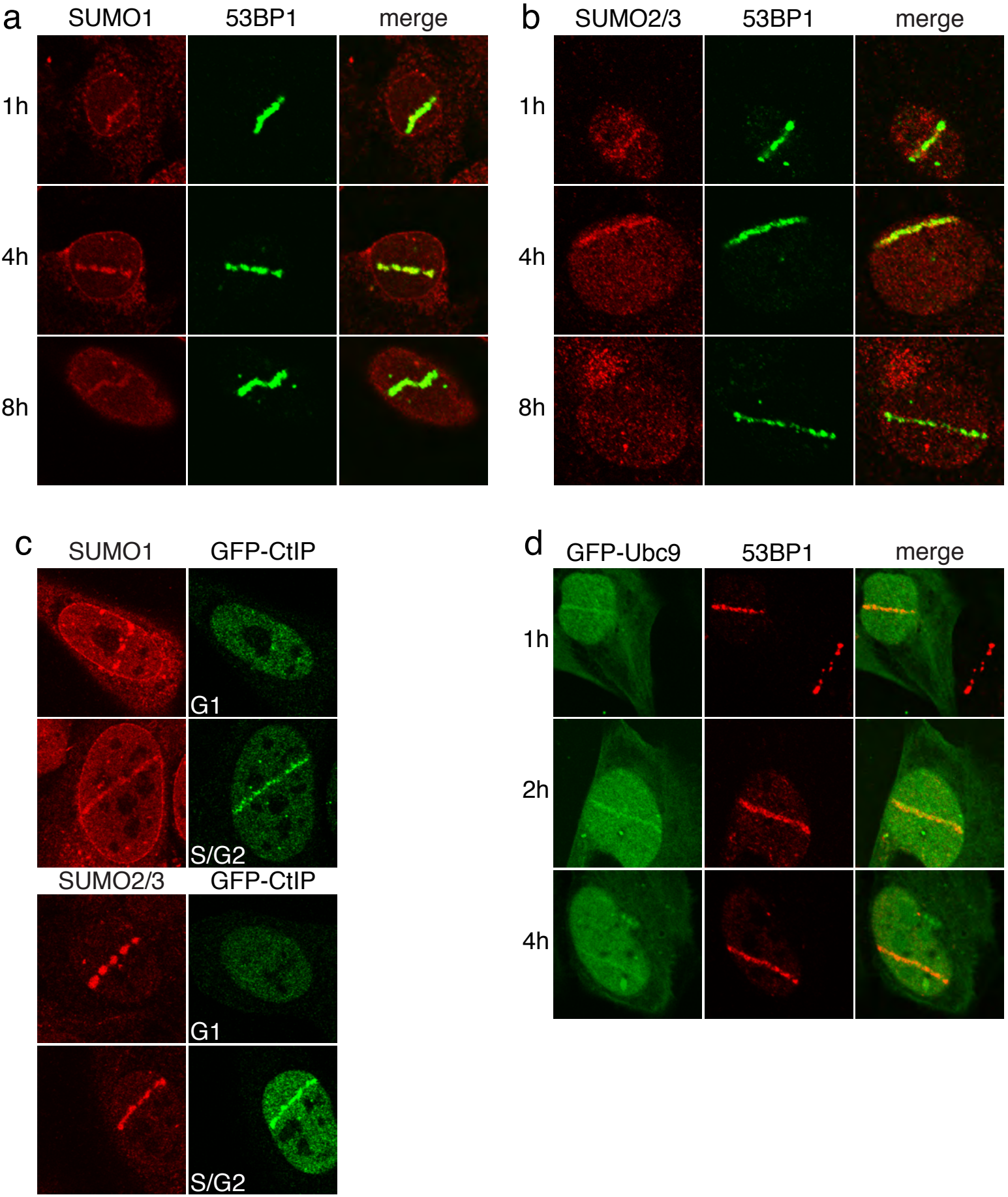

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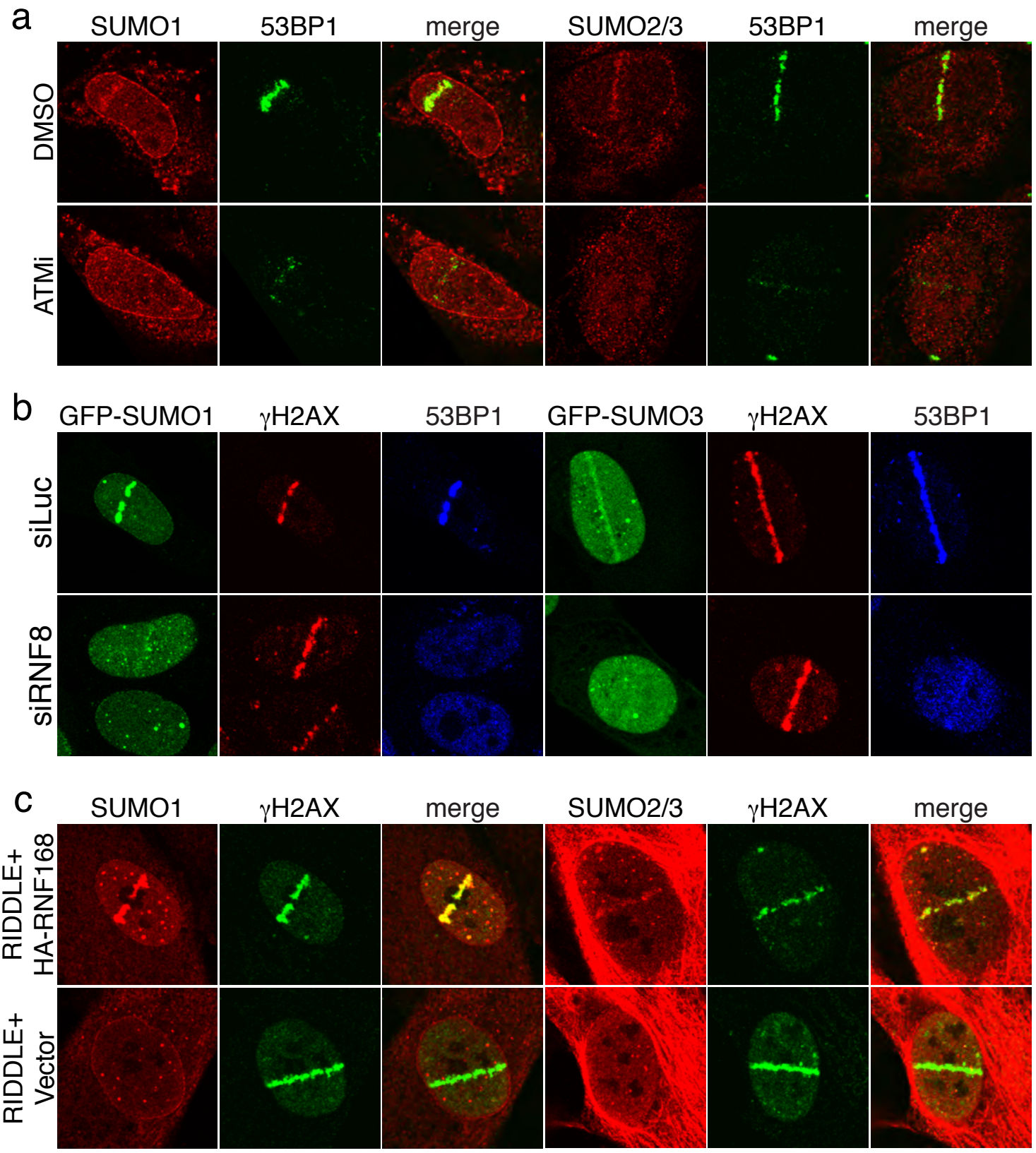

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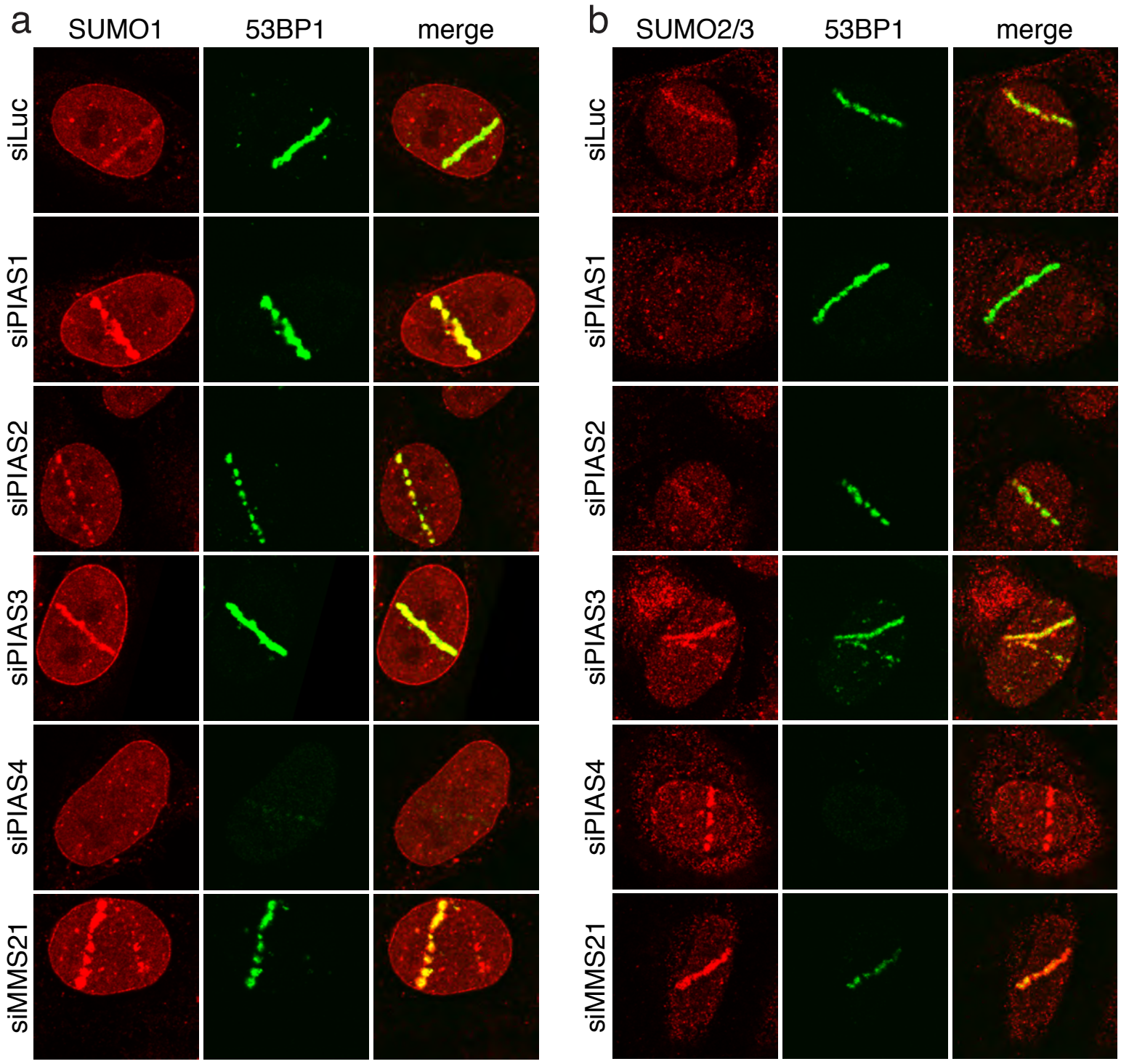

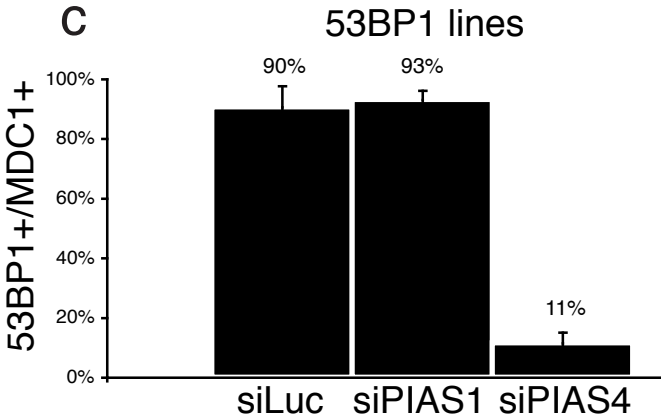

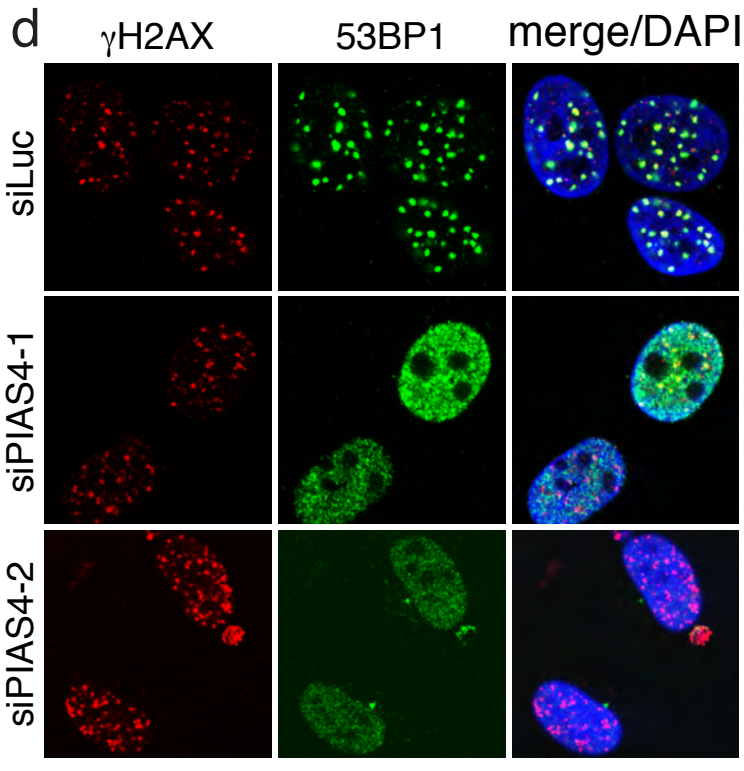

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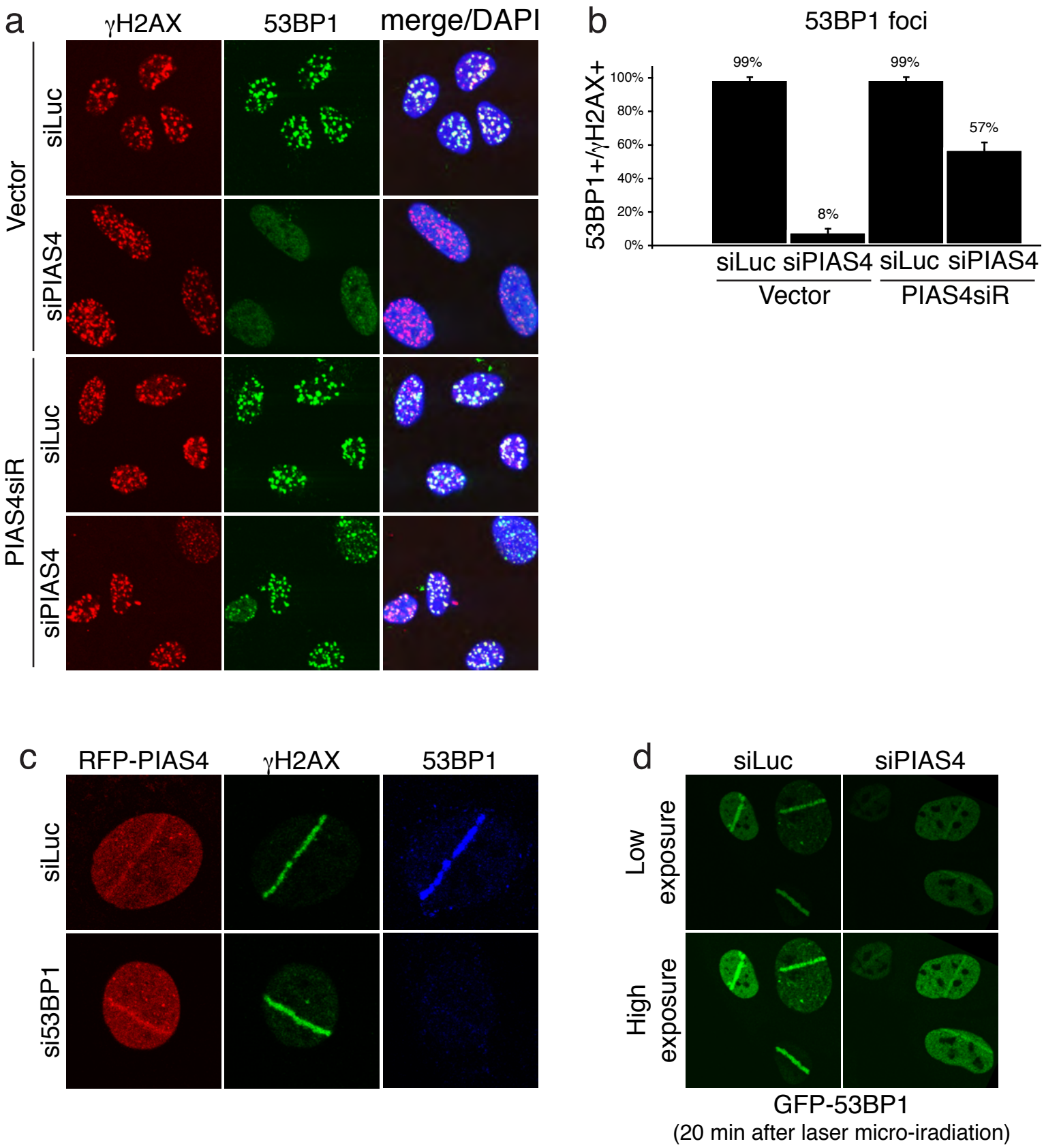

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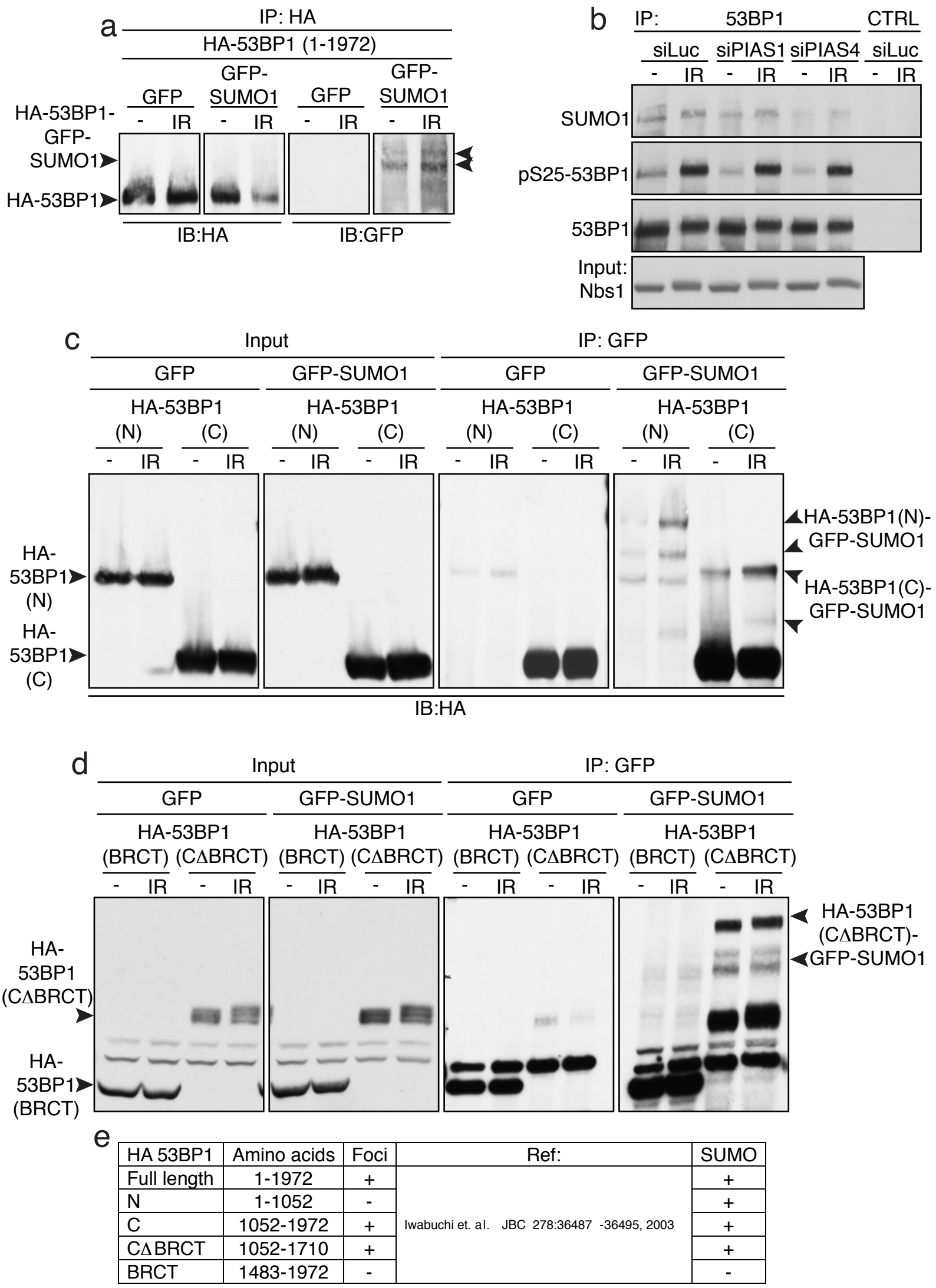

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HA 53BP1	Amino acids	Foci	Ref:	SUMO
Full length	1-1972	+	Iwabuchi et. al. JBC 278:36487 -36495, 2003	+
N	1-1052	-		+
C	1052-1972	+		+
CΔBRCT	1052-1710	+		+
BRCT	1483-1972	-		-

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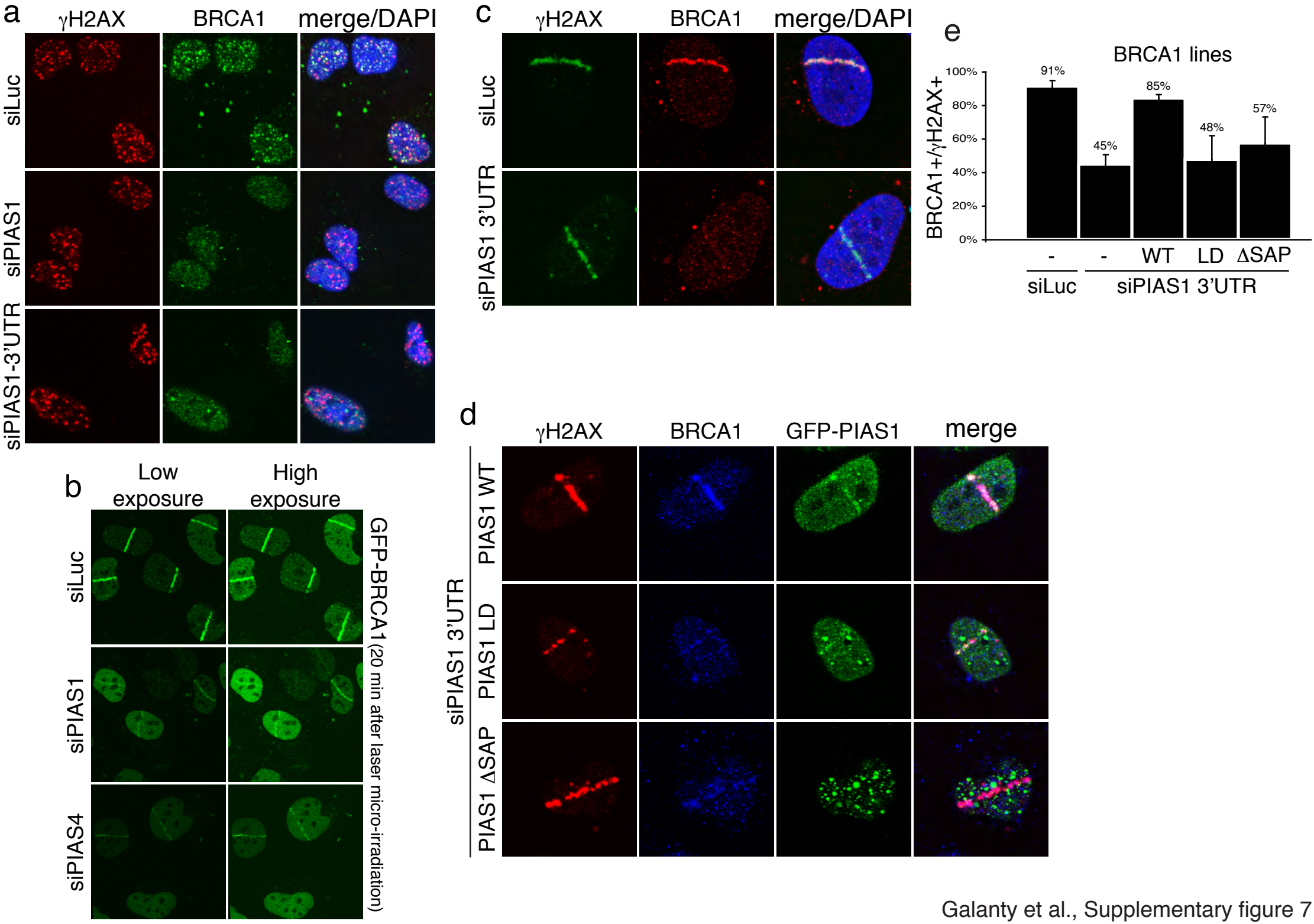

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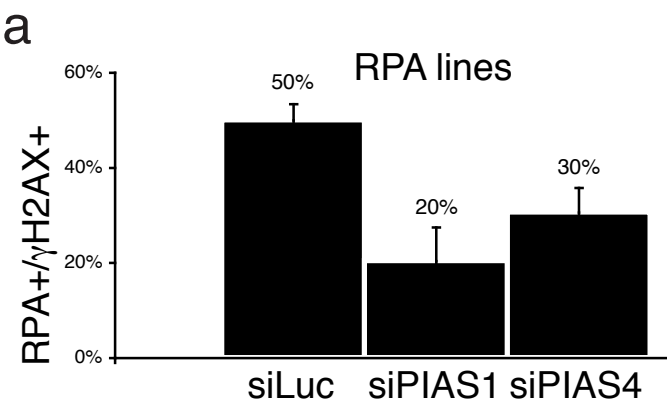

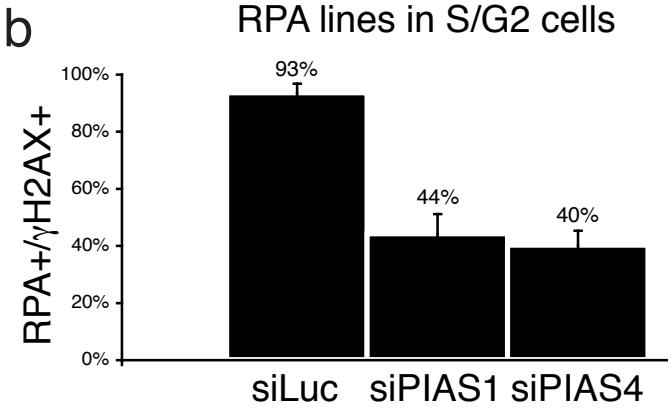

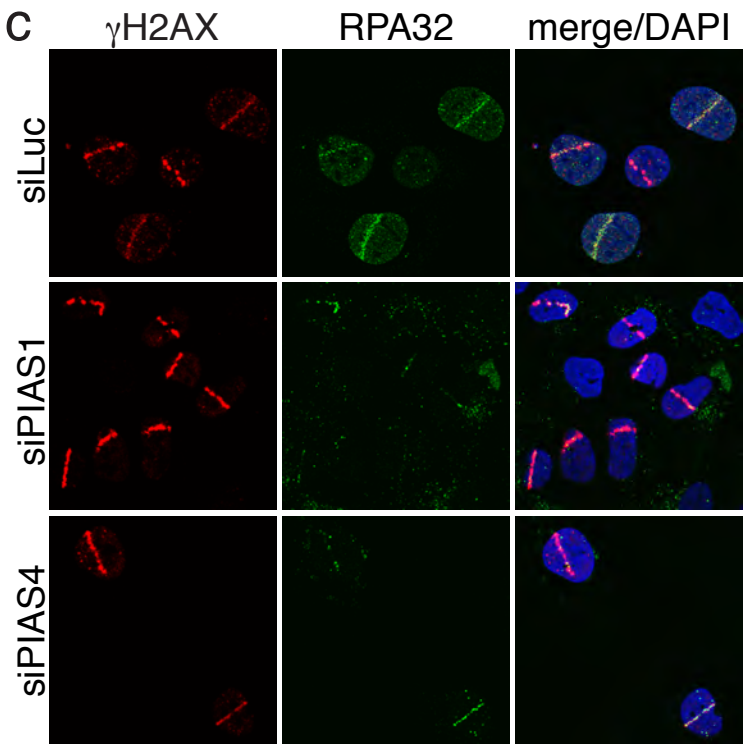

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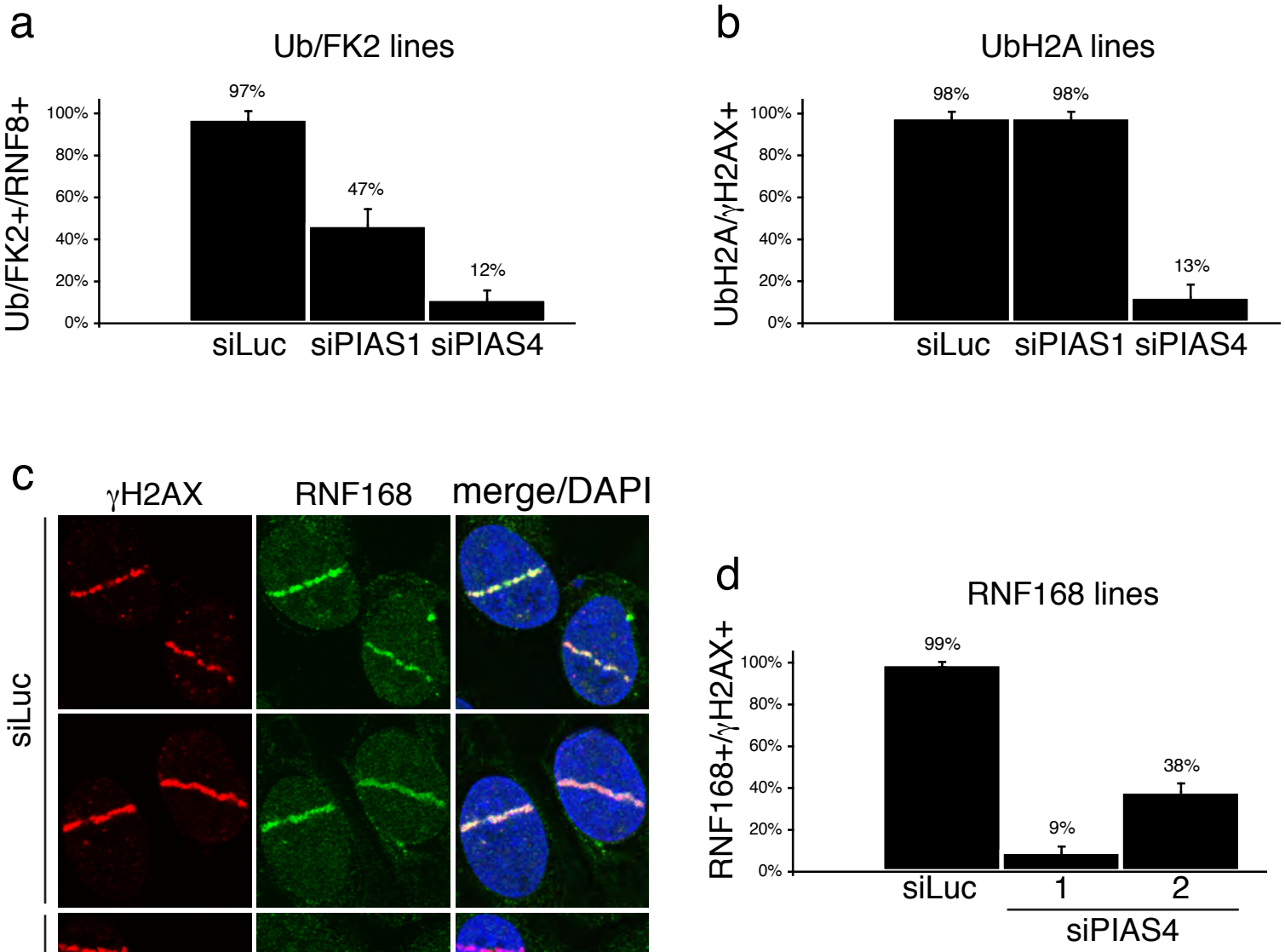

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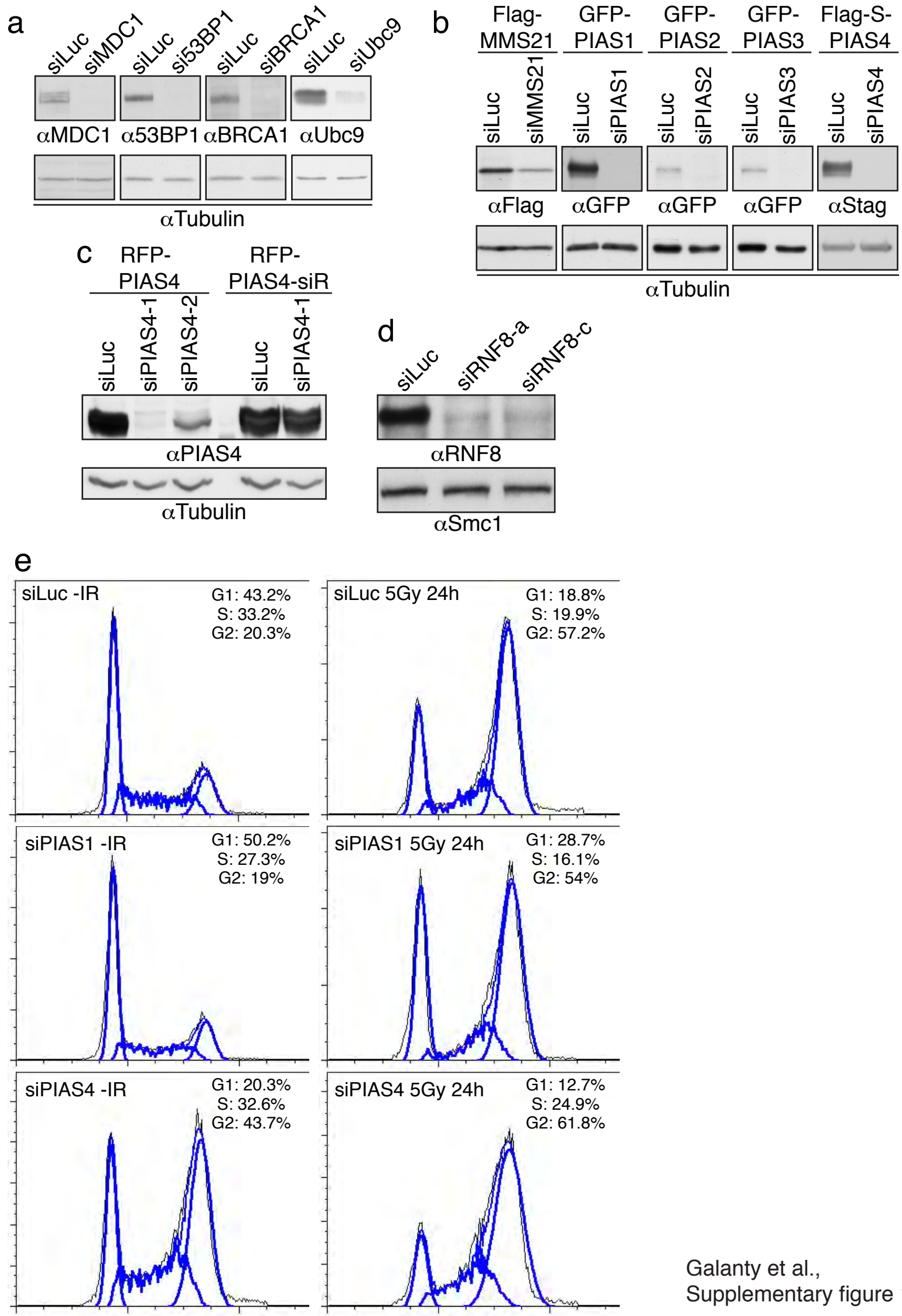

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