Supplementary Information

USP1-trapping lesions as a source of DNA replication stress and genomic instability

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This File includes:

Supplementary Figures 1-7
Supplementary Movies 1-6
Supplementary Tables 1-2
Supplementary Note 1
Supplementary Figure 1
Supplementary Figure 1. Characterization of HCT116 parental and USP1 knock-in mutant cell lines for defects in endogenous replication stress. a, Extracts of HCT116 parental and USP1 mutant cells were incubated with or without HA-Ub-VS DUB probe and analyzed by immunoblotting for USP1. MCM2 serves as a loading control. b, HCT116 cell lines were treated with 1 mM cycloheximide (CHX) for the indicated times to inhibit protein translation. USP1 levels were analyzed by immunoblotting. c, Alternative clones for each USP1 knockout or knock-in mutant cell line were pulse-labeled sequentially with IdU and CldU for 20 min each and subjected to DNA fiber analysis of elongating fork speed. CldU tract lengths of elongating forks are plotted with mean and +/- SD for 3 independent experiments (n=200 elongating forks), and p values were calculated using the Mann-Whitney rank-sum t-test (ns=no significance, *p<0.05, **p<0.01, ****p<0.0001, two-tailed). d, HCT116 cell lines were treated with control or Polk siRNA for 72 hours and analyzed by immunoblotting with the indicated antibodies. e, CldU tract lengths were measured for left and right forks emanating from the same origin of replication (n=100 bi-directional forks (origins)), as in Figure 1E. If the difference in fork length was more than 30% (outside of the red lines) they were classified as asymmetric forks. Percentages of asymmetric forks from each sample are indicated.
Supplementary Figure 2
**Supplementary Figure 2. Cell cycle effects of USP1 autocleavage mutants.**

**a**, HCT116 cell lines were treated with or without 2 mM HU for 4 hours and subjected to immunoblotting analysis with the indicated antibodies. **b**, Schematics of different classes of replication structures from DNA fiber analysis used to determine the frequency of origins for each of the indicated HCT116 cell lines. The percentage of new origin firing was calculated as the sum of both bi-directional red-green-red tracts and red only tracks over the total number of replication structures. Data for % origin firing are represented by mean and +/- SD for three independent experiments (n=200 events) and p-values were calculated using t-test with Welch’s correction (ns=no significance, *p<0.05, **p<0.01, two-tailed). **c**, USP1 WT and mutant cells were pulse-labeled with 10 µM EdU for 30 min and analyzed by flow cytometry for DNA synthesis (EdU) and DAPI staining for DNA content (10,000 events per sample). Percentages of cells in G1, S, and G2/M phases are graphed for three biological replicates. Error bars indicate SEM. **d**, Asynchronously growing HCT116 cell lines were fixed and co-stained for Cyclin A and 53BP1 (counterstained with DAPI). Representative images for each cell line are shown. Cells that were Cyclin A negative with 53BP1 nuclear bodies were quantified and graphed with mean and +/- SD for three independent experiments (n=300 Cyclin A negative cells per experiment). P-values were calculated using t-test with Welch’s correction (ns=no significance, **p<0.01, ***p<0.001, two-tailed). Scale bar = 20 µM **e**, Cells were treated with either control or Polκ siRNA for 72 hours and subjected to DNA fiber analysis. Replication origins firing during the second labeling period (red only tracts) were analyzed for replication fork speed. CldU tract lengths of elongating forks are plotted with mean and +/- SD for 3 independent experiments (n=100 red only origins), and p values were calculated using the Mann-Whitney rank-sum t-test (ns=no significance, *p<0.05, ***p<0.001, **p<0.0001), two-tailed).
Supplementary Figure 3. A representative sample of genomic DNA fractionation of HCT116 cells for Ok-seq analysis. 

a, Alkaline gel image showing sucrose gradient of HCT116 USP1C90S#1 genomic DNA. Fractions 1-9 (below >300bp) as indicated within the red box are concentrated for further processing to generate the adaptor-ligated Okazaki fragments library. 

b, Representative PCR gel image of amplified libraries of Okazaki fragments for HCT116 USP1C90S#1 and HCT116 USP1C90S#2 cell lines. 

c, Tapestation image of amplified libraries in (b) after removal of any adaptor-dimers and primer-dimers as part of quality control.
Supplementary Figure 4
Supplementary Figure 4. Alternative clones of USP1 mutant cell lines show similar delocalized patterns of replication initiation and termination within long genes. a, (upper panel) Schematic showing anticipated Okazaki fragment distributions around replication origins with strong (blue) versus weak (red) localized firing efficiencies. (lower panel) Schematic representation of Okazaki fragment distributions arising from replication termination at TTS under normal conditions (black) versus delocalized termination under replication stress (red). b, Percentage of replication forks moving left to right around TSS sorted by total RNA-seq read depth quartile (FPKM), (from ref64), for the indicated cell lines. P values were calculated using the Kruskal-Wallis test, using the regions 50-30kb upstream and 1-10kb downstream of the TSS. Effect sizes, shown as eff, were calculated using the same regions. The values were calculated comparing the USP1 mutant cell lines against the USP1 wild-type cell line. All statistics are presented in the Methods. c, Percentage of replication forks moving left to right around TSS of actively transcribed genes (FPKM>median), binned by gene length according to quartiles for transcribed genes. d, Same analysis as in (c), binned for long genes of 50-100 kb (upper panel) or >100 kb (lower panel). e, Percentage of replication forks moving left to right around TTS binned by RNA-seq read depth quartile for the indicated cell lines. f, Percentage of replication forks moving left to right around TTS of actively transcribed genes (FPKM>median), binned by gene length according to quartiles for transcribed genes. g, Same analysis as in (f), binned for long genes of 50-100 kb (upper panel) or >100 kb (lower panel).
Supplementary Figure 5
Supplementary Figure 5. Incubation of Halotag ligand alone in Mock samples did not cause unspecific background localizations. a, Average USP1 density per nucleus. (mean -/+/ std, n=166,183,76, 109 nuclei for Mock, USP1-WT, USP1-C90S, and USP1-GG/AA, respectively, p-values were calculated using the student’s t-test). Boxes represent the 1st (25%) and 3rd (75%) percentile. b, Measurements of the average EdU density around USP1 (left), and the average USP1 density around EdU (right) are shown, based on at least two independent experiments (mean -/+/ std, left: n= 183, 188, 71, 113 nuclei for Mock, WT, C90S, and GG/AA respectively; right n=207, 188, 71, 113 nuclei for Mock, WT, C90S and GG/AA respectively, p-values were calculated using the student’s t-test). Boxes represent the 1st (25%) and 3rd (75%) percentile. c, Plots of the EdU density as a function of the USP1 density of the same nucleus (black dots, n = 183, 76, 109 nuclei for WT, C90S, and GG/AA, respectively). The linear regression (red line) is performed for all the samples, and no significant correlation is observed for all the samples.
Supplementary Figure 6

(a) Nuclear soluble and Chromatin

(b) iPOND and input
Supplementary Figure 6. GG/AA mutant protein is enriched at replication forks by iPOND analysis. a, Immunoblotting analysis of nuclear soluble and chromatin-bound fractions prepared from USP1 WT and mutant cell lines. Histone H3 was used as a positive control marker for the chromatin fraction. Relative levels of USP1 (compared to WT=1 ) in the nuclear soluble and chromatin fractions were quantified by densitometry analysis. Mean with SD are plotted for 3 independent experiments and p-values were calculated using t-test with Welch’s correction (*p<0.05, **p<0.01, two-tailed). b, HCT116 and USP1-GG/AA cells were pulse-labeled for 10 min with 10 µM EdU and then either collected directly (+EdU) or treated with 10 uM thymidine for 1 hr (+EdU + Chase) or 2 mM HU for 3 hours (+EdU +HU). iPOND and input samples were analyzed by immunoblotting with the indicated antibodies.
Supplementary Figure 7
Supplementary Figure 7. SPRTN depletion causes chromatin-enrichment of the USP1-C90S protein. a, HCT116 cell lines were treated with or without an alternative siRNA for SPRTN (siSPRTN-7) and subjected to DNA fiber analysis of fork speed, as previously described. CldU tract lengths of elongating forks are plotted with mean and +/- SD for 3 independent experiments (n=200 elongating forks), and p values were calculated using the Mann-Whitney rank-sum t-test (ns=no significance, *p<0.05, **p<0.01, ****p<0.0001, two-tailed). b, HCT116 USP1-C90S cells were treated with either siCtrl or siSPRTN-1 siRNAs and complemented with siRNA-resistant Flag-SPRTN constructs prior to immunoblotting analysis for the indicated proteins. c, HCT116 USP1-GG/AA#1 cells overexpressing Flag-SPRTN-WT were compared to EV for measurements of elongating CldU Tract length. CldU tract lengths are plotted for 3 independent experiments (n=200 elongating forks) with mean +/- SD indicated and p values were calculated using the Mann-Whitney rank-sum t-test. d, U2OS Cerulean-PCNA cells expressing siRNA-resistant HaloTag-USP1 fusion proteins were treated with or without siUSP1-1 and ML323 as indicated and subjected to immunoblotting analysis. e, (left graph) Average USP1 localizations per nucleus as a function of time (mean +/- SEM, n= 16, 33, 45, and 33 nuclei for WT, C90S, GG/AA, andQ672A, respectively). Lines are the fitted single-exponential decay model. (right graph) The fitted bulk tau_on of different USP1 molecules as indicated. Mean +/- std is the fitting result of the time course of tau_on in left graph. f, Cells were treated with or without siSPRTN-1 siRNA for 72 h and subjected to immunoblotting analysis of prepared nuclear soluble and chromatin fractions. Relative levels of chromatin-bound USP1 were quantified using densitometry analysis (normalized to WT=1) and the mean with SD are plotted for 3 independent experiments. P-values were calculated using t-test with Welch’s correction (ns=no significance, *p<0.05, two-tailed).
**Supplementary Movie 1** (see Source Files). Representative SR blinking movies for the same cell shown for AF647 for EdU signal as SR movie. Scale bar = 2000 nm. Only 500 frames are shown in order to reduce the file size.

**Supplementary Movie 2** (see Source Files). Representative SR blinking movies for the same cell shown for JF549 for HaloTag USP1 signal as SR movie. Scale bar = 2000 nm. Only 500 frames are shown in order to reduce the file size.

**Supplementary Movie 3** (see Source Files). **No significant HaloTag single-molecule signal for Mock-transfected cells.** Representative live-cell movie of U2OS cells using JF646-Halo ligand incubation without transfection of USP1 HaloTag constructs (Mock sample). Incubation time is same as for others. Left video panel is depicting the JF646 channel (USP1); Right video panel is CFP-PCNA as a marker for nucleus in S-phase.

**Supplementary Movie 4-6** (see Source Files). Representative live-cell movies of U2OS cells stably expressing HaloTag-USP1 WT (Video 4), C90S (Video 5), and GG/AA (Video 6) fusion proteins.
### Supplementary Table 1 – Key Resources Table

#### Antibodies

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| USP1                | Bethyl | A301-698A  |
| MCM2                | Bethyl | A300-191A  |
| PCNA [PC10]         | Abcam  | ab29       |
| Chk1                | Abcam  | ab2845     |
| Histone H3          | Abcam  | ab1791     |
| Ubiquityl PCNA (Lys 164) | Cell Signaling Technologies | 13439S |
| Chk1 (Phospho Ser345) | Cell Signaling Technologies | 2348S |
| α-Tubulin           | CalBiochem | CP06     |
| Pol                 | Santa Cruz Biotechnology | sc-166667 |
| MCM7                | Santa Cruz Biotechnology | sc-9966 |
| Flag [M2]           | Sigma-Aldrich | F1804 |
| Mouse anti-IdU [B44] | BD Biosciences | 347580 |
| rat anti-CldU [BU1/75 (ICR1)] | Abcam | ab6326 |
| 53BP1               | Abcam  | ab175933   |
| Cyclin A2           | Calbiochem | CC17     |
| Goat anti-mouse IgG (H+L) Alexa Fluor 488 | Thermo Fisher | A11001 |
| Goat anti-rat IgG (H+L) Alexa Fluor 594 | Thermo Fisher | A11007 |
| Goat anti-mouse IgG (H+L) Alexa Fluor 546 | Thermo Fisher | A11003 |
| Goat anti-rabbit IgG Alexa Fluor 488 | Thermo Fisher | A11008 |
| Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) | Jackson Labs | 115-035-003 |
| Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) | Jackson Labs | 111-035-003 |

#### Chemicals, Peptides, and Recombinant Proteins

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| HA-Ubiquitin-Vinyl Sulphone | Boston Biochem | U-212 |
| USP1-UAF1 inhibitor, ML323 | Calbiochem | 531131 |
| Cycloheximide       | Calbiochem | 239765 |
| Biotin PEG-Azide    | ThermoFisher Scientific | B10184 |
| Biotin TEG-Azide    | Berry & Associates | BT-1085 |
| CuSO4 – click chemistry grade | Jena Biosciences | M1004-50 |
| Ascorbic acid       | Sigma-Aldrich | A92902-100G |
| Streptavidin, agarose conjugate beads | EMD Millipore | 16-126 |
| Dynabeads MyOne Streptavidin T1 | ThermoFisher Scientific | 65601 |
| Hydroxyurea         | Sigma-Aldrich | H8627 |
| cOmplete Mini-Protease Inhibitor Cocktail | Roche | 11836170001 |
| 5’-ethynyl-2-deoxyuridine (EdU) | Sigma-Aldrich | 900584 |
| Thymidine (dT)      | Sigma-Aldrich | T1895 |
| 5’-lodo-2’deoxyuridine (IdU) | Sigma-Aldrich | I7125 |
| Reagent or Resource                          | Source         | Identifier    |
|---------------------------------------------|----------------|---------------|
| 5’-chloro-2’deoxyuridine (CldU)              | Sigma-Aldrich  | C6891         |
| Janelia Fluor 549 HaloTag ligand            | Promega        | GA1110        |
| Janelia Fluor 646 HaloTag ligand            | Promega        | GA1120        |
| HaloTag TMRDirect Ligand                    | Promega        | G2991         |
| Cisplatin                                   | EMD Millipore  | 232120-50MG   |
| Lipofectamine 3000 transfection reagent     | Thermo Fisher  | L3000015      |
| Lipofectamine RNAiMAX                       | Thermo Fisher  | 13778150      |
| Opti-MEM                                    | Gibco          | 31985-062     |
| McCoy’s 5A Medium                           | Gibco          | 16600-082     |
| DMEM                                        | Gibco          | 11995-065     |
| FBS                                         | R&D Systems    | S11150H       |
| Prolong Gold Antifade Reagent               | Cell Signaling Technologies | 9071S       |
| Parafomaldehyde 16% solution                | Electron Microscopy Sciences | 15710     |
| Phenol:chloroform:isoamyl alcohol           | Thermo Fisher  | 15593049      |
| T4 DNA ligase                               | Enzymatics     | L6030-LC-L    |
| T4 polynucleotide kinase                    | New England Biolabs | M0201L   |
| Proteinase K                                | Roche          | 3115828001    |

**Critical Commercial Assays/Kits**

| Reagent or Resource                          | Source         | Identifier    |
|---------------------------------------------|----------------|---------------|
| CellTiter-Glo Luminescent Cell Viability Assay | Promega        | G7570         |
| Click-iT PLUS EdU Imaging Kit, Alexa Fluor 647 dye | Thermo Fisher  | C10640        |
| Click-iT Plus EdU Alexa Fluor 594 Flow Cytometry assay kit | Thermo Fisher  | C10646        |
| Subcellular Protein Fractionation Kit for Cultured Cells | Thermo Fisher  | 78840         |

**Experimental Models – Cell Lines**

| Reagent or Resource                          | Source         | Identifier            |
|---------------------------------------------|----------------|-----------------------|
| HCT116                                      | ATCC           |                       |
| HEK293T                                     | ATCC           |                       |
| U2OS                                        | ATCC           |                       |
| HCT116 USP KO #1                           | This Study     | Applied Stem Cell     |
| HCT116 USP1 KO #2                          | This Study     | Applied Stem Cell     |
| HCT116 USP1-C90S #1                         | This Study     | Applied Stem Cell     |
| HCT116 USP1-C90S #2                         | This Study     | Applied Stem Cell     |
| HCT116 USP1-GG/AA #1                        | This Study     | Applied Stem Cell     |
| HCT116 USP1-GG/AA #2                        | This Study     | Applied Stem Cell     |
| HCT116 USP1-Q672V #1                        | This Study     | Applied Stem Cell     |
| HCT116 USP1-Q672V #2                        | This Study     | Applied Stem Cell     |
**U2OS NLS-mCerulean-PCNA**
Gift from Huijun Xue (E. Rothenberg Lab); See Materials & Methods

**U2OS HaloTag-USP1-WT (siRes) + NLS-mCerulean-PCNA**
This Study
See Materials & Methods

**U2OS HaloTag-USP1-C90S (siRes) + NLS-mCerulean-PCNA (siRes)**
This Study
See Materials & Methods

**U2OS HaloTag-USP1-GGAA (siRes) + NLS-mCerulean-PCNA**
This Study
See Materials & Methods

**U2OS HaloTag-USP1-Q672A (siRes) + NLS-mCerulean-PCNA**
This Study
See Materials & Methods

**Oligonucleotides**

| Reagent or Resource | Source | Sequence |
|--------------------|--------|----------|
| All-Star Negative Control siRNA | Qiagen | Proprietary |
| PolK siRNA (siPolK-5) | Qiagen | 5’-TGGAATTAGAACAAAGCCGAA-3’ |
| USP1 siRNA (siUSP1-1) | Qiagen | 5’-TCGGCAATCTTGCTATCTTA-3’ |
| SPRTN siRNA (siSPRTN-1; siSPRTN-7) | Qiagen | #1, 5’-AGCCAATATAACGGTATACCA-3’, #7, 5’-ACAGTTGGGAACATCCCTAAA-3’ |
| USP1 siRNA-resistant-F primer | IDT | 5’-GTGGGACTGAATAATCTTGGAAACGTGTTACTTGAATAGTACTTCAGG-3’ |
| USP1 siRNA-resistant-R primer | IDT | 5’-CCTGAAGTATACATTTCAAGTAACACGTGTTTCCAAGATTATTCTCCGA-3’ |
| SPRTN siRNA-resistant-F primer | IDT | 5’-CATCAACAGCGCTGACTGGAGCAAACATCAAGCGGTACCATACTTTCAAGATG-3’ |
| SPRTN siRNA-resistant-R primer | IDT | 5’-CATCGTGAAAAGTATGGTACACGGTGATGTGTGCTCCAGTACGGTGTTGATG-3’ |
| SPRTN UBZ_F primer | IDT | 5’-AGCAAAATGGTTAATGGCCCAGTGTTGATGTCAGAATGAAGTTCTG-3’ |
| SPRTN PIPm_F primer | IDT | 5’-AATGTTCTAAGCAACCGCGCTCCTAGAGATCTATTCTCG-3’ |
| SPRTN PIPm_R primer | IDT | 5’-CAATGATACTCTTAGAGACCGCGCTTGCTTAGAACATT-3’ |

**Recombinant DNA**

| Reagent or Resource | Source | Identifier |
|--------------------|--------|------------|
| pHTrn-HaloTag (EV) | Promega | G7721 |
| pHTrn-HaloTag-USP1-WT | This Study | See Materials & Methods |
### gRNA sequences for generation of CRISPR knock-in cell lines

| gRNA name  | Sequence (5’→3’)                                                                 | Notes                                                                 |
|------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------|
| hUSP1_g1   | TTTGTCGGGACTGAATAATCT                                                           | Used to generate C90S mutation                                        |
| hUSP1_g2   | CTATCTTAATAGTATACTTC                                                            | Used to generate C90S mutation                                        |
| hUSP1_g3   | GAAGCTATGGAACATTGTCAATTAGAGTTGGTGGAAATA                                         | Used to generate GG670-671AA and Q672V mutations                     |
| hUSP1_g4   | GTAGAAGCTATGGAACATTGTCAATTAGAGTTGGTGGAAATA                                      | Used to generate GG670-671AA and Q672V mutations                     |

### Single-stranded deoxynucleotide donors for generation of CRISPR knock-in cells

| Name                                                   | Sequence (5’→3’)                                                                 |
|--------------------------------------------------------|---------------------------------------------------------------------------------|
| hUSP1-C1539_GG670-671AA.g3.ssODN                       | GGTTGAGAATTATAATGATGAGAAGAGTGCAATTAGAGTTGGTGGAAATA CACAGCCAAGTAGAAAATGTA CAAAAAGCAAAAGCAGATTATAGAGCATTACACAAAGGCTTC TAATCCTGATAGGTTGCTAGTACAGCGTTGCTGAAGAAATTCT |
| Software                | Source                           |
|-------------------------|----------------------------------|
| ImageJ v1.52a           | https://imagej.nih.gov/ij        |
| FlowJo v10              | https://www.flowjo.com/          |
| GraphPad Prism v8       | https://www.graphpad.com         |
| MatLab (v2017b)         | https://www.mathworks.com/       |
| OriginLab (2018)        | https://www.originlab.com        |
Supplementary Note 1. Representative Pair- and Cross-Correlation Data and Fitted Curves.

Auto- (A) and Cross- (B) correlation fitting examples. The auto correlation was fitted to Equation 5 and the cross correlation was fitted to a symmetric-gauss model as explained below.

Considering the cross correlation between a P-Q pair, for each P, the Qs distribute at \( r_0 \) around P with some variance. Note that such probability distribution might expand to the ‘\(-r\)’ axis due to the relatively large variance (A). Because the dumbbell-like P-Q pairs are randomly orientated in the nucleus, such distribution should identical from \( \theta = 0 \sim 2\pi \). Note that the distribution at \( \theta = \phi + \pi \) may expand from the ‘\(-r\)’ axis to the \( r \) axis (B). Therefore, after averaging from 0 to \( 2\pi \) along \( \theta \), the final radial function (cross-correlation as a function of distance) is contributed by the symmetric distribution along \( r \), shown in C as solid parts in both blue and orange colors.

In our data, a symmetric Gauss model perfectly approximates the final radial cross-correlation profile.

\[
C(r) = A \exp \left[ - \frac{(r - r_0)^2}{2\sigma^2} \right] + A \exp \left[ - \frac{(r + r_0)^2}{2\sigma^2} \right]
\]

Description of Equation 5 (how cluster densities are calculated). Equation 5 is the definition of the cross-correlation function \( C_{PQ}(d) \) (where equation 4 is its analytical model). More rigorously, it should be written in the vector format

\[
C_{PQ}(d) = \frac{\langle \rho_P(R) \rho_Q(R + d) \rangle_R}{\langle \rho_P(R) \rangle_R \langle \rho_Q(R) \rangle_R}
\]
Where $\rho_{P/Q}(R)$ is the local density of species P/Q at a given location R in the image; $\langle \cdots \rangle_R$ denotes the average of all the locations R across the image. As the P-Q pairs in the nucleus are randomly oriented, the 2D $C_{PQ}(d) = C_{PQ}(d, \theta)$ is integrated across $\theta$ from 0 to $2\pi$, resulting in the 1D radial distribution function $C_{PQ}(d)$.

$\langle \rho_{P}(R)\rho_{Q}(R + d)\rangle_R$ is the pairing density at distance d, and thus $\langle \rho_{P}(R)\rho_{Q}(R + d)\rangle_R / \langle \rho_{P}(R)\rangle_R$, or equivalently $C_{PQ}(d)\langle \rho_{Q}(R)\rangle_R$, denotes the average local density of Q that pairs with each P at distance d, and vice versa. The amplitude, $A$, of the cross-correlation (equation 4) is the $C(d = r_0)$ where the cross-correlation reaches the maximum, and $A\langle \rho_{Q}(R)\rangle_R$ is used to estimate the average local density of Q pairing with each P. Note that if P and Q are randomly distributed (no cross-correlation signal) around each other, the local pairing density is 0. This distinguishes from direct counting the local density around each other, which would result in high local surrounding density when P and Q are densely but randomly distributed around each other.