Expanded View Figures

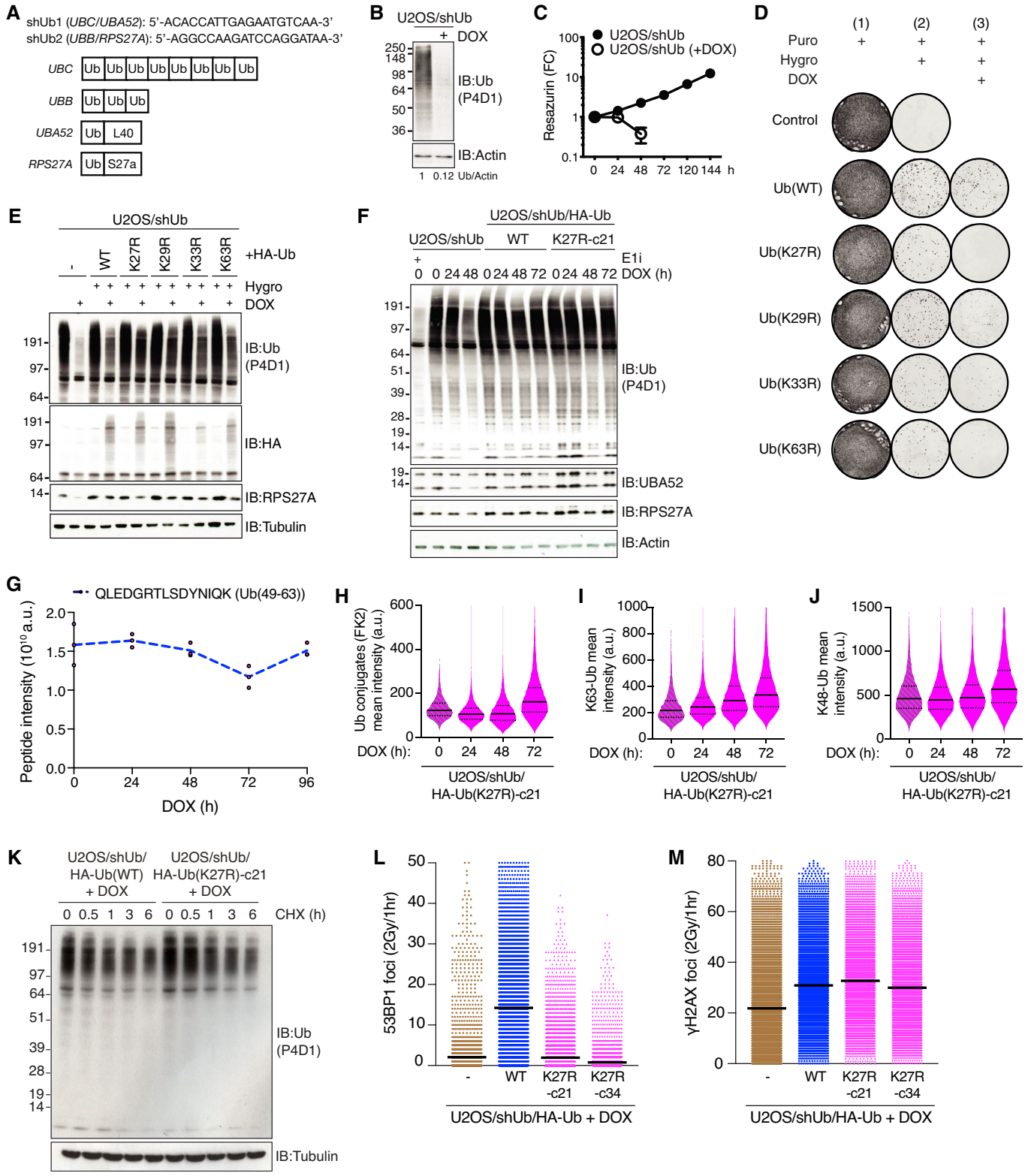

Figure EV1.

Figure EV1. (related to Fig 1) Generation and characterization of Ub replacement cell lines.

A Short hairpin RNA (shRNA) target sequences cloned into pPuro-shUb (top) and schematic depicting mammalian genes encoding Ub (bottom).
B Immunoblot analysis of Ub depletion in U2OS/shUb cells after 48 h of DOX treatment.
C Viability of U2OS/shUb cells exposed or not to DOX, as determined by resazurin metabolic assay (mean ± SD; $n = 3$ independent experiments).
D Representative images of clonogenic rescue assay to restore viability to U2OS/shUb cells after DOX treatment (Fig 1A–C). Where indicated, integration of the Ub replacement cassettes was selected with Hygromycin B, and expression was induced with DOX.
E Immunoblot analysis of cells in (D).
F Immunoblot analysis of stable U2OS/shUb/HA-Ub Ub replacement cell lines treated with DOX for the indicated times or Ub E1 inhibitor (E1i) for 1 h.
G Mass spectrometry-based quantification of native Ub(49-63) peptide spanning after Trypsin digestion. Data represent triplicate technical replicate samples taken over a 96-h DOX induction time course of U2OS/shUb/HA-Ub(K27R)-c21 cells.
H U2OS/shUb/HA-Ub(K27R) cells treated with DOX for the indicated times were fixed and immunostained with Ub conjugate-specific antibody (FK2). Levels of Ub conjugates were quantified using quantitative image-based cytometry (QIBC) (solid lines, median; dashed lines, quartiles (a.u., arbitrary units); > 5,000 cells analyzed per condition).
I As in (H), but using Ub-K63 antibody.
J As in (H), but using Ub-K48 antibody.
K Immunoblot analysis of U2OS/shUb/HA-Ub cell lines treated or not with cycloheximide (CHX) for the indicated times.
L Nuclear 53BP1 foci in DOX-treated U2OS/shUb, U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K27R) cell lines exposed to IR (2 Gy), fixed 1 h later and immunostained with 53BP1 antibody was quantified by QIBC (black bars, mean; > 5,000 cells analyzed per condition).
M As in (L), except cells were stained with γ-H2AX antibody (black bars, mean; > 5,000 cells analyzed per condition).

Data information: Data are representative of three (B,D-F,H-J) and two (K-M) independent experiments with similar outcome.

Figure EV2. (related to Fig 2) K27-linked ubiquitylation is required for cell proliferation and cell cycle progression.

A, B Immunoblot analysis of U2OS/shUb and U2OS/shUb/HA-Ub cell lines treated with DOX for the indicated times or Ub E1 inhibitor (E1i) for 1 h.
C U2OS/shUb/HA-Ub replacement cell lines were treated with DOX for 72 h where indicated, fixed and stained with K29-Ub specific synthetic antigen binder sAB-K29 (Yu *et al*, 2021). Levels of K29-Ub conjugates were quantified using QIBC (solid lines, median; dashed lines, quartiles (a.u., arbitrary units); > 5,000 cells analyzed per condition).
D Viability of U2OS/shUb, U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K27R) cell lines without DOX treatment, as determined by Resazurin metabolic assay (mean ± SEM; $n = 3$ independent experiments).
E As in (D), but U2OS/shUb, U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K29R) cell lines are shown. (mean ± SEM; $n = 3$ independent experiments).
F As in (D), but U2OS/shUb/HA-Ub(WT), U2OS/shUb/HA-Ub(K27R) and U2OS/shUb/HA-Ub(WT hypomorph) cell lines with or without DOX treatment are shown (mean ± SEM; $n = 3$ independent experiments).
G Immunoblot analysis of U2OS/shUb/HA-Ub(WT), U2OS/shUb/HA-Ub(WT hypomorph) and U2OS/shUb/HA-Ub(K27R) cell lines treated or not with DOX for 72 h.
H, I Cell cycle profiles of Ub replacement cell lines treated with DOX for the indicated times were determined by QIBC analysis of DAPI and either EdU (H) or PCNA (I) signal intensities (> 2,000 cells analyzed per condition).
J Quantification of EdU incorporation in DOX-treated Ub replacement cell lines at the indicated times after release from a single thymidine block (solid lines, median; dashed lines, quartiles; > 2,000 S phase cells analyzed per condition).

Data information: Data are representative of five (H,I) and three (A-C,G,J) independent experiments.

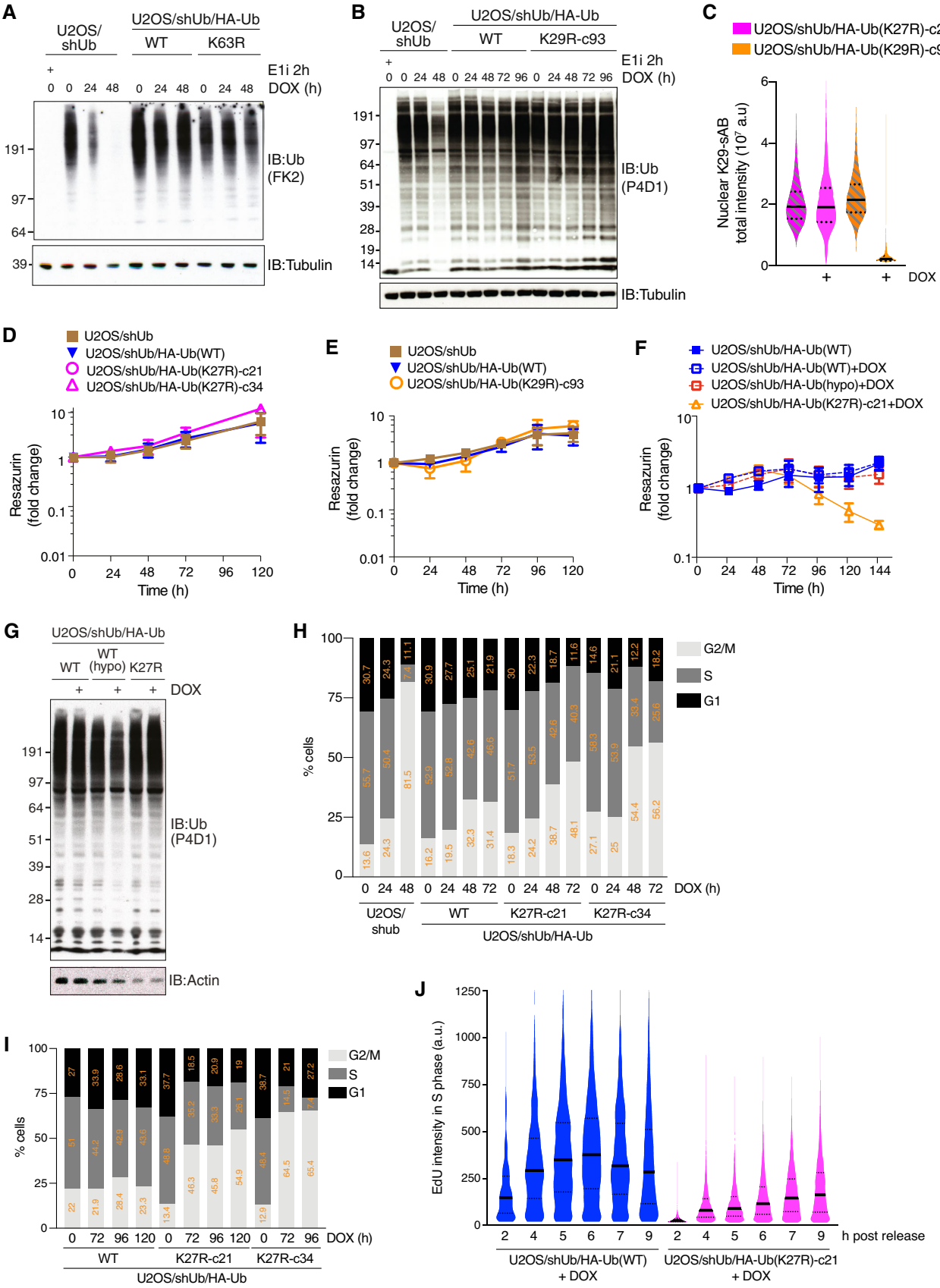
A
U2OS/shUb
U2OS/shUb/HA-Ub
WT
K63R
E1i 2h
DOX (h)
IB:Ub (FK2)
IB:Tubulin
B
U2OS/shUb
U2OS/shUb/HA-Ub
WT
K29R-c93
E1i 2h
DOX (h)
IB:Ub (P4D1)
IB:Tubulin
C
U2OS/shUb/HA-Ub(K27R)-c21
U2OS/shUb/HA-Ub(K29R)-c93
Nuclear K29-sAB total intensity (10^7 a.u)
DOX
D
U2OS/shUb
U2OS/shUb/HA-Ub(WT)
U2OS/shUb/HA-Ub(K27R)-c21
U2OS/shUb/HA-Ub(K27R)-c34
Resazurin (fold change)
Time (h)
E
U2OS/shUb
U2OS/shUb/HA-Ub(WT)
U2OS/shUb/HA-Ub(K29R)-c93
Resazurin (fold change)
Time (h)
F
U2OS/shUb/HA-Ub(WT)
U2OS/shUb/HA-Ub(WT)+DOX
U2OS/shUb/HA-Ub(hypo)+DOX
U2OS/shUb/HA-Ub(K27R)-c21+DOX
Resazurin (fold change)
Time (h)
G
U2OS/shUb/HA-Ub
WT
WT (hypo)
K27R
DOX
IB:Ub (P4D1)
IB:Actin
H
% cells
G2/M
S
G1
DOX (h)
U2OS/shub
WT
K27R-c21
K27R-c34
U2OS/shUb/HA-Ub
I
% cells
G2/M
S
G1
DOX (h)
WT
K27R-c21
K27R-c34
U2OS/shUb/HA-Ub
J
EdU intensity in S phase (a.u.)
h post release
U2OS/shUb/HA-Ub(WT) + DOX
U2OS/shUb/HA-Ub(K27R)-c21 + DOX

Figure EV2.

Figure EV3. (related to Fig 3) K27-linked ubiquitylation predominantly occurs in the nucleus and affects the integrity of the p97 machinery.

A U2OS/shUb and DOX-treated U2OS/shUb/HA-Ub cell lines were lysed, subjected to HA IP under denaturing conditions and immunoblotted with indicated antibodies.
B Representative images of U2OS cells co-immunostained with antibodies to K27-linked Ub (Ub-K27) and total Ub conjugates (FK2). Scale bar, 10 μm.
C Immunoblot analysis of HA-Ub conjugates from U2OS/shUb/HA-Ub cell lines for MS analysis (Fig 3E; Dataset EV1) isolated as shown in Fig 3D.
D Correlation analysis comparing label-free quantitation values of protein groups from individual replicate HA-Ub conjugate samples isolated as in Fig 3D.
E Relative intensity of peptide detection for indicated proteins in U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K27R) whole cell extract analyzed by MS (black bars, median; $n = 3$ technical replicates). Full proteome data are shown in Dataset EV2.
F DOX-treated U2OS/shUb (48 h) and U2OS/shUb/HA-Ub (72 h) cell lines were lysed, subjected to HA IP under denaturing conditions and immunoblotted with indicated antibodies as in Fig 3F and G.
G DOX-induced U2OS/shUb/HA-Ub cell lines treated or not with p97i for the indicated times were fixed and immunostained with Ub conjugate-specific antibody (FK2). Levels of Ub conjugates in the cytoplasm were quantified using QIBC (solid lines, median; dashed lines, quartiles; > 1,000 cells analyzed per condition).
H DOX-induced U2OS/shUb and U2OS/shUb/HA-Ub cell lines were pre-extracted, fixed and immunostained with p47 antibody, and analyzed by QIBC (solid lines, median; dashed lines, quartiles; > 3,000 cells analyzed per condition).
I As in (H), except cells were immunostained with UFD1 antibody.
J As in (H), except cells were immunostained with p97 antibody.

Data information: Data (A,B,F-J) are representative of three independent experiments with similar outcome.

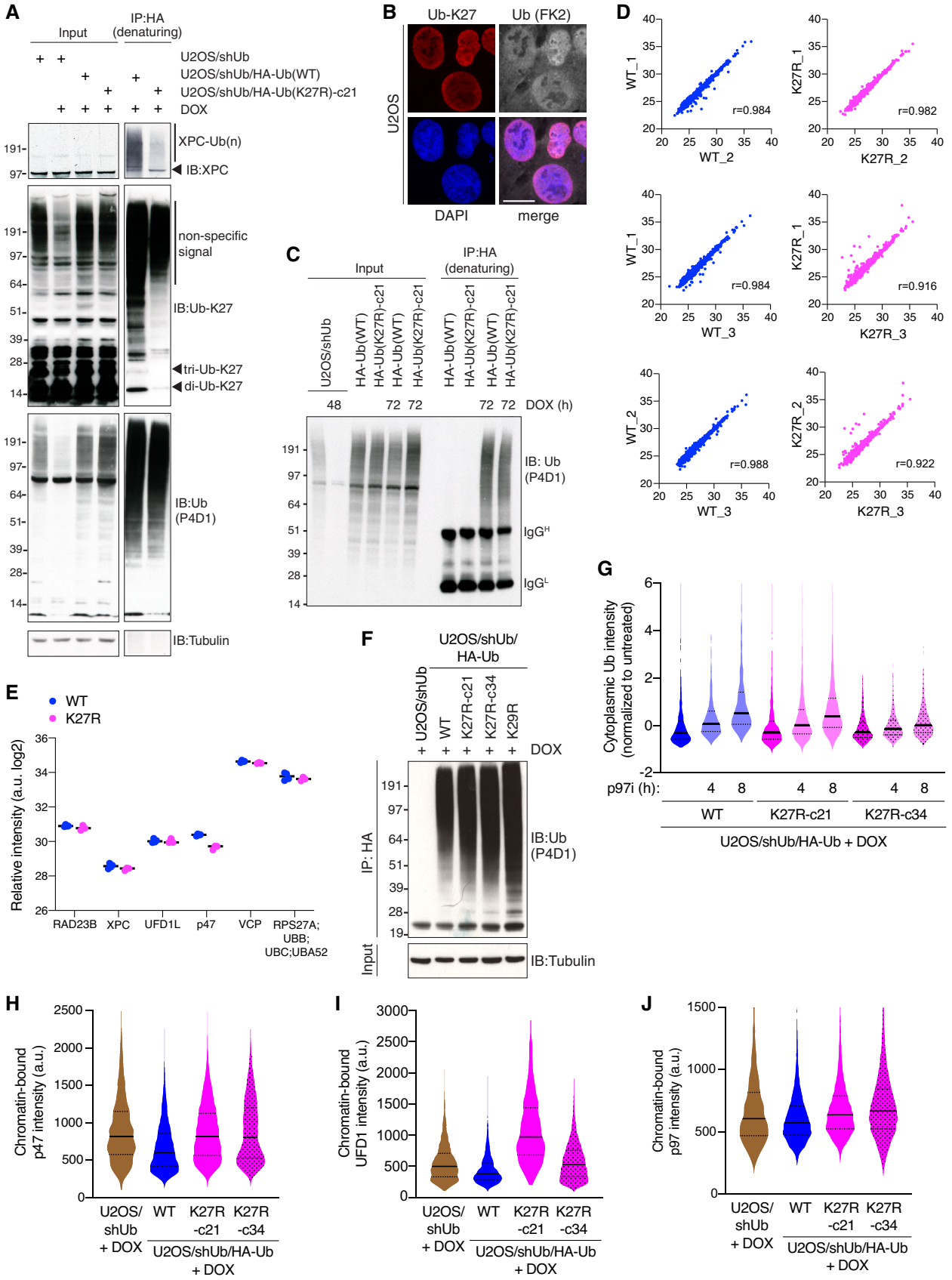
A
Input
IP:HA (denaturing)
U2OS/shUb
U2OS/shUb/HA-Ub(WT)
U2OS/shUb/HA-Ub(K27R)-c21
DOX
XPC-Ub(n)
IB:XPC
non-specific signal
IB:Ub-K27
tri-Ub-K27
di-Ub-K27
IB:Ub (P4D1)
IB:Tubulin
B
Ub-K27
Ub (FK2)
U2OS
DAPI
merge
C
Input
IP:HA (denaturing)
U2OS/shUb
HA-Ub(WT)
HA-Ub(K27R)-c21
HA-Ub(WT)
HA-Ub(K27R)-c21
HA-Ub(WT)
HA-Ub(K27R)-c21
HA-Ub(WT)
HA-Ub(K27R)-c21
48
72 72
72 72
DOX (h)
IB: Ub (P4D1)
IgG^H
IgG^L
D
WT_1
WT_2
K27R_1
K27R_2
WT_3
K27R_3
r=0.984
r=0.982
r=0.984
r=0.916
r=0.988
r=0.922
E
WT
K27R
Relative intensity (a.u. log2)
RAD23B
XPC
UFD1L
p47
VCP
RPS27A; UBB; UBC;UBA52
F
U2OS/shUb/ HA-Ub
+ U2OS/shUb
WT
K27R-c21
K27R-c34
K29R
DOX
IP: HA
IB:Ub (P4D1)
Input
IB:Tubulin
G
Cytoplasmic Ub intensity (normalized to untreated)
p97i (h):
4 8 4 8 4 8
WT
K27R-c21
K27R-c34
U2OS/shUb/HA-Ub + DOX
H
Chromatin-bound p47 intensity (a.u.)
U2OS/ shUb + DOX
WT
K27R -c21
K27R -c34
U2OS/shUb/HA-Ub + DOX
I
Chromatin-bound UFD1 intensity (a.u.)
U2OS/ shUb + DOX
WT
K27R -c21
K27R -c34
U2OS/shUb/HA-Ub + DOX
J
Chromatin-bound p97 intensity (a.u.)
U2OS/ shUb + DOX
WT
K27R -c21
K27R -c34
U2OS/shUb/HA-Ub + DOX

Figure EV3.

Figure EV4. (related to Fig 4) Disabling K27-linked ubiquitylation impairs p97-dependent substrate processing and is functionally epistatic with p97 inhibition.

A Uninduced U2OS/shUb/HA-Ub replacement cell lines stably expressing shUb-resistant Ub(G76V)-GFP reporter were treated or not with p97i as indicated, and nuclear Ub(G76V)-GFP signal intensity was analyzed by QIBC (black bars, mean; 150–1,000 GFP-positive cells analyzed per condition).

B DOX-treated U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K29R) cell lines were transfected with shUb-resistant Ub(G76V)-GFP construct and treated or not with p97i for 4 h. Cell extracts were subjected to GFP IP followed by immunoblotting with indicated antibodies.

C U2OS/Ub(G76V)-GFP-c1 cells treated with p97i (4 h) or MG132 (6 h) where indicated were subjected to GFP IP under denaturing conditions and analyzed by silver staining.

D DOX-treated U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K27R) cell lines were transfected with the indicated Ub(G76V)-GFP expression constructs for 24 h, and nuclear GFP signal was analyzed by QIBC (black bars, mean; > 1,000 GFP-positive cells were analyzed per condition).

E Immunoblot analysis of DOX-treated U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K27R) cells transfected with non-targeting control (CTRL) or p97 siRNA.

F, G DOX-treated U2OS/shUb/HA-Ub cell lines were subjected to local UV irradiation through pore filters in the presence or absence of p97i, fixed at the indicated times and co-immunostained with XPC and DDB2 antibodies. XPC and DDB2 enrichment at local UV spots was quantified (black bars, mean; > 150 cells with UV spots analyzed per condition; $n = 4$ independent experiments; $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant, unpaired t-test).

H Representative images of DOX-treated Ub replacement cell lines shown in (F,G). Scale bar, 10 μm.

Data information: Data are representative of four (F,G), three (A) and two (B-E) independent experiments with similar outcome.

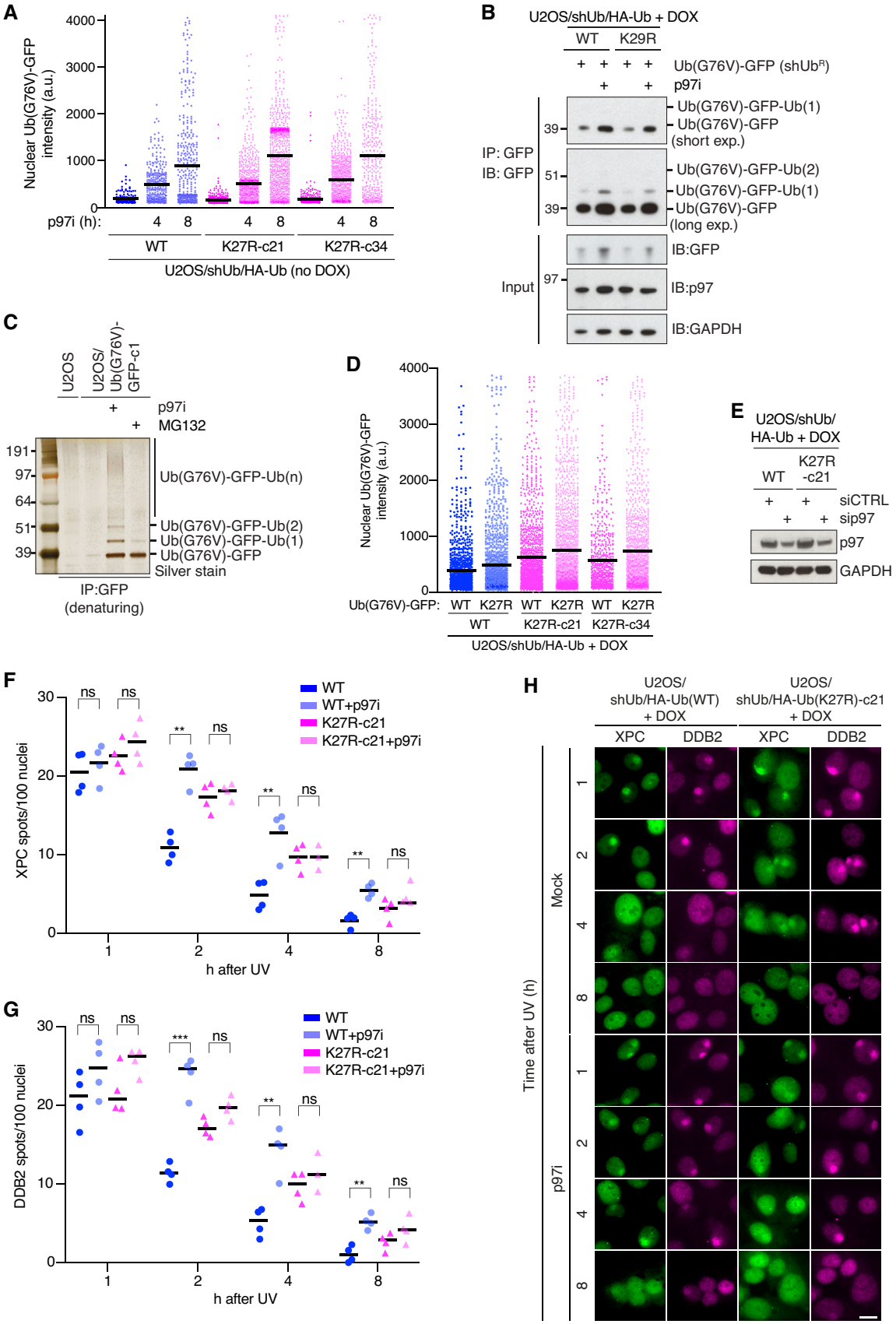
A
Nuclear Ub(G76V)-GFP intensity (a.u.)
4000
3000
2000
1000
0
p97i (h):
4 8 4 8 4 8
WT
K27R-c21
K27R-c34
U2OS/shUb/HA-Ub (no DOX)
B
U2OS/shUb/HA-Ub + DOX
WT
K29R
Ub(G76V)-GFP (shUbR)
p97i
IP: GFP
IB: GFP
39
51
39
Ub(G76V)-GFP-Ub(1)
Ub(G76V)-GFP
(short exp.)
Ub(G76V)-GFP-Ub(2)
Ub(G76V)-GFP-Ub(1)
Ub(G76V)-GFP
(long exp.)
Input
97
IB:GFP
IB:p97
IB:GAPDH
C
U2OS
U2OS/ Ub(G76V)-GFP-c1
p97i
MG132
191
97
64
51
39
Ub(G76V)-GFP-Ub(n)
Ub(G76V)-GFP-Ub(2)
Ub(G76V)-GFP-Ub(1)
Ub(G76V)-GFP
Silver stain
IP:GFP
(denaturing)
D
Nuclear Ub(G76V)-GFP intensity (a.u.)
4000
3000
2000
1000
0
Ub(G76V)-GFP: WT K27R WT K27R WT K27R
WT
K27R-c21
K27R-c34
U2OS/shUb/HA-Ub + DOX
E
U2OS/shUb/ HA-Ub + DOX
WT
K27R -c21
siCTRL
sip97
p97
GAPDH
F
XPC spots/100 nuclei
30
20
10
0
ns ns ** ns ** ns ** ns
WT
WT+p97i
K27R-c21
K27R-c21+p97i
1 2 4 8
h after UV
G
DDB2 spots/100 nuclei
30
20
10
0
ns ns *** ns ** ns ** ns
WT
WT+p97i
K27R-c21
K27R-c21+p97i
1 2 4 8
h after UV
H
U2OS/ shUb/HA-Ub(WT) + DOX
U2OS/ shUb/HA-Ub(K27R)-c21 + DOX
XPC
DDB2
XPC
DDB2
Time after UV (h)
Mock
p97i
1
2
4
8
1
2
4
8

Figure EV4.

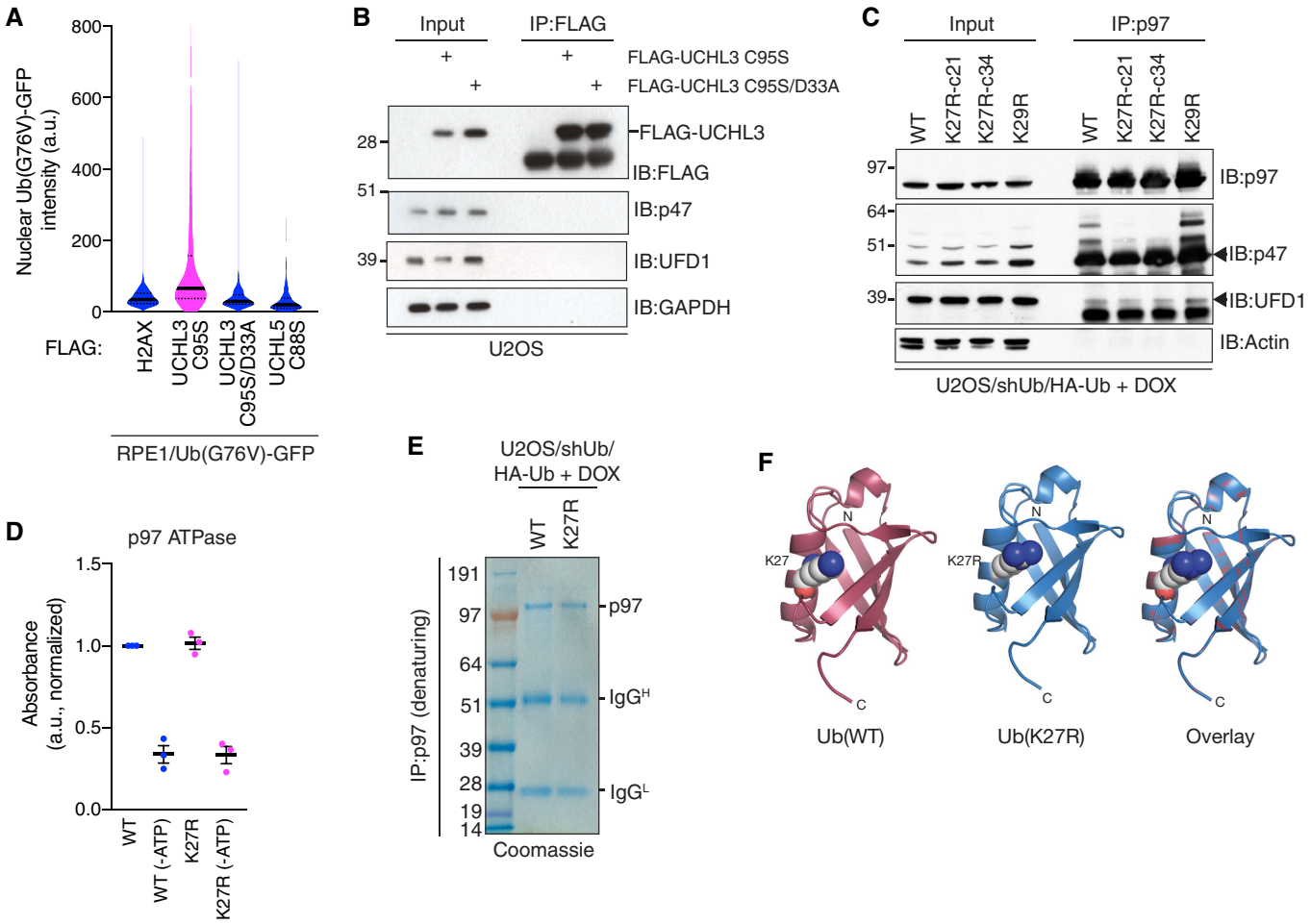

Figure EV5. (related to Fig 5) Blocking the decoding of K27-linked ubiquitylation signals impairs p97 substrate turnover.

A RPE-1 cells stably expressing Ub(G76V)-GFP were transfected with indicated FLAG-tagged expression constructs, fixed and immunostained with FLAG antibody. Levels of Ub(G76V)-GFP in FLAG-positive cells were quantified using QIBC (solid lines, median; dashed lines, quartiles; > 1,000 transfected cells analyzed per condition).

B U2OS cells transfected with FLAG-UCHL3 constructs were subjected to FLAG IP followed by immunoblotting with indicated antibodies.

C DOX-treated Ub replacement cell lines were subjected to p97 IP followed by immunoblotting with the indicated antibodies.

D ATPase activity of p97 immobilized by IP under denaturing conditions from U2OS/shUb/HA-Ub(WT) or U2OS/shUb/HA-Ub(K27R) (mean ± SEM; n = 3 independent experiments).

E Coomassie staining of p97 IPs in (D).

F Crystal structure of human Ub (PDB ID: 5UJL; Castaneda *et al*, 2016) (left), modeled structure of a Ub(K27R) mutant (middle) using the PyMol mutagenesis tool, and their overlay (right). Amino acids at the Ub K27 position are highlighted by space-filling representation.

Data information: Data are representative of three (A,D,E) and two (B,C) independent experiments with similar outcome.