Molecular Cell, Volume *81*

Supplemental Information

The ubiquitin ligase RFWD3 is required for translesion DNA synthesis

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Supplemental Figure Legends

Figure S1. RFWD3 is essential for TLS across peptide adducts. Related to Figure 1. (**A**) Left panel, schematic of *Xenopus laevis* RFWD3 protein indicating the residues used to generate RFWD3-N and RFWD3-F antibodies. Right panel, mock- and RFWD3-N or RFWD3-F depleted extracts were blotted with the indicated antibodies. Asterisk indicates a non-specific band. (**B**) pMH was replicated in mock- or RFWD3-F depleted extracts. Samples were digested and analyzed as in Figure 1E. (**C**) Whole proteome MS analysis of mock- versus RFWD3-depleted egg extracts. The volcano plot shows the difference in abundance of proteins between the mock reaction and RFWD3-depleted samples (with either N or F antibodies) (x-axis), plotted against the *p*-value resulting from two-tailed Student's *t*-testing (y-axis). Proteins significantly down-regulated (FDR<5%) in RFWD3-depleted extracts are represented in red. *n*=4 biochemical replicates, FDR<5% corresponds to a permutation-based FDR-adjusted *q*-value of <0.05. (**D**) Extracts depleted with either RFWD3-N or RFWD3-N were compared to a mock depletion dilution series and blotted with the indicated antibodies. Note that none of the blotted proteins appear as significantly depleted. (**E**) A plasmid containing a site-specific Fpg crosslink (depicted in the left scheme) was replicated in mock- or RFWD3-depleted extracts and analyzed as in Figure 1C (top panel) or digested and analyzed as in Figure 1E alongside a sequencing ladder (lower panels). Red arrowheads indicate the accumulation of open circular molecules observed in the absence of RFWD3. (**F**) pMH^{ssDNA} or $pMH^{ssDNA\text{-}PK}$ were incubated in mock- or RFWD3-depleted non-licensing extracts in the presence of [α-^{32}P]dATP. Samples were digested with PvuII and NdeI and analyzed on a denaturing polyacrylamide gel. The upper scheme depicts the extension products generated by PvuII and NdeI digest.

Figure S2. Replication of pFPG requires TLS. Related to Figure 2. (**A**) Mock-, Polη-, REV1- or Polη and REV1-depleted extracts were blotted with the indicated antibodies. (**B**) pMH was replicated in Polη-depleted extracts supplemented with buffer or recombinant Polη. Samples were digested and analyzed as in Figure 1E. (**C**) pMH^{ssDNA} was incubated in mock-, Polη- or REV1-depleted non-licensing extracts. Samples were digested and analyzed as in Figure S1E. (**D**) Scheme comparing the different crosslinking chemistries between M.HpaII and Fpg. (**E**) pFpg was replicated in mock-, Polη- or REV1-depleted extracts and analyzed as in Figure 1C. (**F**) Samples from (E) were digested and analyzed as in Figure 1E. Note that the different requirements of TLS polymerases to bypass Fpg and M.HpaII adducts are likely dictated by their different crosslinking chemistry. (**G**) Quantification of mutation frequencies measured after replication of pFpg in mock- or Polη-depleted

egg extracts. Replication samples were amplified by PCR and analyzed by next generation sequencing (see Materials and Methods). The 0 position corresponds to the location of the protein adduct, which is linked to an abasic site. Note that the mutation frequencies across the Fpg crosslink are 10 times higher than for HpaII crosslink. This is because Fpg is crosslinked to an abasic site, which carries no base information. (**H**) Distribution of nucleotide misincorporation from the data generated in (G). Misincorporation is based on the assumption that a G was originally paired to C. In cells, G can be oxidized to 8-oxoguanine and converted to an abasic site via Ogg1 or Fpg. During this process, the glycosylase can become irreversibly crosslinked to the open ring abasic site intermediate.

Figure S3, RFWD3 is essential for TLS during pICL repair. Related to Figure 3. (**A**) Simplified model of pICL^{Pt} repair in egg extracts. (**B**) pICL^{pt} was replicated in mock-, REV1- or RFWD3-depleted extracts and reaction samples were blotted with the indicated antibodies. The % of ubiquitylated FANCD2 in each lane was calculated as the fraction of the intensities of the ubiquitylated band divided by the sum of the ubiquitylated and non-ubiquitylated bands. (**C**) Scheme depicting the products generated upon HincII and SapI digest in the pICL repair assay. (**D-E**) pICL^{pt} was replicated in mock- or RFWD3-depleted extracts (with either RFWD3-N or RFWD3-F). Samples were isolated and digested with either HincII alone (D) or with HincII and SapI (E), and analyzed by agarose gel electrophoresis. pQuant is used as a recovery control. The percentage of repair of the ICL was quantified and plotted in the lower graph.

Figure S4. RFWD3 regulates PCNA ubiquitylation. Related to Figure 4. (**A**) pICL^{Pt} was replicated in mock- or RFWD3-depleted extracts and the reaction samples were blotted against the indicated antibodies. Note that ubiquitylation of FANCD2 and phosphorylation of CHK1 occur with normal kinetics in the absence of RFWD3. (**B**) pMH^{PK} and pICL^{pt} were replicated in the presence or absence of a p97 inhibitor (NMS873) and analyzed as in Figure 1C. Note that upon p97 inhibition, CMG unloading is severely inhibited during replication-coupled repair of pICL^{pt} and replication intermediates are stabilized (Fullbright et al., 2016). (**C**) pMH^{PK} samples from (B) were digested and analyzed as in Figure 1E. (**D**) pMH^{ssDNA}, which triggers the damage-dependent destruction of CDT1, was incubated in non-licensing extracts in the presence or absence of ubiquitin E1 inhibitor (MLN7243), and total extracts were blotted with the indicated antibodies at the indicated time point. PSA3 was used as loading control. Note that MLN7243 inhibits the ubiquitin dependent destruction of CDT1. (**E**) pMH or pMH^{PK} were replicated in egg extracts in the presence or absence of ubiquitin E1 inhibitor (MLN7243) to block *de novo* ubiquitylation. Samples were analyzed by agarose gel

electrophoresis as in Figure 1C. Red arrows indicate persisting OC molecules. (**F**) pMH was replicated in extracts in the presence of 1 mg/mL of the indicated ubiquitin mutant. Radiolabeled samples were digested and analyzed as in Figure 1E. (**G**) Mock- or UBC13-depleted extracts were blotted with the indicated antibody. Asterisk indicates an unspecific band. (**H-I**) pMH was replicated in Mock- or UBC13-depleted extracts in the presence of radiolabeled [α-^{32}P]dATP and reaction products were resolved on an agarose gel (**H**) or digested and resolved on a denaturing polyacrylamide gel (**I**). Red arrowheads indicate persistent OC molecules in the UBC13-depleted reaction. (**J**) Sperm chromatin was either untreated or treated with 2000 J/m^2 of UV-C and then incubated in non-licensing mock-, UBC13- or RFWD3-depleted extracts for 30 min. Chromatin was recovered via chromatin spindown and samples were blotted with the indicated antibodies. Note that UBC13 depletion specifically abrogates PCNA poly-ubiquitylation. (**K**) Sperm chromatin was either untreated or treated with 2000 J/m^2 of UV-C and then incubated in non-licensing extracts in the presence of ubiquitin E1 inhibitor (MLN7243) or 1 mg/mL of the indicated ubiquitin mutants. Samples were blotted with the indicated antibodies. Red dots indicate PCNA ubquitylated species, black dot indicates SUMOylated PCNA. (**L**) Sperm chromatin was either untreated or treated with 20 J/m^2 of UV-C and then replicated in mock- or RFWD3-depleted extracts. Branched and branch-free DNA molecules are indicated according to (Hashimoto et al., 2010). (**M**) pCTRL was replicated in either mock or RFWD3-depleted extracts. Reactions were subjected to plasmid pull-down and samples were blotted with the indicated antibodies. Red dots correspond to PCNA ubiquitylation. Black dot corresponds to mono-SUMOylated PCNA. (**N**) pMH^{Leads} was replicated in mock- or RFWD3-depleted extracts. Radiolabeled samples were analyzed as in Figure 1C. Red arrowheads indicate persistent OC molecules.

Figure S5. Contribution of E3 ubiquitin ligases to PCNA ubiquitylation in human cells. Related to Figure 5. (**A**) UO2S cells expressing Strep-HA-PCNA transfected with the indicated siRNAs were treated with UV light and PCNA was recovered via pull-down under denaturing conditions as in Figure 5A. (**B**) U2OS or U2OS cells expressing Strep-HA-PCNA were transfected with RFWD3 and the indicated siRNAs. Proteins were recovered and analyzed as in Figure 5A.

Figure S6. RFWD3 does not regulate SUMO levels on UV damaged chromatin. Related to Figure 6. (**A**) MS analysis of protein recruitment to UV-treated sperm chromatin compared to untreated sperm chromatin in egg extracts. The volcano plot shows the difference in abundance of proteins between the two sample conditions (x-axis), plotted against the *p*-value resulting from two-tailed Student's *t*-testing (y-axis). Proteins significantly down- or up-regulated (FDR<5%) upon UV

treatment are represented in red or blue, respectively. *n*=4 biochemical replicates, FDR<5% corresponds to a permutation-based FDR adjusted *q*-value of <0.05. Note that different isoforms of the same protein can sometimes be detected. Proteins in dark blue or red were also significantly affected by depletion of RFWD3 (shown in Figure 6B). (**B**) Mock-, REV1- or RFWD3-depleted extracts were blotted with the indicated antibodies. Note that depletion of REV1 leads to substantial co-depletion of REV7 but not Polκ. (**C**) Quantification of SUMO1, SUMO2 and SUMO3 on sperm chromatin, directly identified by MS/MS, and quantified in a label-free manner. Control, mock-depleted extracts incubated with undamaged chromatin; UV, mock-depleted extracts incubated with UV-treated chromatin; RFWD3Δ-UV, RFWD3-depleted extracts incubated with UV-treated chromatin; REV1Δ-UV, REV1-depleted extracts incubated with UV-treated chromatin. *n*=4 biochemical replicates, error bars represent SEM. * $p<0.05$, via two-tailed Student's *t*-testing.

Figure S7. RFWD3 ubiquitylates proteins on ssDNA. Related to Figure 7. (**A**) pFpg or pFpg^{ssDNA} were incubated in non-licensing extracts depleted of SPRTN and plasmid pull-down was performed at the indicated time points. Samples were blotted with the indicated antibodies. (**B**) pFpg^{ssDNA} was incubated in non-licensing SPRTN-depleted extracts and DPC pull-down was performed at the indicated time points as in Figure 7A. At each time point the pull-down samples were split and either untreated or treated with the indicated specific deubiquitylating enzymes (Boston Biochem) before western blot analysis. Otubain1, cleaves lysine K48-linked ubiquitin chains, while AMSH cleaves lysine K63-linked ubiquitin chains. (**C**) pMH^{ssDNA} was incubated in mock- or RFWD3-depleted non-licensing extracts (also depleted of SPRTN) and reaction samples processed as in Figure 7A. (**D-E**) Cross-complementation of RFWD3 depletion (RFWD3-F antibody) with RFWD3 protein eluted from immunoprecipitated egg extracts using RFWD3-N antibody. (**D**) schematic of the experimental setup and western blot showing the level of RFWD3 in the extract in the different indicated conditions. (**E**) pFpg^{ssDNA} was incubated in mock- or RFWD3-depleted (RFWD3-F antibody) non-licensing extract and supplemented with peptide eluates of IgG- or RFWD3-immunoprecipitated egg extracts. Reaction samples were processed as in (A). Asterisk denotes non-specific bands.

Figure S1

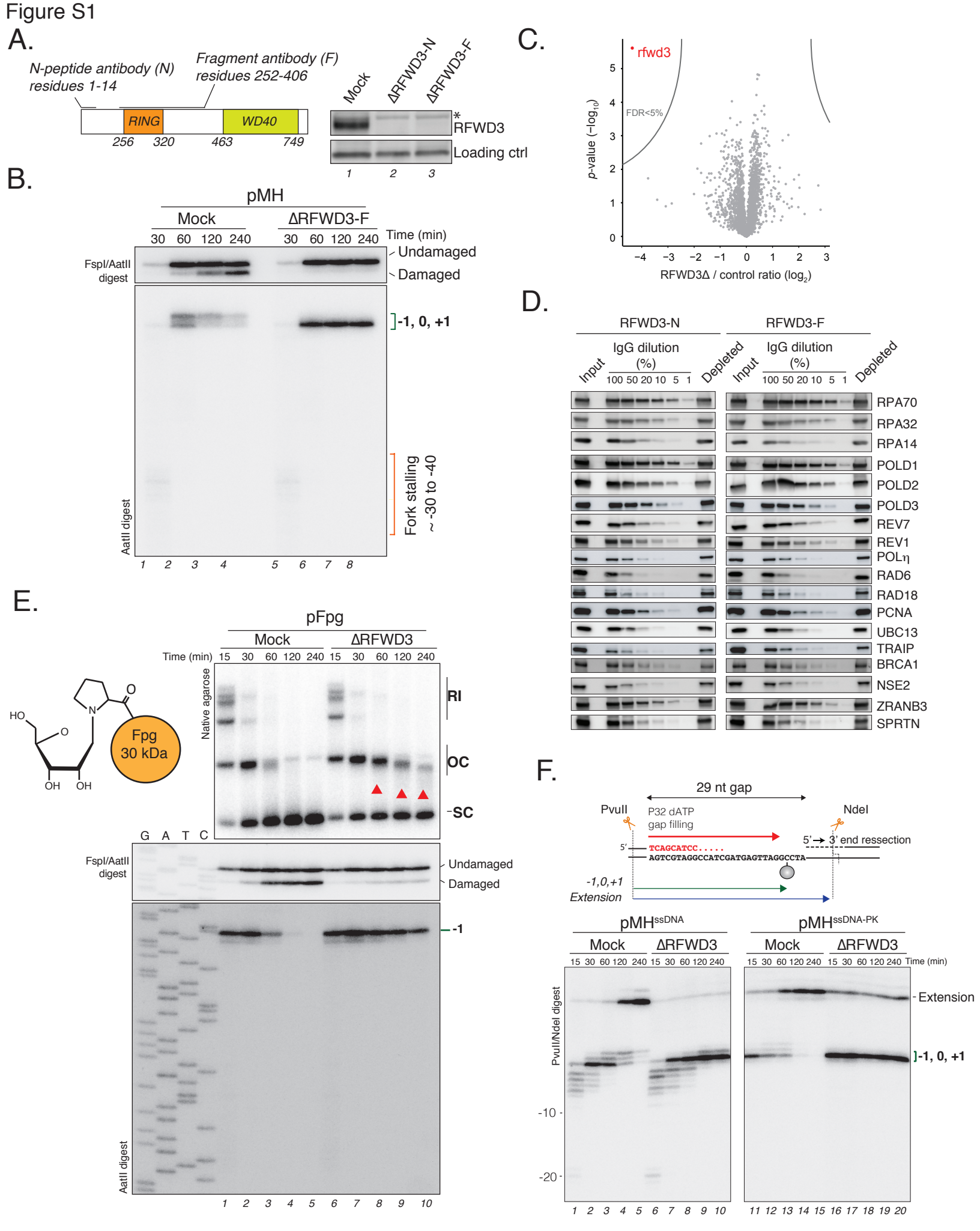

A.
N-peptide antibody (N)
residues 1-14
Fragment antibody (F)
residues 252-406
RING
WD40
256
320
463
749
Mock
ΔRFWD3-N
ΔRFWD3-F
*
RFWD3
Loading ctrl
1
2
3
B.
pMH
Mock
ΔRFWD3-F
30 60 120 240
30 60 120 240
Time (min)
FspI/AatII digest
Undamaged
Damaged
-1, 0, +1
AatII digest
Fork stalling
~ -30 to -40
1 2 3 4 5 6 7 8
C.
rfwd3
FDR<5%
p-value (−log10)
RFWD3Δ / control ratio (log2)
D.
RFWD3-N
RFWD3-F
Input
IgG dilution (%)
100 50 20 10 5 1
Depleted
RPA70
RPA32
RPA14
POLD1
POLD2
POLD3
REV7
REV1
POLη
RAD6
RAD18
PCNA
UBC13
TRAIP
BRCA1
NSE2
ZRANB3
SPRTN
E.
pFpg
Mock
ΔRFWD3
Time (min)
15 30 60 120 240 15 30 60 120 240
Native agarose
RI
OC
SC
Fpg
30 kDa
G A T C
FspI/AatII digest
Undamaged
Damaged
-1
AatII digest
1 2 3 4 5 6 7 8 9 10
F.
29 nt gap
PvuII
P32 dATP
gap filling
NdeI
5'→ 3' end ressection
TCAGCATCC.....
AGTCGTAGGCCATCGATGAGTTAGGCCTA
-1,0,+1
Extension
pMHssDNA
pMHssDNA-PK
Mock
ΔRFWD3
15 30 60 120 240 15 30 60 120 240
Time (min)
PvuII/NdeI digest
-10
-20
Extension
-1, 0, +1
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20

Figure S2

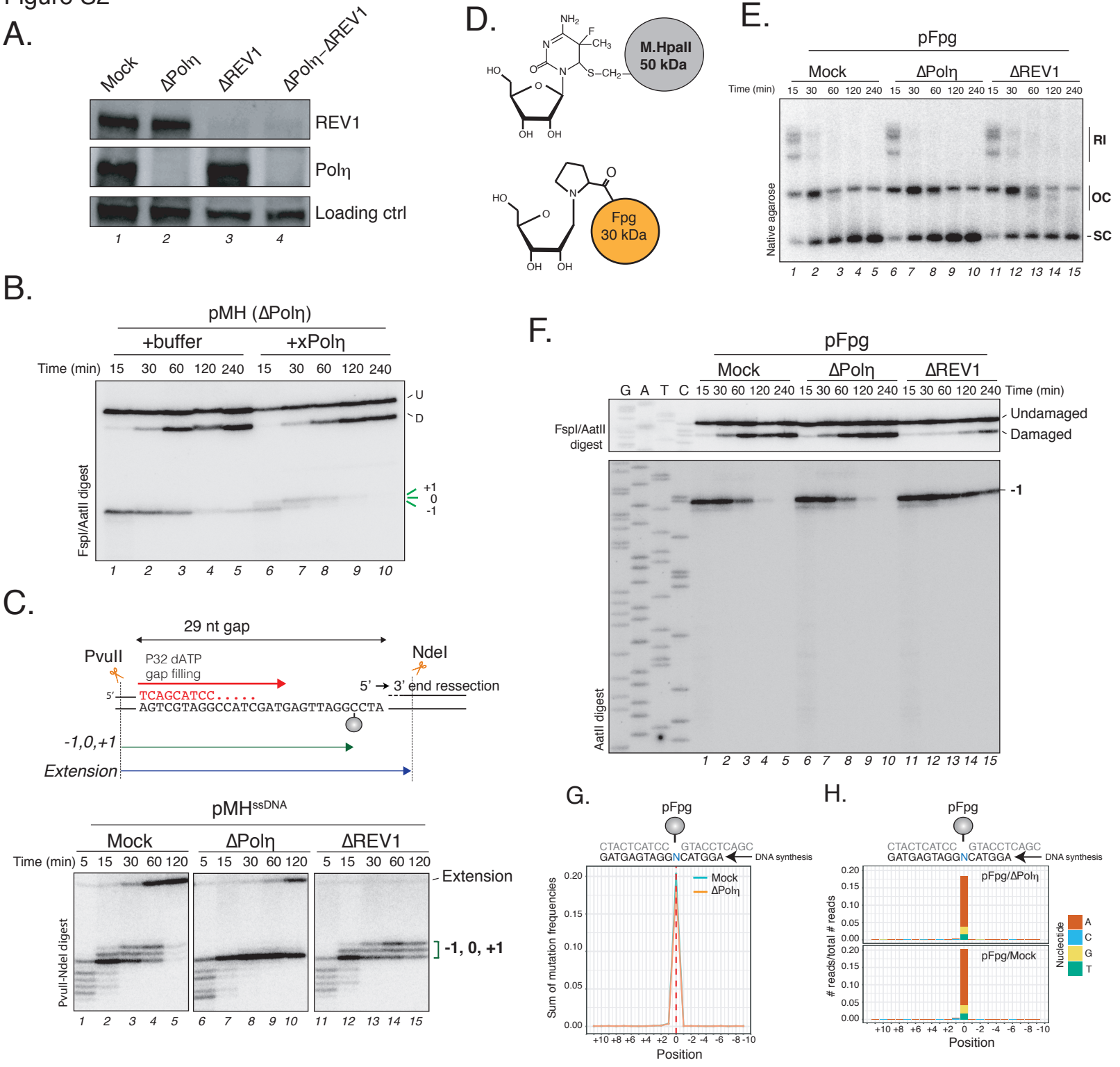

A.
Mock
ΔPolη
ΔREV1
ΔPolη−ΔREV1
REV1
Polη
Loading ctrl
1 2 3 4
D.
M.HpaII
50 kDa
Fpg
30 kDa
E.
pFpg
Mock
ΔPolη
ΔREV1
Time (min) 15 30 60 120 240 15 30 60 120 240 15 30 60 120 240
Native agarose
RI
OC
SC
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B.
pMH (ΔPolη)
+buffer
+xPolη
Time (min) 15 30 60 120 240 15 30 60 120 240
FspI/AatII digest
U
D
+1
0
-1
1 2 3 4 5 6 7 8 9 10
C.
29 nt gap
PvuII
NdeI
P32 dATP
gap filling
5' → 3' end ressection
5'
TCAGCATCC.....
AGTCGTAGGCCATCGATGAGTTAGGCCTA
-1,0,+1
Extension
pMHssDNA
Mock
ΔPolη
ΔREV1
Time (min) 5 15 30 60 120 5 15 30 60 120 5 15 30 60 120
PvuII-NdeI digest
Extension
-1, 0, +1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
F.
pFpg
Mock
ΔPolη
ΔREV1
G A T C 15 30 60 120 240 15 30 60 120 240 15 30 60 120 240 Time (min)
FspI/AatII digest
Undamaged
Damaged
AatII digest
-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
G.
pFpg
CTACTCATCC GTACCTCAGC
GATGAGTAGGNCATGGA
DNA synthesis
Sum of mutation frequencies
Mock
ΔPolη
0.20
0.15
0.10
0.05
0.00
+10 +8 +6 +4 +2 0 -2 -4 -6 -8 -10
Position
H.
pFpg
CTACTCATCC GTACCTCAGC
GATGAGTAGGNCATGGA
DNA synthesis
reads/total # reads
pFpg/ΔPolη
pFpg/Mock
Nucleotide
A
C
G
T
0.20
0.15
0.10
0.05
0.00
+10 +8 +6 +4 +2 0 -2 -4 -6 -8 -10
Position

Figure S3

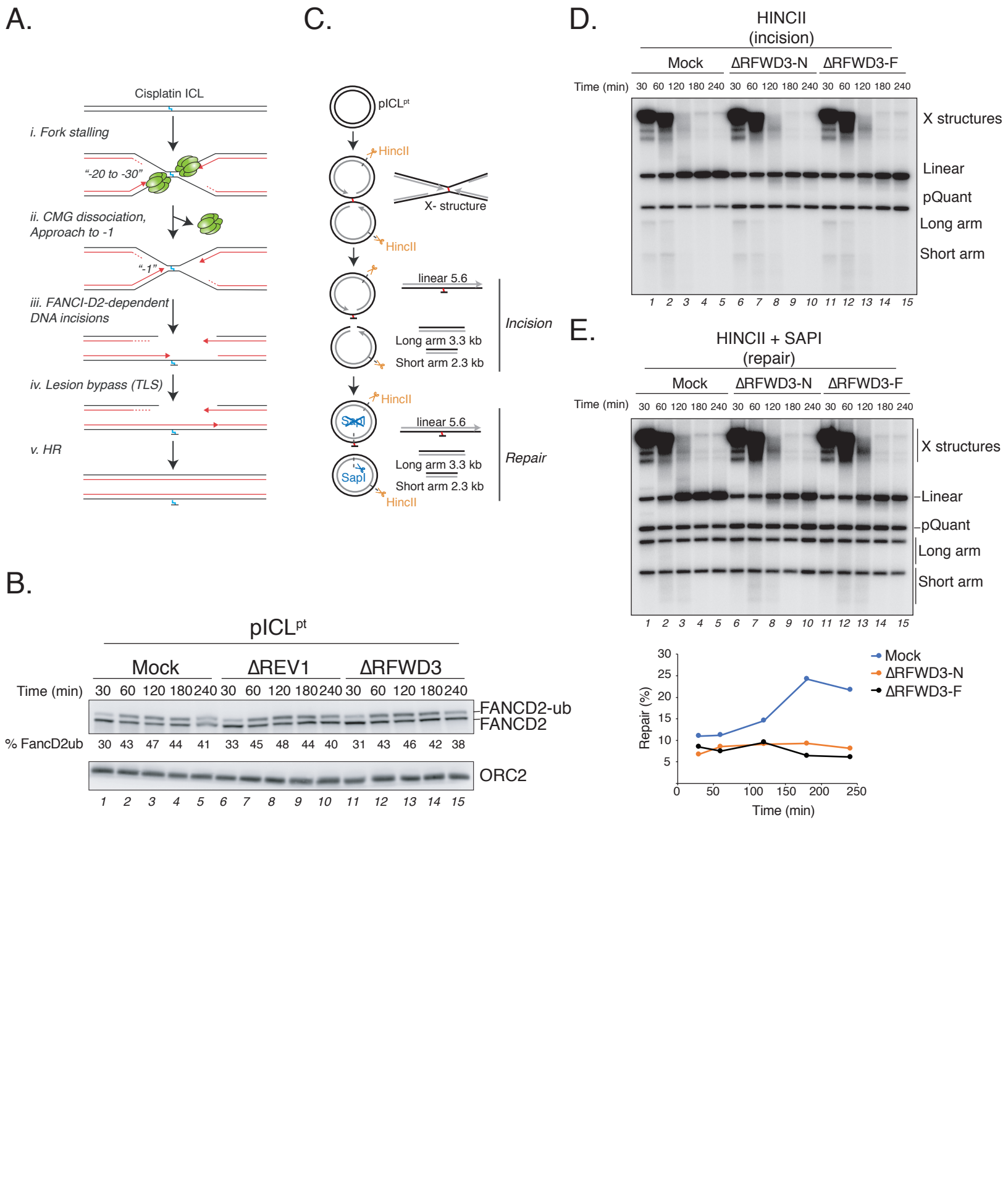

A.
Cisplatin ICL
i. Fork stalling
"-20 to -30"
ii. CMG dissociation, Approach to -1
"-1"
iii. FANCI-D2-dependent DNA incisions
iv. Lesion bypass (TLS)
v. HR
C.
pICLpt
HincII
X- structure
HincII
linear 5.6
Long arm 3.3 kb
Short arm 2.3 kb
Incision
HincII
SapI
linear 5.6
Long arm 3.3 kb
Short arm 2.3 kb
SapI
HincII
Repair
D.
HINCII (incision)
Mock
ΔRFWD3-N
ΔRFWD3-F
Time (min) 30 60 120 180 240 30 60 120 180 240 30 60 120 180 240
X structures
Linear
pQuant
Long arm
Short arm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
E.
HINCII + SAPI (repair)
Mock
ΔRFWD3-N
ΔRFWD3-F
Time (min) 30 60 120 180 240 30 60 120 180 240 30 60 120 180 240
X structures
Linear
pQuant
Long arm
Short arm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Repair (%)
30
25
20
15
10
5
0 50 100 150 200 250
Time (min)
Mock
ΔRFWD3-N
ΔRFWD3-F
B.
pICLpt
Mock
ΔREV1
ΔRFWD3
Time (min) 30 60 120 180 240 30 60 120 180 240 30 60 120 180 240
FANCD2-ub
FANCD2
% FancD2ub 30 43 47 44 41 33 45 48 44 40 31 43 46 42 38
ORC2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure S4

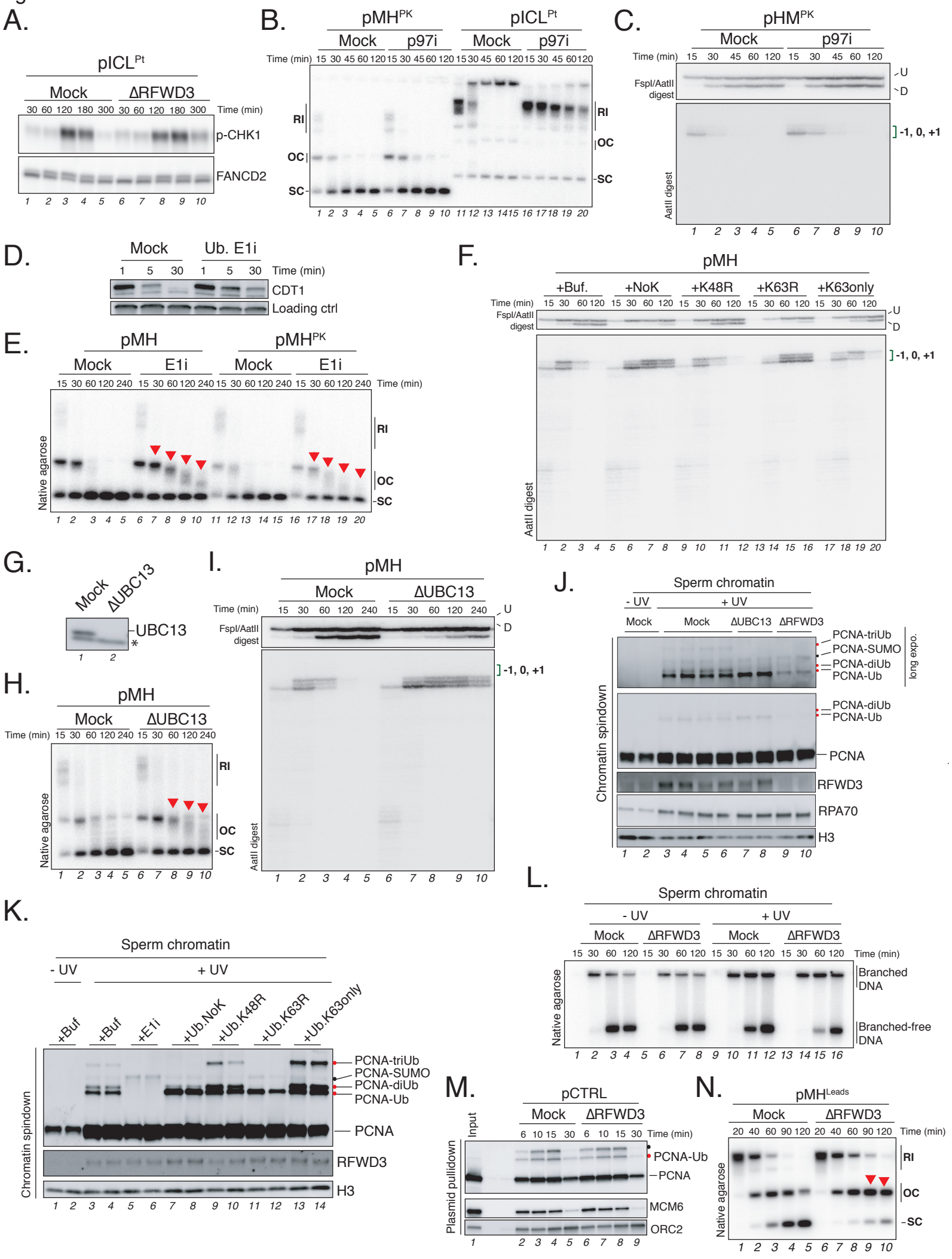

A.
pICL^Pt
Mock
ΔRFWD3
Time (min)
p-CHK1
FANCD2
B.
pMH^PK
pICL^Pt
Mock
p97i
Time (min)
RI
OC
SC
C.
pHM^PK
Mock
p97i
Time (min)
FspI/AatII digest
U
D
AatII digest
-1, 0, +1
D.
Mock
Ub. E1i
Time (min)
CDT1
Loading ctrl
E.
pMH
pMH^PK
Mock
E1i
Time (min)
Native agarose
RI
OC
SC
F.
pMH
+Buf.
+NoK
+K48R
+K63R
+K63only
Time (min)
FspI/AatII digest
AatII digest
-1, 0, +1
G.
Mock
ΔUBC13
UBC13
H.
pMH
Mock
ΔUBC13
Time (min)
Native agarose
RI
OC
SC
I.
pMH
Mock
ΔUBC13
Time (min)
FspI/AatII digest
AatII digest
-1, 0, +1
J.
Sperm chromatin
- UV
+ UV
Mock
Mock
ΔUBC13
ΔRFWD3
PCNA-triUb
PCNA-SUMO
PCNA-diUb
PCNA-Ub
long expo.
Chromatin spindown
PCNA
RFWD3
RPA70
H3
K.
Sperm chromatin
- UV
+ UV
+Buf
+Buf
+E1i
+Ub.NoK
+Ub.K48R
+Ub.K63R
+Ub.K63only
PCNA-triUb
PCNA-SUMO
PCNA-diUb
PCNA-Ub
Chromatin spindown
PCNA
RFWD3
H3
L.
Sperm chromatin
- UV
+ UV
Mock
ΔRFWD3
Mock
ΔRFWD3
Time (min)
Native agarose
Branched DNA
Branched-free DNA
M.
pCTRL
Mock
ΔRFWD3
Input
Time (min)
Plasmid pulldown
PCNA-Ub
PCNA
MCM6
ORC2
N.
pMH^Leads
Mock
ΔRFWD3
Time (min)
Native agarose
RI
OC
SC

Figure S5

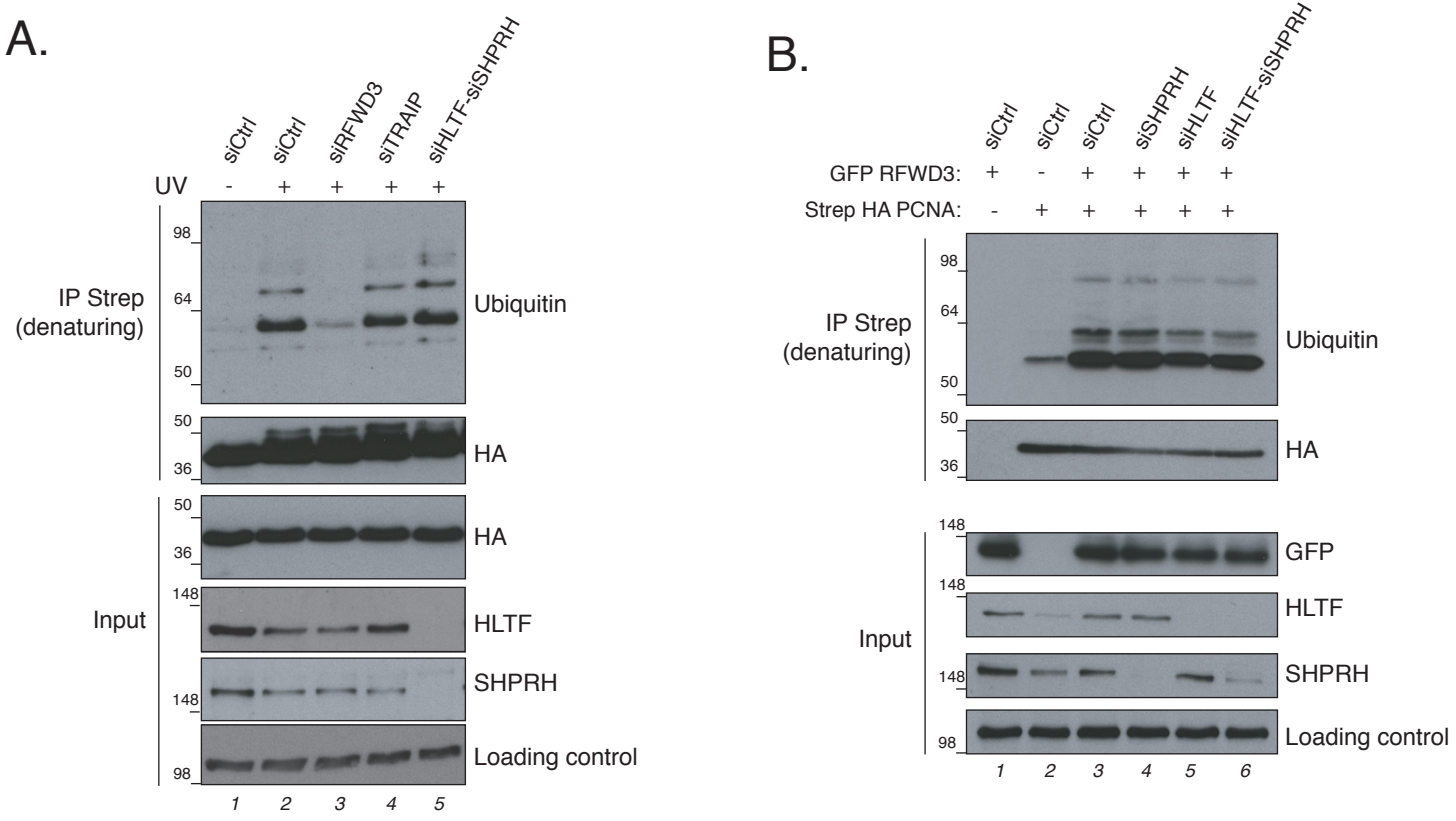
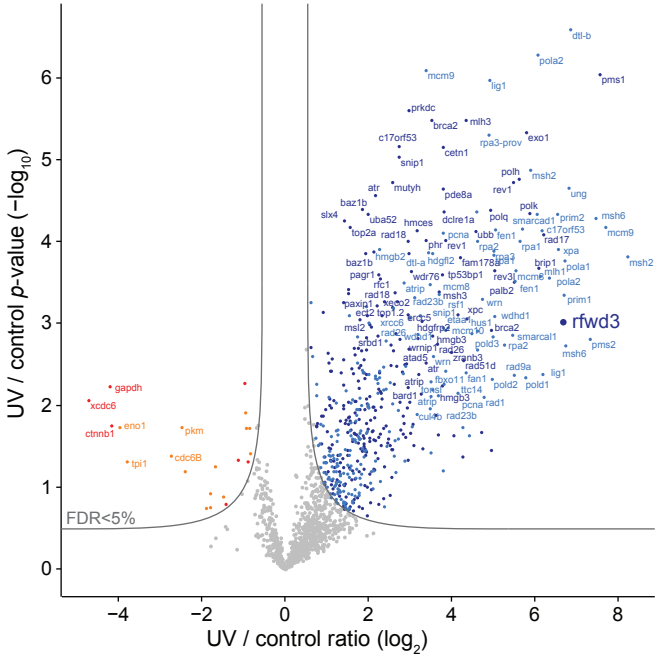
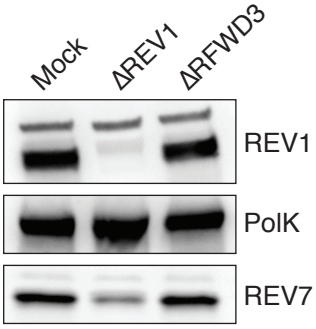

A.
siCtrl
siCtrl
siRFWD3
siTRAIP
siHLTF-siSHPRH
UV - + + + +
IP Strep (denaturing)
98
64
50
Ubiquitin
50
36
HA
Input
50
36
HA
148
HLTF
148
SHPRH
98
Loading control
1 2 3 4 5
B.
siCtrl
siCtrl
siCtrl
siSHPRH
siHLTF
siHLTF-siSHPRH
GFP RFWD3: + - + + + +
Strep HA PCNA: - + + + + +
IP Strep (denaturing)
98
64
50
Ubiquitin
50
36
HA
Input
148
GFP
148
HLTF
148
SHPRH
98
Loading control
1 2 3 4 5 6

Figure S6

A.



B.



C.

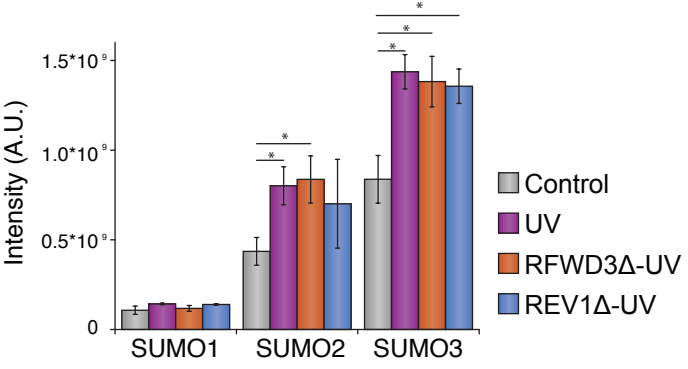
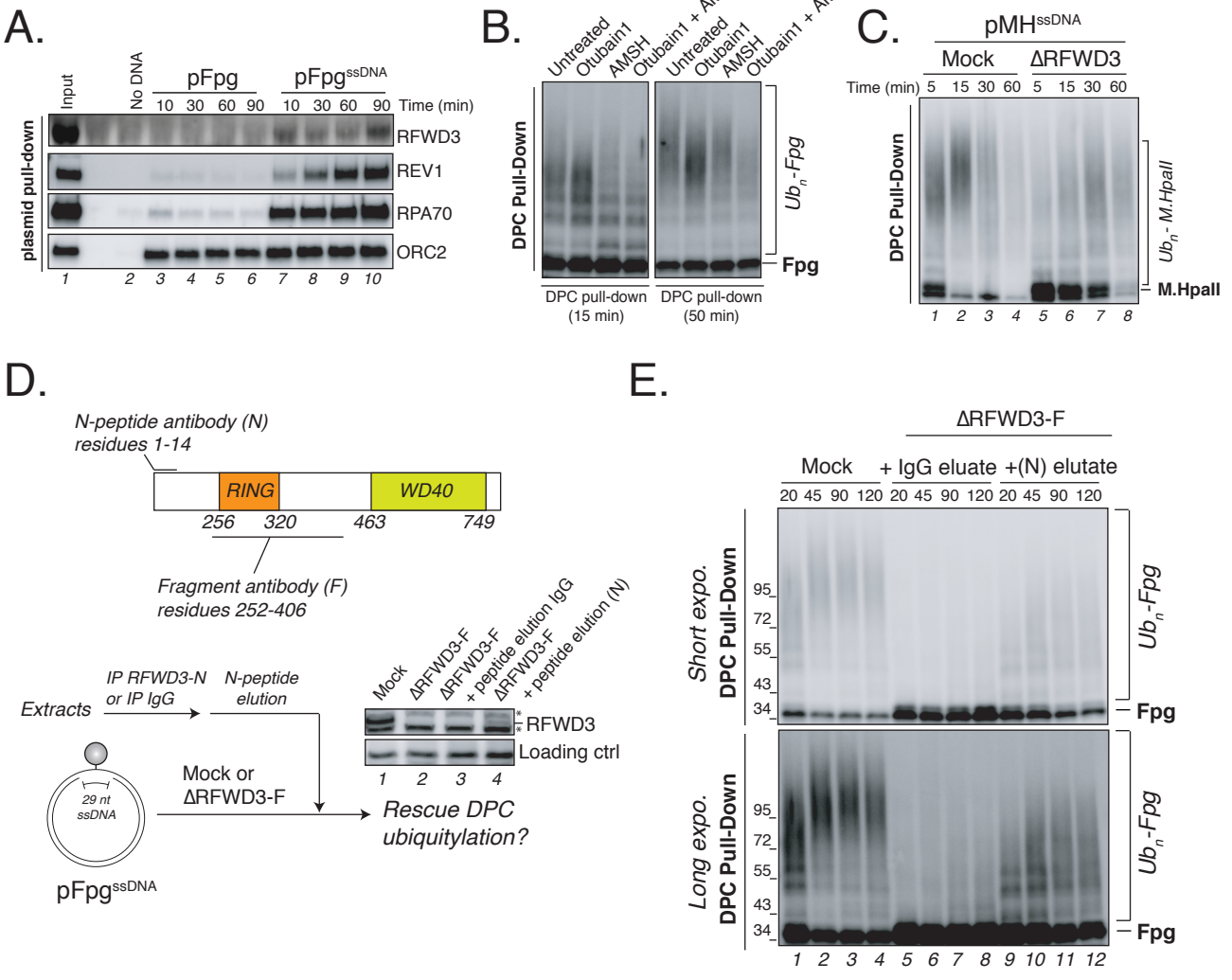


Figure S7


A.
Input
No DNA
pFpg
pFpgssDNA
10 30 60 90 10 30 60 90 Time (min)
plasmid pull-down
RFWD3
REV1
RPA70
ORC2
1 2 3 4 5 6 7 8 9 10
B.
Untreated
Otubain1
AMSH
Otubain1 + AMSH
DPC Pull-Down
Ubn-Fpg
Fpg
DPC pull-down (15 min)
DPC pull-down (50 min)
C.
pMHssDNA
Mock
ΔRFWD3
Time (min) 5 15 30 60 5 15 30 60
DPC Pull-Down
Ubn - M.HpaII
M.HpaII
1 2 3 4 5 6 7 8
D.
N-peptide antibody (N)
residues 1-14
RING
WD40
256 320 463 749
Fragment antibody (F)
residues 252-406
Extracts
IP RFWD3-N or IP IgG
N-peptide elution
Mock
ΔRFWD3-F
ΔRFWD3-F + peptide elution IgG
ΔRFWD3-F + peptide elution (N)
RFWD3
Loading ctrl
1 2 3 4
29 nt ssDNA
Mock or ΔRFWD3-F
Rescue DPC ubiquitylation?
pFpgssDNA
E.
ΔRFWD3-F
Mock
+ IgG eluate
+(N) elutate
20 45 90 120 20 45 90 120 20 45 90 120
Short expo.
DPC Pull-Down
95
72
55
43
34
Ubn-Fpg
Fpg
Long expo.
DPC Pull-Down
95
72
55
43
34
Ubn-Fpg
Fpg
1 2 3 4 5 6 7 8 9 10 11 12