SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids- The RAD6 gene coding sequence was PCR-amplified from *S. cerevisiae* genomic DNA and cloned into pFastBacDual (Invitrogen) under the control of the P10 promoter. Subsequently, the RAD18 gene coding sequence was PCR-amplified and cloned under control of the PH promoter in pFastBacDual[Rad6].

Proteins- Rad6 and Rad18-expressing Sf21 cells were lysed in 50 mM Tris pH 8.0, 0.5 M NaCl, 2 mM β-mercaptoethanol, and 0.02% Triton X-100. Clarified lysate was loaded onto a 15 mL MonoQ column (Qiagen) and bound proteins were washed with 50 mM Tris pH 8.0, 1 mM β-mercaptoethanol, 200 mM NaCl. Rad6 and Rad18 were eluted in wash buffer supplemented with 350 mM NaCl. Rad6 and Rad18 containing fractions were then diluted 1:3 in 50 mM Tris pH 8.0, 2 mM β-mercaptoethanol and loaded onto a 5 mL MonoS column. Bound proteins were washed in mM Tris pH 8.0, 1 mM β-mercaptoethanol, 100 mM NaCl, and Rad6-Rad18 were eluted in wash buffer supplemented with 300 mM NaCl.

Loading and Monoubiquitination of PCNA by Rad6-Rad18- 30 μL reactions contained 40 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 100 μM ATP, 0.1 mM DTT in the presence of 20 ng of PCNA, 20 ng of RFC, and 1 pmol of 75/31-nt partial-heteroduplex DNA containing biotin-streptavidin at each end of the 75-nt oligomer at 37°C for 1 hr. Then 1 μg of Rad6-Rad18, 5 μg of Ub, and 0.01 μg E1 were added to the loaded PCNA reactions, and incubated similarly for the indicated times.

Polyubiquitination assays containing Rad6-Rad18- These assays were conducted as described in the experimental procedures except that Rad6-Rad18 was added at a concentration equivalent to the (Ubc13-Mms2)-Rad5 concentration.

SUPPLEMENTAL FIGURES AND LEGENDS

Fig. S1. Rad18 does not influence polyUb synthesis by Ubc13-Mms2. A) Rad6-Rad18 promotes monoubiquitination of loaded PCNA as reported previously (31). B) Standard free chain conjugation reactions were performed in the presence of Ubc13-Mms2, E1, wild-type Ub, and with no E3 (lanes 1-3), 2 μM Rad5 (lanes 4-6), 2 μM Rad6-Rad18 (lanes 7-9), or 2 μM Rad5 and 2 μM Rad6-Rad18 (lanes 10-12). Products were resolved by SDS-PAGE and visualized with Coomassie Blue. C) Standard free polyUb chain synthesis reactions containing E1, Ubc13-Mms2, Rad5, ^{125}I-Ub(K63R) and increasing amounts of Ub-D77 were performed with or without Rad6-Rad18 (2 μM). Reactions were separated by SDS-PAGE and ^{125}I-Ub_2 products were quantified by γ-counting. The initial velocities vs. Ub-D77 concentrations are shown. Rates were measured in triplicate and fit by the Michaelis-Menten equation using non-linear regression. Rad6-Rad18 did not change the Vmax (9.1 μM/min) or Km (127 μM) of (Ubc13-Mms2)-Rad5 catalyzed free chain synthesis. Error bars represent +/- SEM.

Fig. S2. Slow exchange of subunits in monoUb_1*-PCNA. MonoUb_1*-PCNA (20 μM) was incubated at 37ºC in 50 mM Tris HCl 7.6, 100 mM NaCl, and 1 mM DTT. Aliquots at the indicated times were frozen in liquid nitrogen. The distributions of PCNA trimers in the aliquots were evaluated by native PAGE and Coomassie staining.

Fig. S3. Rad5 stimulates polyubiquitination of monoUb*-PCNA. Polyubiquitination assays were done with (lanes 1-6) or without (lanes 7-15) Rad5. Reaction products were separated by SDS-PAGE and detected by immunoblotting with anti-FLAG antibodies.

Fig. S4. MonoUb^*_3-PCNA is efficiently loaded onto DNA. RFC was used to load monoUb*3-(FLAG-) PCNA onto a biotinylated ss/dsDNA fragment bound to streptavidin-coated magnetic beads. The DNA-loaded monoUb^*_3-PCNA was separated from unloaded monoUb*3-PCNA via magnetic pull-down of the beads. Proteins were separated by SDS-PAGE and detected by immunoblotting with anti-FLAG antibodies. Input (I), Unloaded (U), Loaded (L).

Fig. S5. Polyubiquitination of DNA-loaded monoUb^*_3-PCNA does not depend on Rad6-Rad18. Assays were similar to previous polyubiquitination assays except that the DNA-loaded monoUb^*_3-PCNA was polyubiquitinated by 0.5 μM (Ubc13-Mms2)-Rad5, E1, and 117 μM 125I-Ub(K63R) with or without 0.5 μM Rad18. The monoUb^*_3-PCNA was A) 10 μM or B) 1 μM.

Figure S.1

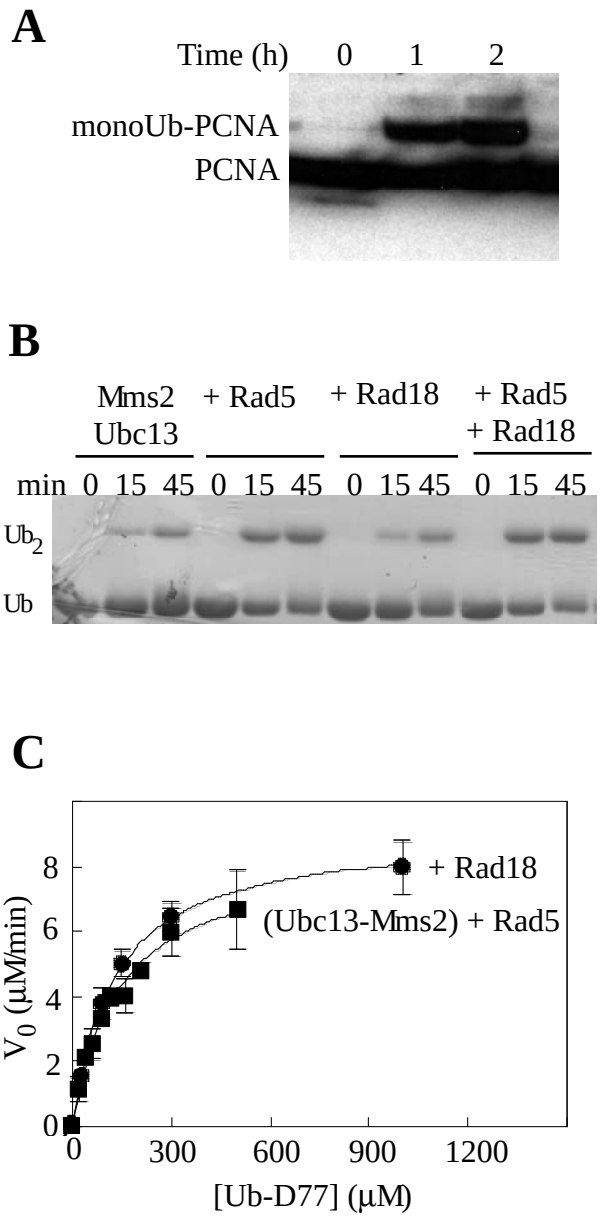

A
Time (h) 0 1 2
monoUb-PCNA
PCNA
B
Mms2
Ubc13
+ Rad5
+ Rad18
+ Rad5
+ Rad18
min 0 15 45 0 15 45 0 15 45 0 15 45
Ub2
Ub
C
+ Rad18
(Ubc13-Mms2) + Rad5
V0 (μM/min)
0
2
4
6
8
0
300
600
900
1200
[Ub-D77] (μM)

Figure S.2

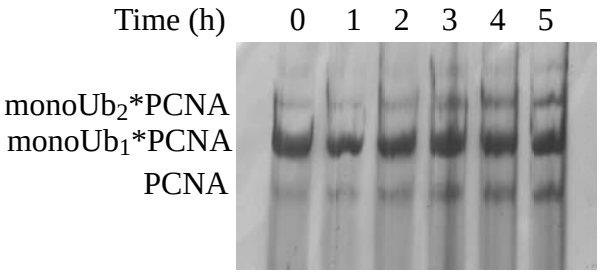


Figure S.3

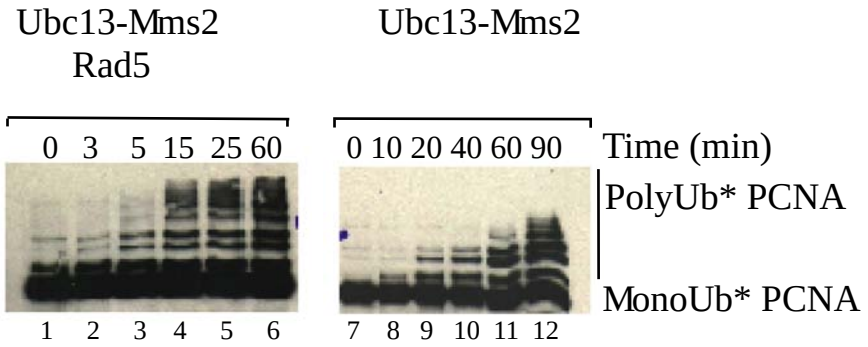

Ubc13-Mms2
Rad5
Ubc13-Mms2
0 3 5 15 25 60
0 10 20 40 60 90
Time (min)
PolyUb* PCNA
MonoUb* PCNA
1 2 3 4 5 6
7 8 9 10 11 12

Figure S.4

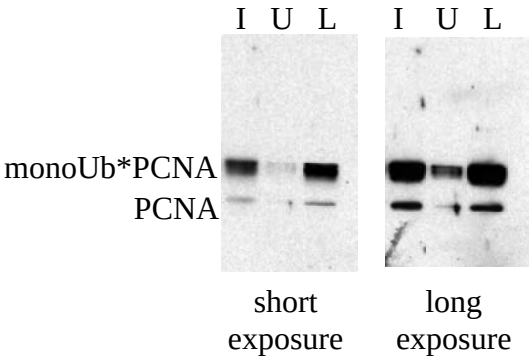
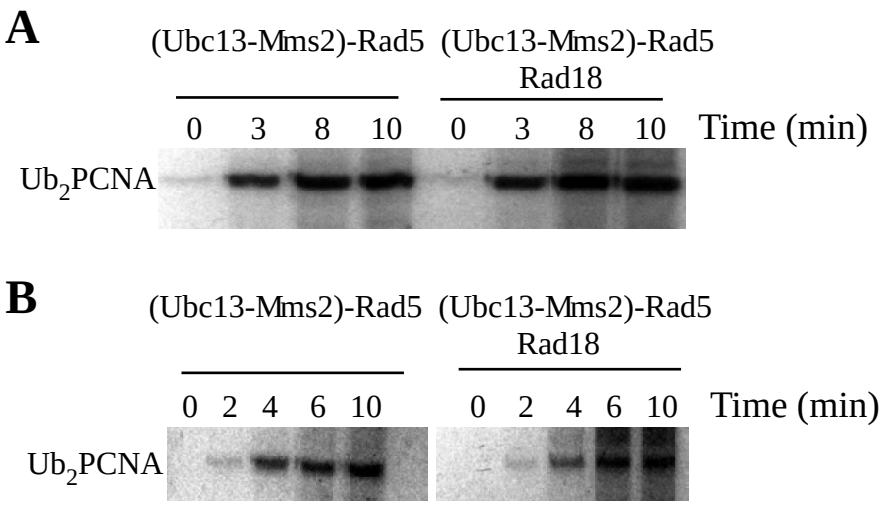


Figure S.5


A
(Ubc13-Mms2)-Rad5
(Ubc13-Mms2)-Rad5
Rad18
0 3 8 10 0 3 8 10
Time (min)
Ub2PCNA
B
(Ubc13-Mms2)-Rad5
(Ubc13-Mms2)-Rad5
Rad18
0 2 4 6 10 0 2 4 6 10
Time (min)
Ub2PCNA