SUPPLEMENTAL INFORMATION

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

GST-pulldown assay-GST-fused to the UIM of epsin1 or GST (25 μg each) were incubated with 20 μl of glutathione Sepharose 4B (GE Healthcare) for 120 min in phosphate buffered saline containing 0.1% Triton X-100 (PBSX) and subsequently washed three times with the PBSX. The beads were incubated with 6 μg of K63-polyubiquitin chains or 20 μg of K11-polyubiquitin chains (Enzo Life Science) for 120 min in PBSX and then washed with the same buffer three times. The polyubiquitin chains that remained bound to the beads were released by eluting with reduced glutathione and subjected to SDS-PAGE and immunoblotted with the FK2 Ab.

FIGURE LEGENDS FOR SUPPLEMENTAL FIGURES

Fig. S1. Analysis of CD8-MHC I downregulation by blockage of UbcH5b/c. Control shRNA (Luc) or shRNA targeting UbcH5b and c (UbcH5b/c) were retrovirally transduced into T-REx-MIR2 cells. A, Whole cell lysates were analyzed with anti-UbcH5 and tubulin Abs. B, After incubating the T-REx-MIR2 cells with (open histograms) or without (shaded histograms) Dox for 7 hr, the expression level of CD8-MHC I was examined by flow cytometry. C, In the left panel, transduced T-REx-MIR2 cells were incubated with or without Dox for 6 hr and FITC-conjugated anti-CD8 Ab was added for the last 10 min of culture. Internalized CD8-MHC I was observed by fluorescence microscopy. In the right panel, surface CD8-MHC I was labeled with anti-CD8 Ab after the cells were incubated with (+) or without (-) Dox for 6 hr. After cultivation at 37°C for the indicated times, the expression of the remaining surface CD8-MHC I was examined by staining with PE-conjugated goat anti-mouse IgG. At each time point, the percentage of remaining CD8-MHC I was calculated relative to the value at 0 min. D, The ubiquitination status of surface CD8-MHC I on the transduced T-REx-MIR2 cells was examined as in Figure 1E.

Fig. S2. Analysis of CD8-MHC I-K335R. A, Alignment of protein sequences of the cytoplasmic region of wild type (Wt) and CD8 mutant chimeras. B, In the upper panel, surface molecules on T-REx-MIR2 cells were biotinylated and the cells were incubated with (+) or without (-) Dox for 6 hr. The biotinylated CD8 chimeras were sequentially purified with anti-FLAG (M2) Ab and streptavidin-agarose. Each sample was probed with FK2 Ab (Ubi) or M2 Ab (FLAG). In the lower panel, after cells were incubated with or without Dox for 6 hr, the expression level of the indicated CD8 chimeras was examined by flow cytometry. Data with (open histograms) and without (shaded histograms) Dox are shown. C, In the upper panel, surface CD8 chimeras were labeled with anti-CD8 Ab after the cells were incubated with (+) or without (-) Dox for 6 hr. At the end of the incubation, the downregulation of surface CD8 chimeras was examined by flow cytometry as in Fig. S1C. In the lower panel, the indicated T-REx-MIR2 cells were incubated with or without Dox for 6 hr and FITC-conjugated anti-CD8 Ab was added for the last 10 min of culture. Internalized CD8 chimeras were observed by fluorescence microscopy.

Fig. S3. Clathrin-dependent internalization of CD8-MHC I. A, Control siRNA (scrambled)

or siRNA for clathrin heavy chain (CHC) were transfected into T-REx–MIR2 cells. Whole-cell lysates from the indicated cells were analyzed with anti-CHC and anti-tubulin Abs. B, In the upper panel, after incubation with (open histograms) or without (shaded histograms) Dox for 6hr, the expression level of the surface CD8-MHC I was examined by flow cytometry. In the lower panel, the percentage of the expression level of CD8-MHC I on Dox-treated cells was calculated relative to its expression on Dox-untreated cells. Values represent mean and S.E. (n=3); *P<0.01, compared with the control siRNA. C, Internalization of CD8-MHC I was examined by fluorescence microscopy, as in Figure 1D. D, After knocking down CHC by siRNA, the ubiquitination status of CD8-MHC I on the T-REx-MIR2 cells was examined as in Fig. 1C. E, After T-REx-MIR2 cells were incubated with or without Dox for 6 hr, the protein complexes including CD8-MHC I were obtained by incubation with the M2 mAb (FLAG). The purified protein complexes were examined by western blot analysis with anti-EPS15 Ab, anti-EPS15R Ab or M2 Ab (FLAG) as indicated. The expression levels of EPS15 and EPS15R were examined in whole cell lysates (WCL). F, The effect of the indicated siRNAs was examined as in Figure 4D.

Fig. S4. Epsin 1-dependent internalization of endogenous MHC I. After T-REx-MIR2 cells were incubated with or without Dox for 6 hr in the presence of bafilomycin A1 (2 μM), whole cell lysates were incubated with anti-epsin1 Ab and purified protein complexes were probed with anti-MHC I Ab (HC10) or anti-epsin1 Ab. Whole cell lysates were probed with the indicated antibodies.

Fig. S5. Association of epsin 1 with polyubiquitinated but not with monoubiquitinated CD8-MHC I. After cells were incubated with or without Dox for 6 h in the presence of 2 μM of bafilomycin A1, protein complexes that included the indicated CD8–MHC I chimeras were purified by M2 Ab (FLAG). The purified protein complexes were examined with anti-epsin 1 antibody (Ab) or M2 Ab (FLAG).

Fig. S6. Epsin 1-dependent downregulation of CD8-MHC I. A, In the left panel, an EGFP expression vector and control- or epsin1-shRNA vector were cotransfected in T-REx-MIR2 cells by electroporation. After being incubated with (open histograms) or without (shaded histograms) Dox for 6hr, expression of surface CD8-MHC I was examined in the cells expressing high levels of EGFP. In the right panel, the percentage of the expression level of

CD8-MHC I on Dox-treated cells was calculated relative to its expression level on Dox-untreated cells. Values represent mean and S.E. (n=3); *P<0.01, compared with the control siRNA. B, After epsin 1 knock-down by shRNA, the ubiquitination status of CD8-MHC I in the presence or absence of Dox was examined as in Fig. 1C.

Fig. S7. Inhibition of CD8-MHC I downregulation by ΔUIM, a dominant negative epsin1. A, Each indicated V5-tagged epsin1 was coexpressed with CD8–MHC I 335K and MIR2 in HeLa cells. Protein complexes including CD8-MHC I 335K were purified with M2 Ab (FLAG) and analyzed by western blotting with anti-V5 Ab (epsin1), M2 Ab (FLAG) and FK2 Ab (Ubi). Expression of MIR2, endogenous epsin1 and V5-tagged epsin1 were examined in whole cell lysates with anti-Myc Ab, anti-epsin1 Ab and anti- V5 Ab, respectively. B, In the left panel, wild type (epsin1-wt) and dominant negative (ΔUIM) forms of epsin 1were coexpressed with EGFP in T-REx-MIR2 cells by electroporation. After incubation with (open histograms) or without (shaded histograms) Dox for 6hr, the expression of the surface CD8-MHC I was examined by flow cytometry in the cell population in which EGFP was highly expressed. In the right panel, the percentage of the CD8-MHC I expression level on EGFP-expressing Dox-treated cells was calculated relative to its level on EGFP-expressing Dox-untreated cells. Values represent mean and S.E. (n=3); *P<0.01, compared with the epsin1-Wt.

Fig. S8. Conjugation of endogenous MHC I with K11 and K63 linkages. A, Endogenous MHC I was purified with anti-MHC I Ab (HC10) from control BJAB cells (cont) or Dox-induced T-REx-MIR2 cells (+Dox). The purified MHC I was analyzed with the K63-linkgae-specific Ab (Apu3) and anti-MHC I (HC10). Bands corresponding to ubiquitinated and unmodified MHC I are indicated. B, Denatured endogenous MHC I was purified with anti-MHC I Ab (HC10) from control BJAB cells (cont) or Dox-induced T-REx-MIR2 cells (+Dox). The purified MHC I was analyzed with anti-MHC I Ab (HC70) or stained with Coomassie Blue. The peptide obtained from the band indicated by an asterisk shown in the +Dox lane was analyzed by LC-MS/MS as in Figure 5C.

Fig. S9. Inhibition of mixed polyubiquitin chain formation by Ubi-K11R. HeLa cells were cotransfected with CD8-MHC I 335K, MIR2 and variable amounts of EGFP-tagged Ubi-K11R (600, 200, 66.6, 0 ng). Left panel, the ubiquitinated form of CD8-MHC I-335K was purified with the FK2 Ab (Ubi) and blotted with the M2 Ab (FLAG) as in Figure 5B. Right panel, the

expression level of Ub4 from CD8-MHC I 335K is shown as the intensity relative to that of the Ub3 form in each sample. Values represent mean and S.E. (n=3); *P<0.01, compared with the sample transfected with 600 ng of EGFP-tagged Ubi-K11R-expressing vector (lane 1).

Fig. S10. Inhibition of a mixed polyubiquitin chain formation by Ubi-K11R. The data in Figure 5B were statistically analyzed. The amount of each ubiquitinated form of CD8-MHC I-335K obtained from each sample was expressed as the relative intensity to that of the Ubi-Wt-transfected sample. Values represent mean and S.E. (n=3); *P<0.01, compared with the wild type ubiquitin-expressing sample (Wt).

Fig. S11. Prevention of CD8-MHC I down-regulation by inhibition of K11/K63 linkages. Each indicated EGFP-tagged ubiquitin was expressed in T-REx-MIR2 cells by electroporation. Two days after introduction of each exogenous ubiquitin, the cells were incubated with Dox for 7 hr and the expression level of surface CD8-MHC I was examined in the cells in which EGFP was highly expressed as assessed by flow cytometry. The shaded histograms indicate without (-) Dox and the open histograms indicate with (+) Dox.

Fig. S12. Contribution of the K11 and K63 mixed linkage chain to the association between ubiquitinated MHC I and epsin1. Each indicated EGFP-tagged ubiquitin was coexpressed with CD8-MHC I 335K and MIR2 in HeLa cells. Protein complexes including CD8-MHC I 335K were purified with the M2 Ab (FLAG) and analyzed by western blot with anti-epsin1 Ab and M2 Ab. Expression of epsin1 and MIR2 was examined in whole cell lysates with anti-epsin1 Ab and anti-Myc Ab, respectively.

Fig. S13. Epsin1-UIM binds K63 but not K11 chains. To examine the association between polyubiquitin chains and epsin1-UIM, purified GST or GST-epsin1-UIM was prebound to glutathione beads and incubated with 6 μg of K63-polyubiquitin chains (K63-chain) or 20 μg of K11-polyubiquitin chains (K11-chain). Bound polyubiquitin chains were visualized by immunoblotting with the FK2 Ab (Ubi) (upper panel). The amount of GST or GST-epsin1-UIM was examined with anti-GST Ab (lower panel).

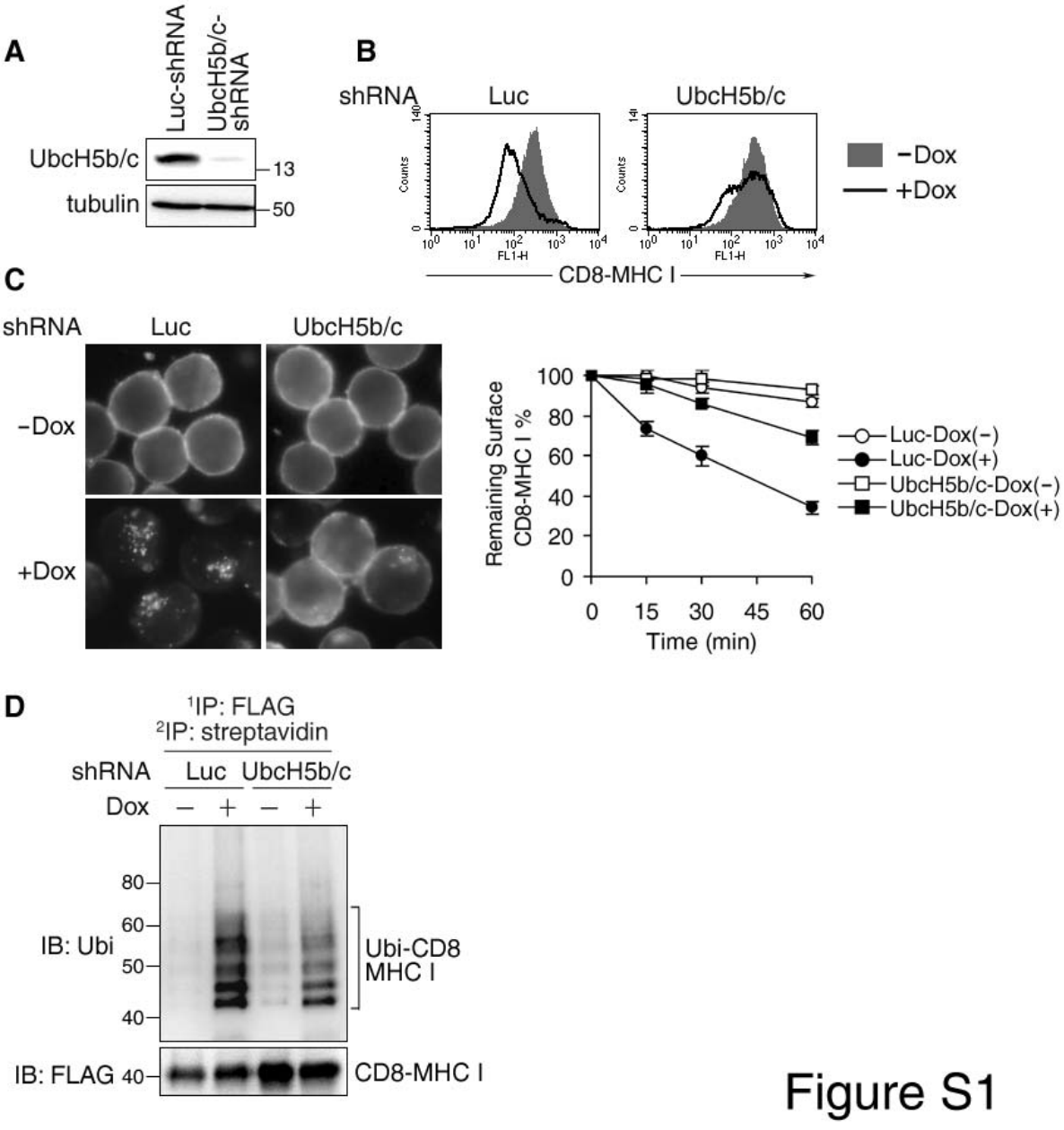

A
Luc-shRNA
UbcH5b/c-shRNA
UbcH5b/c
tubulin
13
50
B
shRNA
Luc
UbcH5b/c
Counts
FL1-H
−Dox
+Dox
CD8-MHC I
C
shRNA
Luc
UbcH5b/c
−Dox
+Dox
Remaining Surface CD8-MHC I %
Time (min)
Luc-Dox(−)
Luc-Dox(+)
UbcH5b/c-Dox(−)
UbcH5b/c-Dox(+)
D
[1]IP: FLAG
[2]IP: streptavidin
shRNA
Luc
UbcH5b/c
Dox
IB: Ubi
Ubi-CD8 MHC I
IB: FLAG
CD8-MHC I

Figure S1

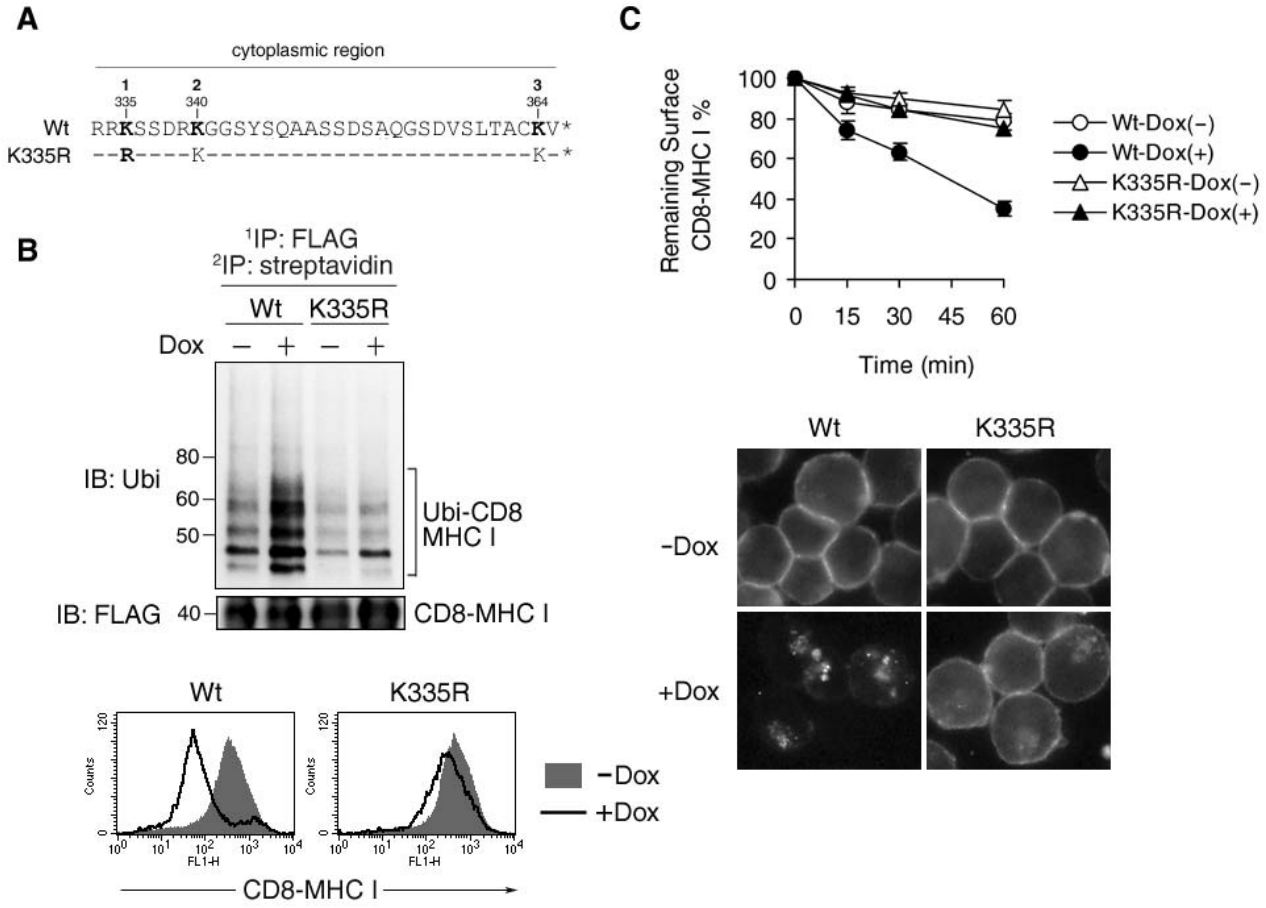

A
cytoplasmic region
1
335
2
340
3
364
Wt RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV*
K335R --R----K----------K-*
B
1IP: FLAG
2IP: streptavidin
Wt
K335R
Dox – + – +
IB: Ubi
80
60
50
Ubi-CD8
MHC I
IB: FLAG
40
CD8-MHC I
Wt
K335R
Counts
FL1-H
−Dox
+Dox
CD8-MHC I
C
Remaining Surface
CD8-MHC I %
100
80
60
40
20
0
0 15 30 45 60
Time (min)
Wt-Dox(−)
Wt-Dox(+)
K335R-Dox(−)
K335R-Dox(+)
Wt
K335R
-Dox
+Dox

Figure S2

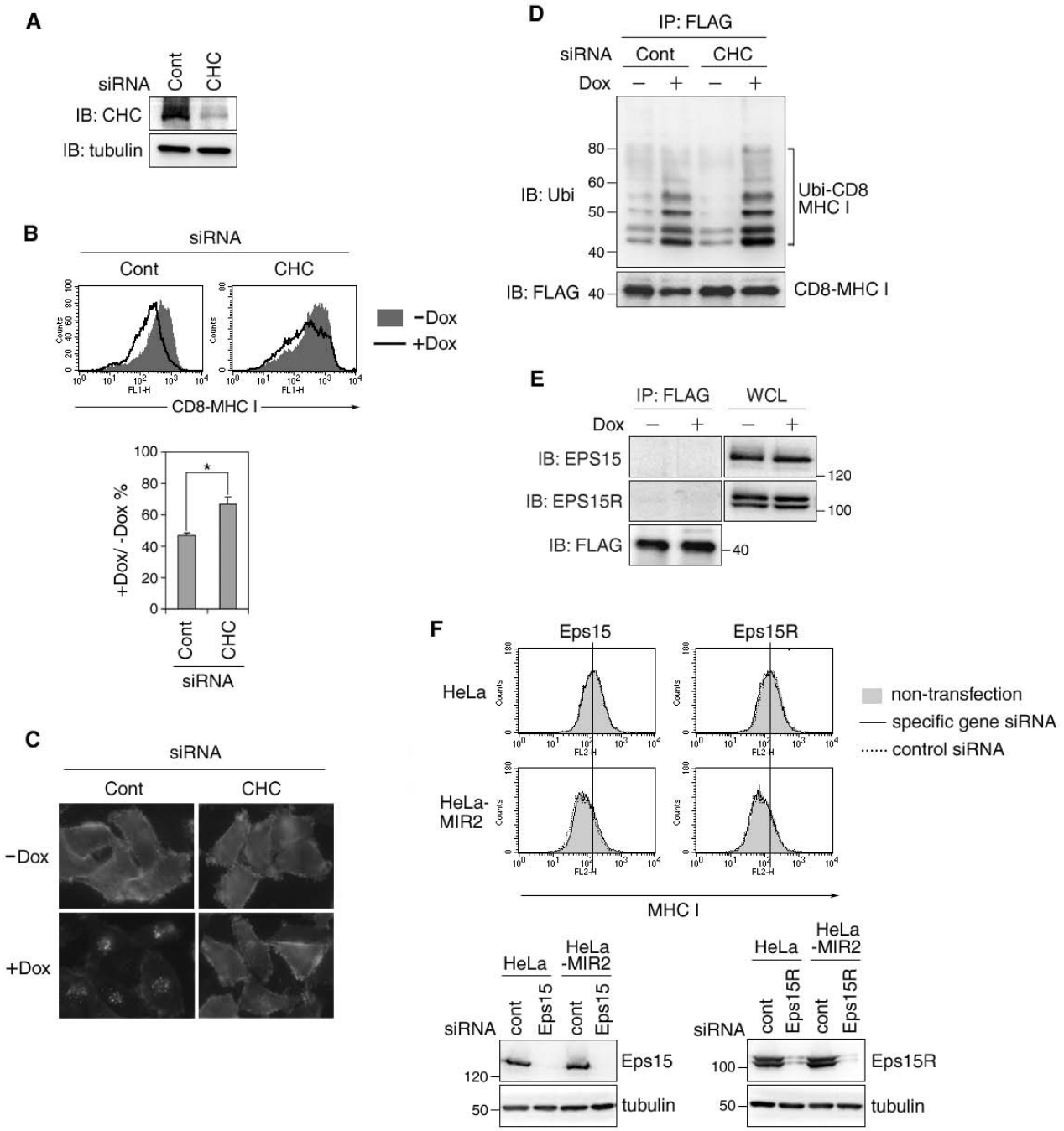

A
siRNA
Cont
CHC
IB: CHC
IB: tubulin
B
siRNA
Cont
CHC
−Dox
+Dox
CD8-MHC I
+Dox/ -Dox %
C
siRNA
Cont
CHC
−Dox
+Dox
D
IP: FLAG
siRNA
Cont
CHC
Dox
IB: Ubi
Ubi-CD8
MHC I
IB: FLAG
CD8-MHC I
E
IP: FLAG
WCL
Dox
IB: EPS15
IB: EPS15R
IB: FLAG
F
Eps15
Eps15R
HeLa
HeLa-MIR2
non-transfection
specific gene siRNA
control siRNA
MHC I
siRNA
cont
Eps15
Eps15R
tubulin

Figure S3

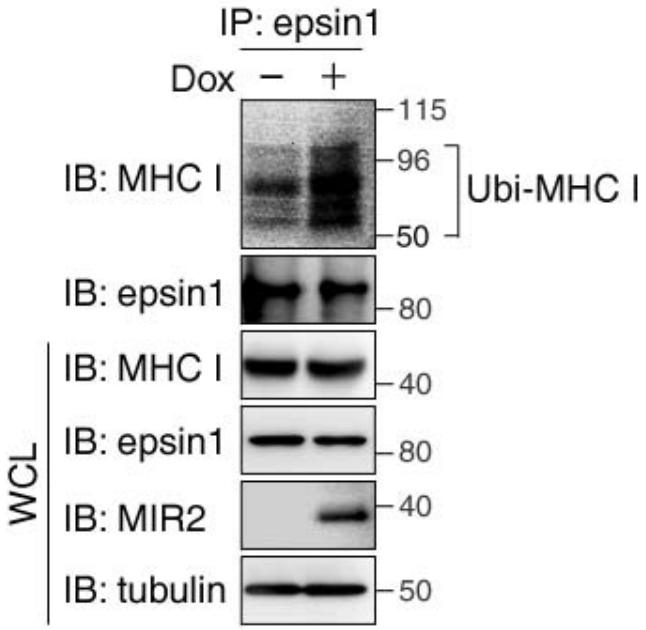

IP: epsin1
Dox − +
115
96
50
Ubi-MHC I
IB: MHC I
IB: epsin1
80
IB: MHC I
40
IB: epsin1
80
IB: MIR2
40
IB: tubulin
50
WCL

Figure S4

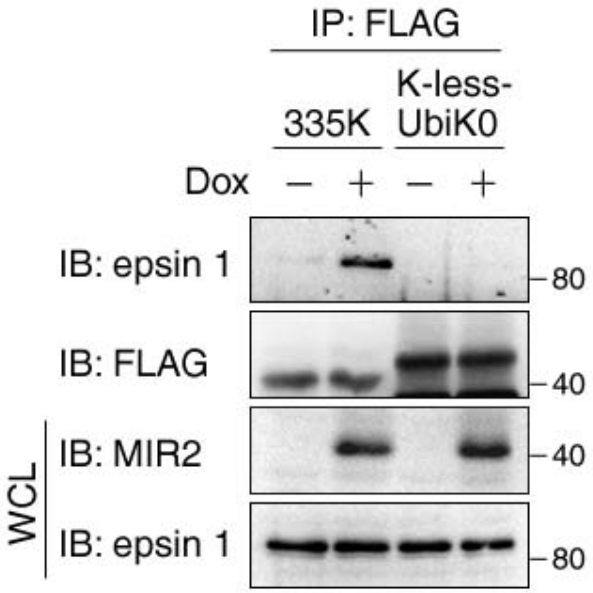

IP: FLAG
335K
K-less-UbiK0
Dox
–
+
–
+
IB: epsin 1
80
IB: FLAG
40
WCL
IB: MIR2
40
IB: epsin 1
80

Figure S5

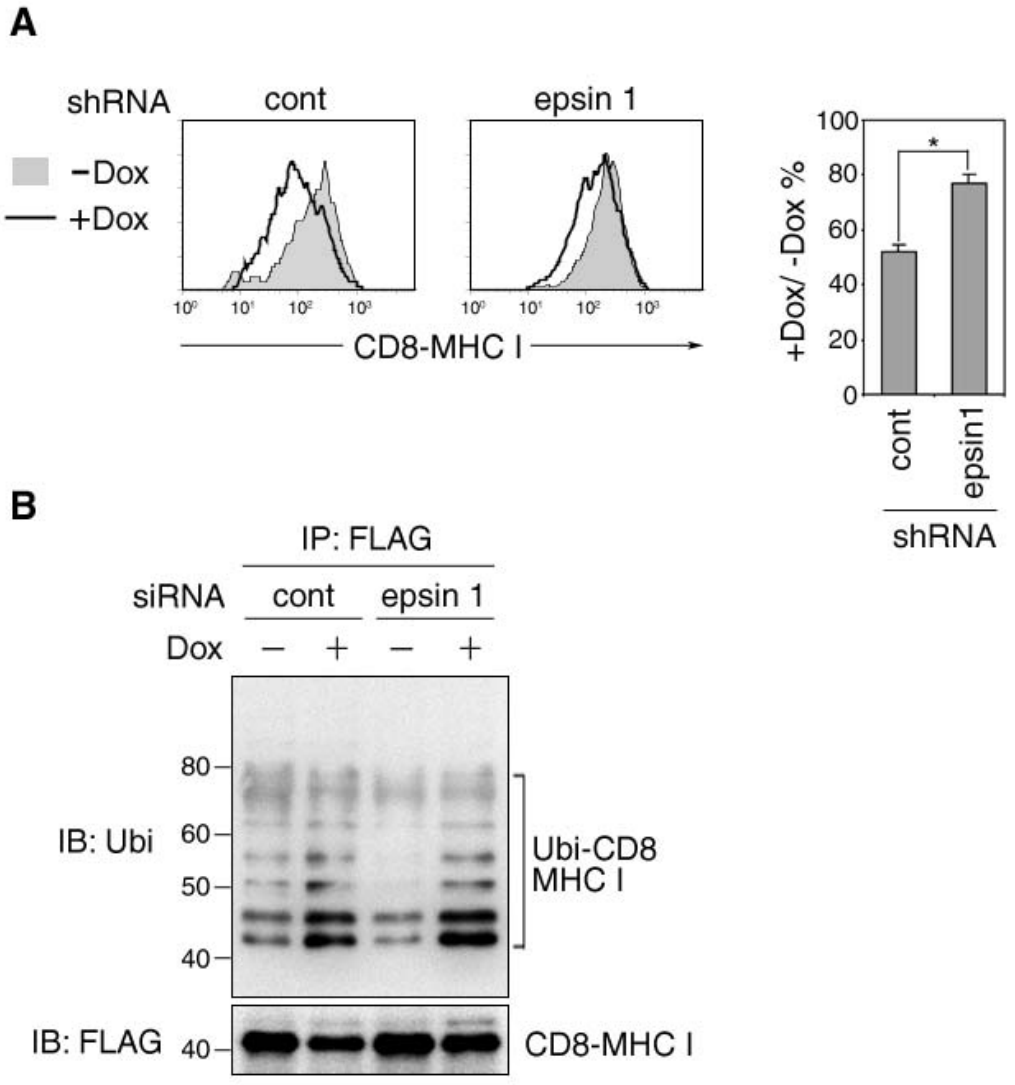

A
shRNA
cont
epsin 1
−Dox
+Dox
CD8-MHC I
+Dox/ -Dox %
100
80
60
40
20
0
*
cont
epsin1
shRNA
B
IP: FLAG
siRNA
cont
epsin 1
Dox
− + − +
80
60
50
40
IB: Ubi
Ubi-CD8
MHC I
IB: FLAG
40
CD8-MHC I

Figure S6

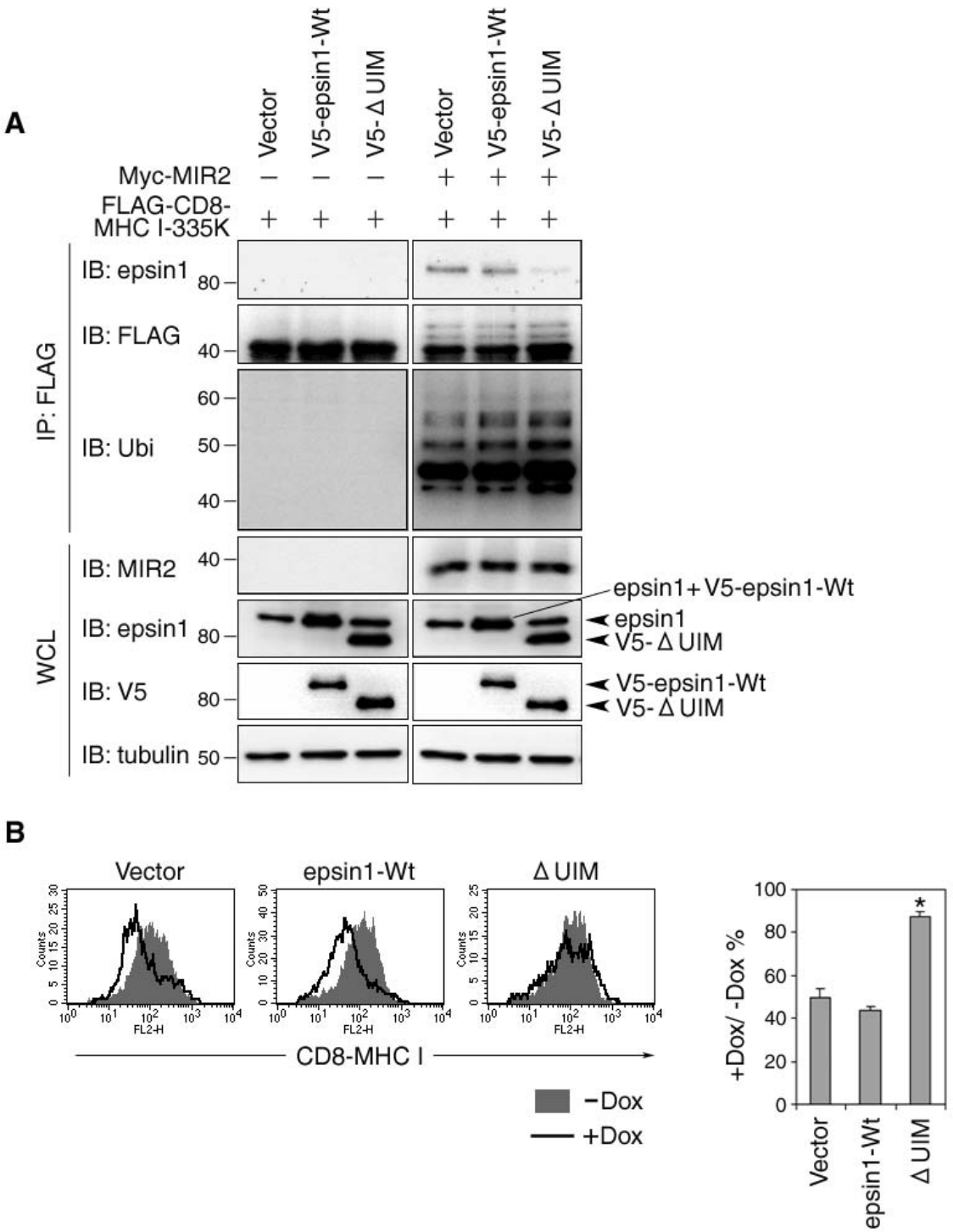

A
Vector
V5-epsin1-Wt
V5-Δ UIM
Vector
V5-epsin1-Wt
V5-Δ UIM
Myc-MIR2
FLAG-CD8-MHC I-335K
IP: FLAG
IB: epsin1
IB: FLAG
IB: Ubi
WCL
IB: MIR2
IB: epsin1
IB: V5
IB: tubulin
80
40
60
50
40
40
80
80
50
epsin1+V5-epsin1-Wt
epsin1
V5-Δ UIM
V5-epsin1-Wt
V5-Δ UIM
B
Vector
epsin1-Wt
Δ UIM
Counts
FL2-H
CD8-MHC I
−Dox
+Dox
+Dox/ -Dox %
Vector
epsin1-Wt
Δ UIM

Figure S7

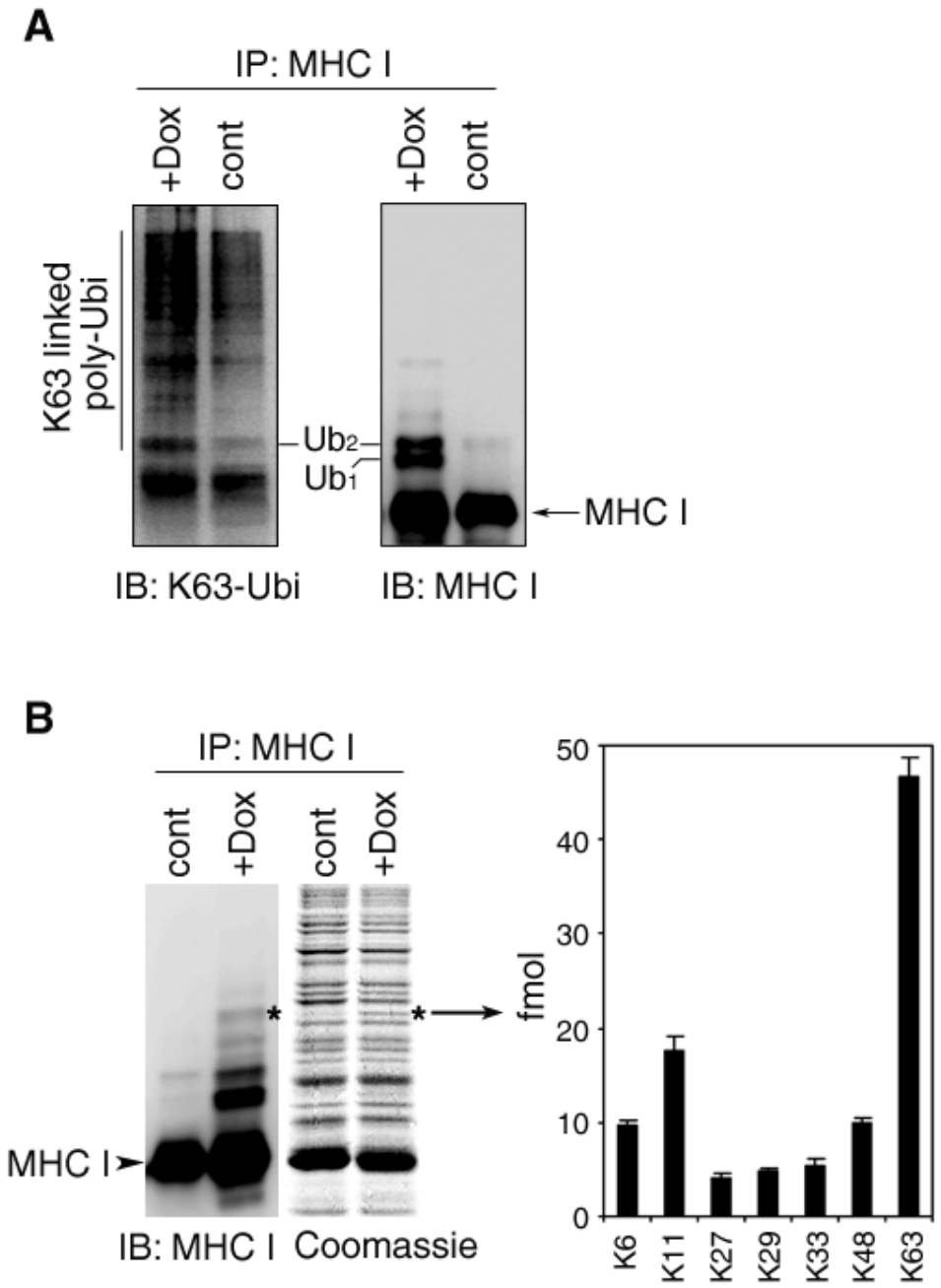
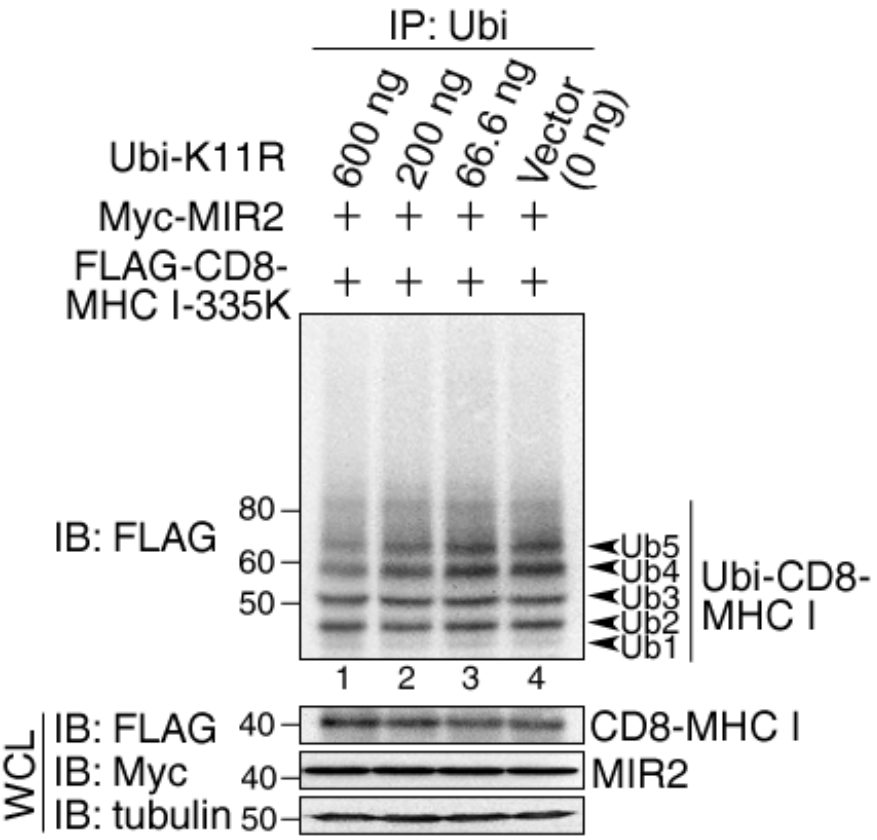

A
IP: MHC I
+Dox
cont
+Dox
cont
K63 linked poly-Ubi
Ub2
Ub1
MHC I
IB: K63-Ubi
IB: MHC I
B
IP: MHC I
cont
+Dox
cont
+Dox
MHC I
IB: MHC I
Coomassie
fmol
50
40
30
20
10
0
K6
K11
K27
K29
K33
K48
K63

Figure S8


IP: Ubi
600 ng
200 ng
66.6 ng
Vector (0 ng)
Ubi-K11R
Myc-MIR2
FLAG-CD8-MHC I-335K
IB: FLAG
80
60
50
Ub5
Ub4
Ub3
Ub2
Ub1
Ubi-CD8-MHC I
1 2 3 4
WCL
IB: FLAG
IB: Myc
IB: tubulin
40
40
50
CD8-MHC I
MIR2

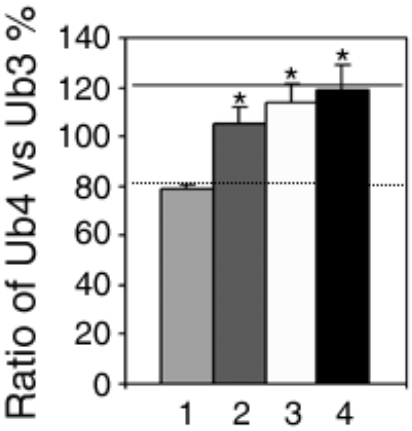

Ratio of Ub4 vs Ub3 %
140
120
100
80
60
40
20
0
1 2 3 4

Figure S9

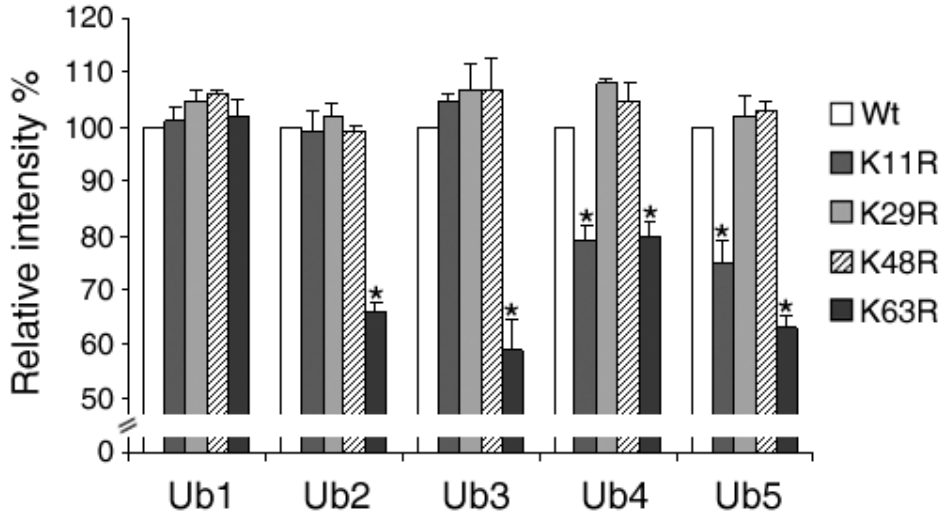

Relative intensity %
120
110
100
90
80
70
60
50
0
Ub1
Ub2
Ub3
Ub4
Ub5
Wt
K11R
K29R
K48R
K63R

Figure S10

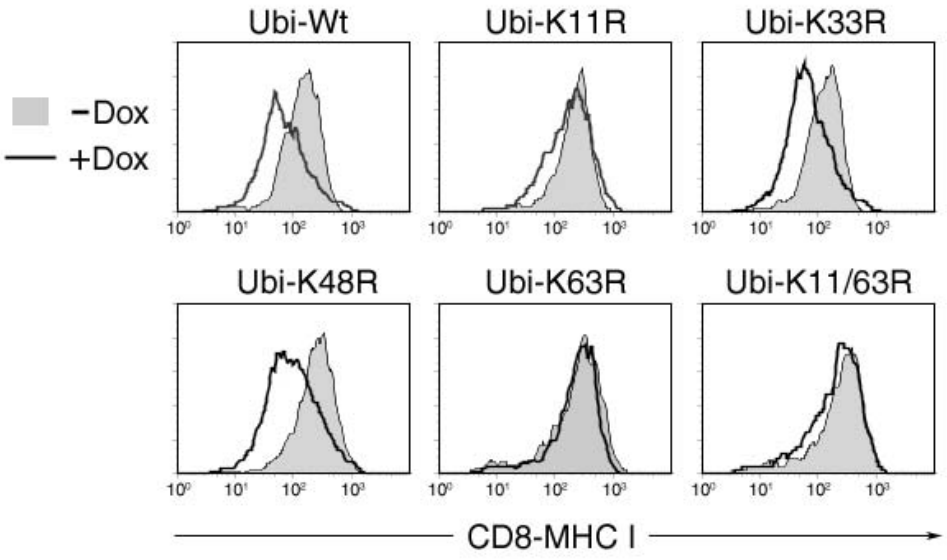

Ubi-Wt
Ubi-K11R
Ubi-K33R
−Dox
+Dox
Ubi-K48R
Ubi-K63R
Ubi-K11/63R
CD8-MHC I

Figure S11

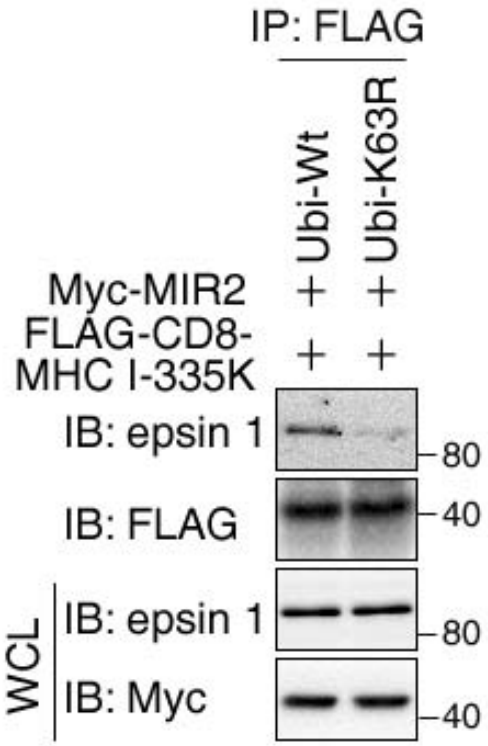

IP: FLAG
+ Ubi-Wt
+ Ubi-K63R
Myc-MIR2
FLAG-CD8-
MHC I-335K
+ +
+ +
IB: epsin 1
80
IB: FLAG
40
WCL
IB: epsin 1
80
IB: Myc
40

Figure S12

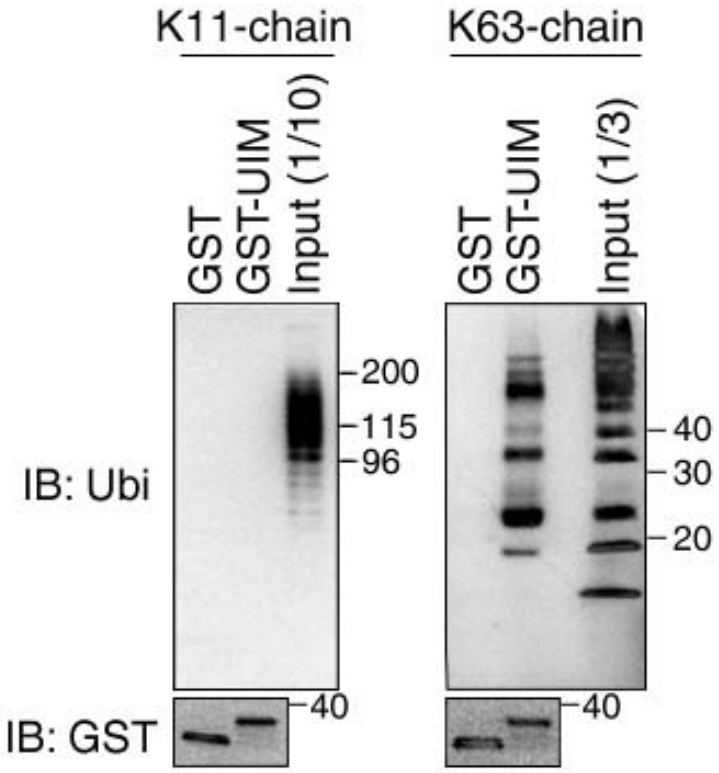

K11-chain
K63-chain
GST
GST-UIM
Input (1/10)
GST
GST-UIM
Input (1/3)
IB: Ubi
200
115
96
40
30
20
IB: GST
40
40

Figure S13