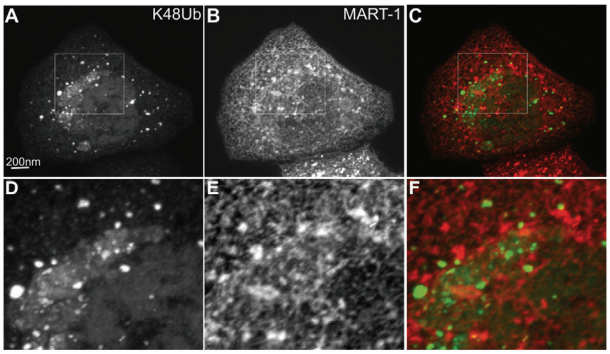
Supplementary Materials and Methods

Yeast strains and growth conditions. Supplementary Table 1 shows the additional yeast strains used for Supplementary Figures.

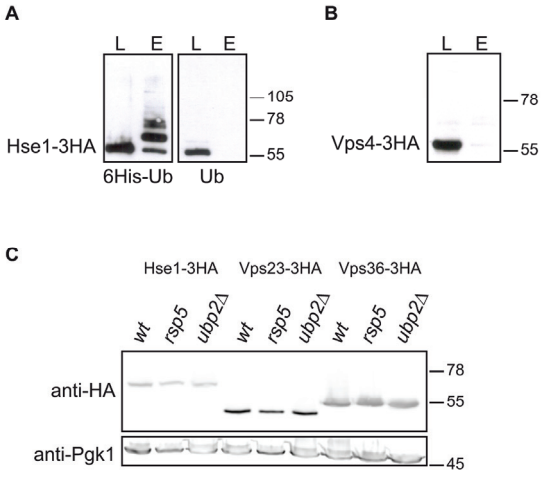
Plasmid construction. Plasmids pRS246-Ub (*CUP1*, 2μ, *URA3*) and m886 (*ADH1*, RGS-His_8-Ub, *LEU2*) were used in Supplementary Figure 2A and 4A, respectively.

Supplementary figure legends



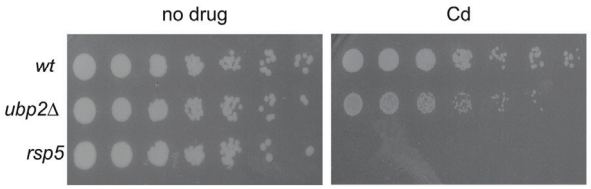
Supplementary Figure 1

Figure S1. The anti-K48Ub antibody does not label MART-1 positive endosomes. HeLa cells transfected with MART-1 were analyzed by IF microscopy with antibodies against MART-1 (B, E) and K48Ub chains (A, D). Overlays are shown in (C) and (F). (D), (E) and (F) correspond to a 1.8 X magnification of the boxed areas in (A), (B) and (C), respectively.



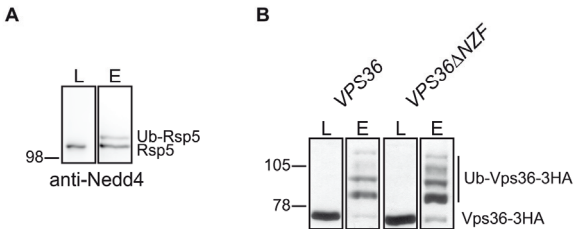
Supplementary Figure 2

Figure S2. ESCRT ubiquitylation is UBD-dependent. (A) Total protein extracts prepared from cells producing chromosome-encoded Hse1-3xHA and overproducing 6His-Ub or untagged Ub were subjected to nickel-column purification. Lysates (L) and eluates (E) were analyzed by SDS-PAGE followed by immunoblotting with anti-HA antibody. Ubiquitylated forms of Hse1-3HA are detected in the eluate of cells producing 6His-Ub, but are absent from the eluate of cells producing untagged Ub. (B) The same experiment as in (A) was performed for wt cells expressing endogenous Vps4-3xHA and overproducing 6His-Ub. No ubiquitylated form of Vps4-3xHA could be detected in the nickel column eluate. (C) **ESCRT ubiquitylation does not regulate protein steady-state levels.** Total protein extracts of wt, *rsp5* or *ubp2Δ* cells producing Hse1-3xHA, Vps23-3xHA or Vps36-3xHA were subjected to SDS-PAGE followed by immunoblotting with an anti-HA antibody. The anti-Pgk1 blot serves as a loading control.



Supplementary Figure 3

Figure S3. *ubp2Δ* cells display cadmium sensitivity. Serial dilutions of liquid cultures of 23344c (wt), 27038b (*npi1/rsp5*) and VA029 (*ubp2Δ*) cells were grown on solid YNB medium plus 2% glucose supplemented with uracil, in the presence or absence of 10 μM cadmium.



Supplementary Figure 4

Figure S4. (A) The E3 ligase Rsp5 is ubiquitylated. Total protein extracts prepared from wt cells producing RGS-His_8-Ub under the control of the *ADH1* promoter were subjected to nickel column purification. Lysate (L) and eluate (E) were immunoblotted with an anti-Nedd4 antibody known to recognize Rsp5. The arrow indicates the monoubiquitylated form of Rsp5, which was abundant in the eluate. **(B) Vps36$^{\Delta NZF}$ is not affected in its ubiquitylation**. We attempted to test one aspect of the coupled ubiquitylation hypothesis with Vps36, using a mutant that abrogates its interaction *in vitro* with Ub without affecting cargo MVB sorting *in vivo* (Shields et al., 2009). Total protein extracts prepared from wt cells expressing Vps36-3xHA and Vps36$^{\Delta NZF}$-3xHA and overproducing 6His-Ub were subjected to Nickel column purification. Lysates (L) and eluates (E) were analyzed with an anti-HA antibody. The ubiquitylation pattern of Vps36 is not altered when the Ub-binding properties of the NZF domain are inhibited. Hence, either Vps36 ubiquitylation does not follow the coupled-ubiquitylation mechanism, or Vps36$^{\Delta NZF}$still binds Ub *in vivo*.

Field Code Changed

Supplementary Table 1. The S. cerevisiae strains used

Strain	***Genotype***	***Source***
RHT517 (*VPS4-3xHA*)	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 VPS4-3xHA::HIS3MX6*	*This study*
RHT518 (*VPS36-3xHA*)	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel VPS36 ::TRP1 ::vps36Δ :HIS3 ::3xHA-KanMX4*	*This study*
RHT519 (*VPS36$^{\Delta NZF}$3xHA*)	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel VPS36$^{\Delta NZF}$ ::TRP1 ::vps36Δ :HIS3 ::3xHA-KanMX4*	*This study*