SUPPLEMENTAL MATERIAL

for

AMSH INTERACTS WITH ESCRT-0 TO REGULATE THE STABILITY AND TRAFFICKING OF CXCR4

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Running head: AMSH controls CXCR4 stability
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SUPPLEMENTAL METHODS

High-density peptide arrays. High-density peptide arrays were synthesized by semi-automated SPOT synthesis on an Intavis MultiSPOT as previously described [56, 64]. The SPOTs membrane was first blocked with 5% milk in TBS-T (0.1M TrisHCl (pH 7.4), 150mM NaCl and 0.1% Tween 20) overnight at 4°C. GST fusion proteins were incubated with the SPOTs membrane containing 1mM DTT for 1.5 hours at room temperature and then washed with TBS-T. GST fusion proteins were detected by immunoblot using an anti-GST antibody (Upstate).

Ubiquitination Assays. For Grb2 ubiquitination HeLa cells were seeded onto 100-mm dishes and allowed to adhere overnight. Cells were co-transfected with HA-tagged ubiquitin and either vector, AMSH or AMSH mutants using Lipofectamine 2000. 24 hours post-transfection, cells were washed with ice-cold PBS and lysed on ice in 900μl of immunoprecipitation buffer (as above). Equal amounts of lysate were incubated with 3μg mouse anti-Grb2 (Cell Signaling Technology, Inc.) overnight at 4°C. Protein G beads (Roche) were added to lysates and further incubated for 3 hours at 4°C. Washed beads were eluted in SDS Sample Buffer, subjected to 10% SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted with mouse anti-HA antibody (Covance) to detect ubiquitinated protein. For Hrs ubiquitination, HeLa cells were transiently co-transfected with HA-ubiquitin and control siRNA or siRNA directed against AMSH using Lipofectamine 2000. Hrs was immunoprecipitated and analyzed for ubiquitination as described above.

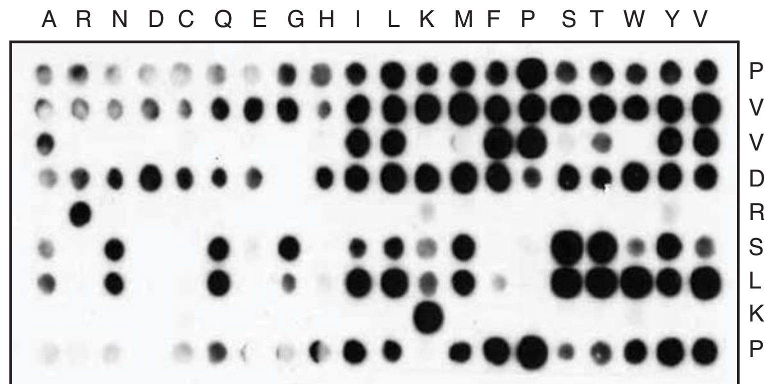
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. An RXXK motif of AMSH is essential for binding of STAM1 SH3 domain recognition.
A, The residues within the extended RXXK motif of AMSH required for the interaction with the SH3 domain of STAM1 were determined using a SPOTs peptide array. Each amino acid of the AMSH binding motif (vertical axis) was substituted with each natural amino acid (horizontal axis). Membrane was probed with GST-STAM1-SH3 fusion protein was detected by immunoblot with anti-GST antibody. *B*, An intact RXXK motif is required for AMSH binding to a subset of SH3 domains. HeLa cells expressing vector or myc tagged wild type AMSH or RXXK mutants were precipitated with GST-SH3 domains from STAM1, STAM2, Grb2 and Gads. Samples were separated by 10% SDS-PAGE and immunoblotted for myc.

Figure S2. Expression of catalytically inactive AMSH does not enhance Grb2 ubiquitination under steady-state conditions.
AMSH dose not affect ubiquitination or stability of Grb2. HeLa cells co-transfected with HA-ubiquitin and vector, wild type AMSH, D348A or AXXA-D348A were immunoprecipitated against endogenous Grb2 by and Grb2 *A,* ubiquitination status was assessed by immunoblot against HA. *B,* Whole cell lysates were analyzed for Grb2 protein abundance by immunoblot.

Figure S3. AMSH depletion results in the hyperubiquitination of ESCRT-0.
HeLa cells transiently co-transfected with HA-ubiquitin and control siRNA (*siControl*) or siRNA directed against human AMSH (*siAMSH*) were immunoprecipitated against endogenous Hrs. *A,* Ubiquitination status of the ESCRT-0 complex was assessed by immunoblotting against HA. Hrs and STAM1 IP controls are shown. *B,* Corresponding whole cell lysates were analyzed for endogenous protein abundance by immunoblot against Hrs, STAM1, AMSH and HA.

Figure S1



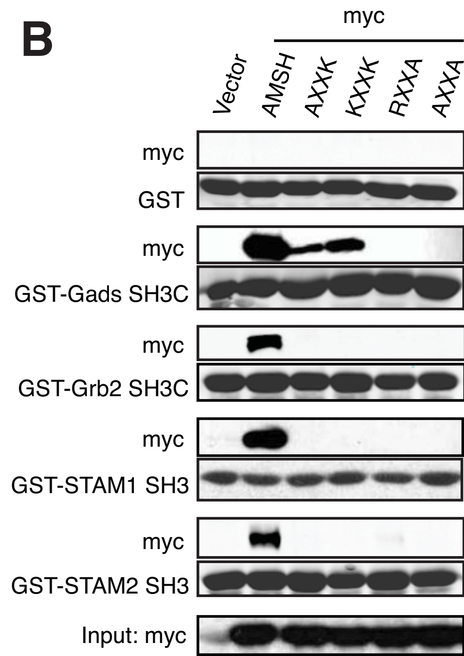


Figure S2

A

AMSH Vector AMSH D348A AXXA-D348A

- + + + + HA-ubiquitin

50 kDa

37 kDa

25 kDa

IB: HA

IB: Grb2

IB: myc-AMSH

B

AMSH Vector AMSH D348A AXXA-D348A

- + + + + HA-ubiquitin

IB: Grb2

IB: AMSH

IB: Actin

Figure S3