Supporting Information

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SI Materials and Methods

Materials. We obtained 25-hydroxycholesterol (25-HC) from Steraloids, Inc. (Wilton, NH), MG-132 from Boston Biochem (Cambridge, MA) and Peptides International (Osaka, Japan), digitonin from Calbiochem, and horseradish peroxidase-conjugated donkey antimouse and antirabbit from Jackson ImmunoResearch Laboratories (West Grove, PA). Other reagents were obtained from previously described sources (1). Lipoprotein-deficient serum (LPDS, $d > 1.215$ g/mL) was prepared from newborn calf serum by ultracentrifugation as described (2).

Expression Plasmids. The following plasmids have been described in the indicated reference: pCMV-HMG-Red-T7 encodes full-length hamster reductase followed by three copies of a T7 epitope tag under transcriptional control of the cytomegalovirus (CMV) promoter (3); pCMV-Insig-1-T7 encoding human Insig-1 fused to three copies of a T7 epitope and pCMV-Insig-2-T7, encoding human Insig-2 followed by three T7 epitopes (4); pCMV-gp78-Myc encodes human gp78 followed by five copies of a c-Myc epitope (5); pCMV-Hrd1-Myc encoding human Hrd1 fused to three copies of the c-Myc epitope and pCMV-Trc8-Myc, which encodes human Trc8, followed by five copies of the c-Myc epitope (6).

Primary Antibodies. Antibodies used for immunoblot analysis include: IgG-9E10, mouse monoclonal antibody against c-Myc purified from the culture medium of hybridoma clone 9E10 (American Type Culture Collection); monoclonal anti-T7 Tag IgG (Novagen); IgG-A9, mouse monoclonal antibody against the catalytic domain of hamster reductase (7); IgG-P4D1, mouse monoclonal antibody against bovine ubiquitin (Santa Cruz Biotechnology); rabbit polyclonal anti-Ubxd8 IgG (Abnova); IgG-740F, rabbit polyclonal antibody against human gp78 (4); IgG-R139, a rabbit polyclonal antibody against hamster Scap (8); and IgG-3D10, a mouse monoclonal antibody against human Trc8 (Abnova). Rabbit polyclonal antibody against human Trc8 (designated IgG-556) was generated by immunizing animals with a recombinant protein consisting of glutathione S-transferase fused to amino acids 545–654 of human Trc8. IgG-17H1 is a mouse monoclonal antibody against human Insig-1 that was generated by immunizing animals with recombinant, His-tagged human Insig-1 (amino acids 1–277) produced in Sf9 cells.

RNA Interference. Duplexes of small interfering RNAs (siRNAs) were designed and synthesized by Dharmacon/Thermo Fisher Scientific. SV-589 cells were set up on day 0 as indicated in the legend to Fig. 2. On day 1, the cells were incubated with 200–600 pmol of siRNA duplexes (GFP, CAGCCACAACGTCTA TATC; Trc8A, CAAUGAAACUCCAGAGGAA; Trc8B, GCUAAGACCAGAAGAGAGA; Trc8C, GGGCAUGAGUGCUGUAAUU; Trc8D, GGGAAUUGAACGAAGAUGA; Trc8E, GAGUUGUAAUGUUUGGAAA; gp78A, CATGCAGAATGTCTCTTAA; gp78B, TGCACACCTTGGCTTTCAT; gp78C, GTTTGGCCCTCTTCGAGTG; gp78D, ATTGCACACCTTGGCTTTCA; Insig-1, CCCACAAATTTAAGAGAGA; Insig-2, CTAAAGTGGATTTCGATAA and TGGCAATGTACGAATGTAA) mixed with Lipofectamine™ RNAiMAX (Invitrogen) diluted in OPTI-MEM I reduced serum medium (Gibco) according to the manufacture's procedure. Following incubation for 6 h at 37 °C, FCS was added to a final concentration of 10%. On day 2 or 3, the RNAi procedure was repeated as described above, except that the cells were incubated for 16 h at 37 °C in medium B containing 10% LPDS, 50 μM compactin and 50 μM mevalonate. The cells were subsequently treated and harvested for analysis as described in the figure legend to Fig. 2.

Real-Time PCR. Total RNA was isolated using RNA STAT60 (Tel-Test) according to manufacture's procedures. Knockdown efficiency was verified by quantitative real-time PCR using each specific primer for human Trc8, gp78, and the control mRNA GAPDH.

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Trc8-Myc (WT)	+		-		-		+		-		-	
Trc8-Myc (1-546)	-		+		-		-		+		-	
Trc8-Myc (545-665)	-		-		+		-		-		+	
Insig1-T7	+		+		+		-		-		-	
Insig2-T7	-		-		-		+		+		+	
25-HC & Mev.	-	+	-	+	-	+	-	+	-	+	-	+
	1	2	3	4	5	6	7	8	9	10	11	12

TRC8

TRC8 (1-546)

TRC8 (545-665)

Pellet

Supernatant

Insig-1 or Insig-2 Pellet

Insig-1 or Insig-2 Supernatant

Fig. S1. Membrane domain of Trc8 mediates association with Insig-1 and Insig-2. CHO-7 cells were set up for experiments at 5×10^5 cells per 60 mm dish in medium A containing 5% LPDS. On day 1, cells were transfected with 100 ng pCMV-Insig-1-Myc or 500 ng pCMV-Insig-2-Myc together with 30 ng pCMV-Trc8-Myc, 30 ng pCMV-Trc8-Myc (1–546), or 300 ng pCMV-Trc8 (545–665) as indicated. Following incubation for 6 h at 37 °C, cells were depleted of sterols through incubation for 16 h in medium A containing 50 μM compactin and 50 μM mevalonate. Cells were then switched to medium A containing 5% LPDS, 10 μM compactin, and 10 μM MG-132 in the absence or presence of 1 μg/mL 25-HC plus 10 mM mevalonate. Following incubation for 5 h at 37 °C, cells were harvested, lysed, and subjected to immunoprecipitation with antiMyc. Pellet and supernatant fractions were subjected to immunoblot analysis with anti-T7 IgG (against Insigs), and IgG-9E10 (against Trc8).