The ubiquitin ligase HACE1 regulates Golgi membrane dynamics during the cell cycle

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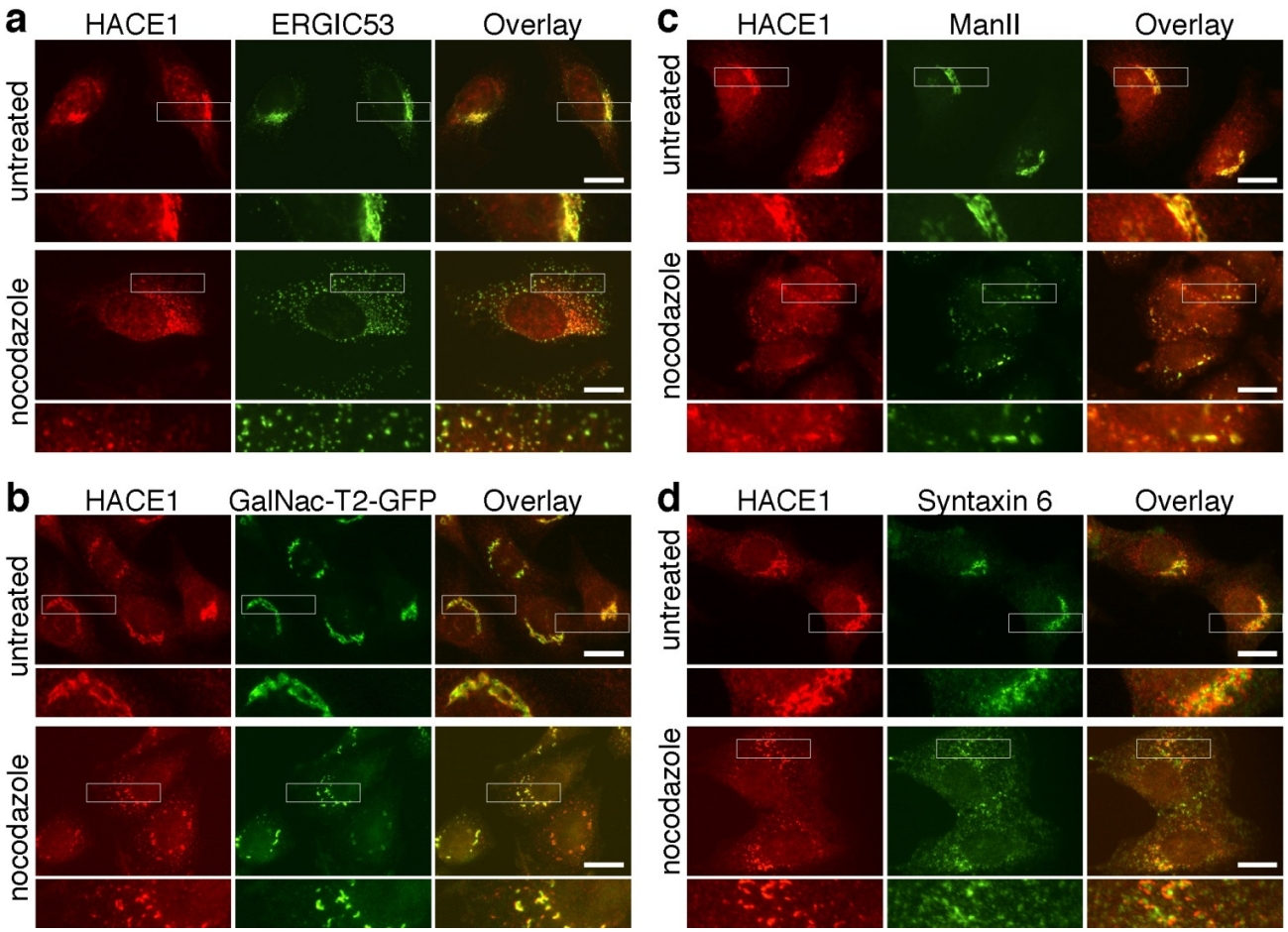
Supplementary Figures



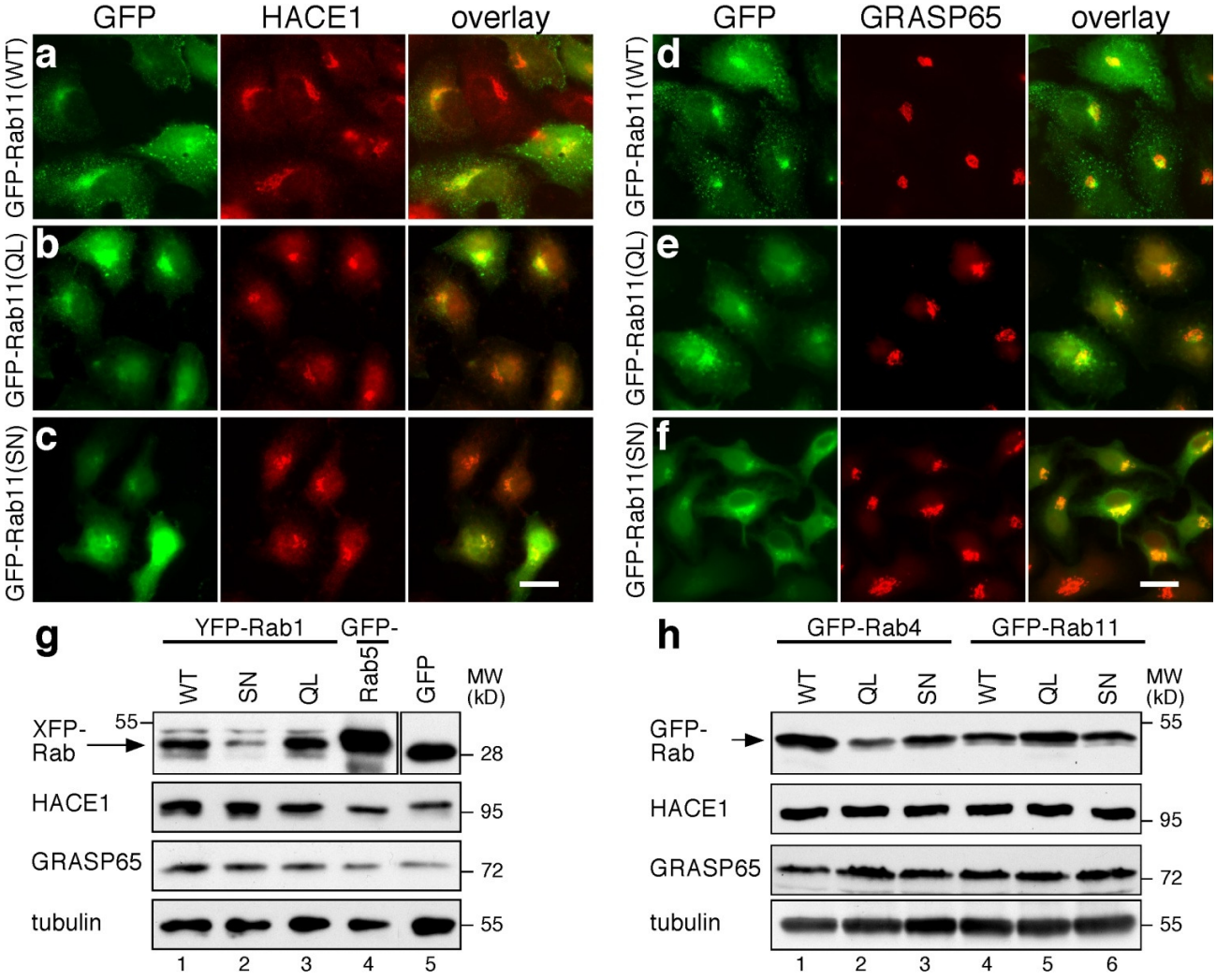
Supplementary Figure S1. HACE1 co-localizes with *cis*-Golgi markers.

(a) Co-localization of HACE1 with ERGIC53, an endoplasmic reticulum-Golgi intermediate compartment (ERGIC) marker. Untreated (upper panels) and nocodazole-treated (lower panels) NRK cells were immunostained with an affinity-purified HACE1 polyclonal antibody (left panels, in red) and a monoclonal ERGIC53 antibody (middle panel, in green). The overlay is shown on the right. Lower panels are enlarged images of the boxed regions.

(b) Co-localization of HACE1 with GFP-tagged N-acetylgalactosaminyltransferase 2 (GalNAc-T2-EGFP). Same as in (**a**) but with the EGFP signal from stably expressed GalNac-T2-EGFP.

(c) Co-localization of HACE1 with mannosidase II (ManII), an enzyme localized in the *medial*-Golgi.

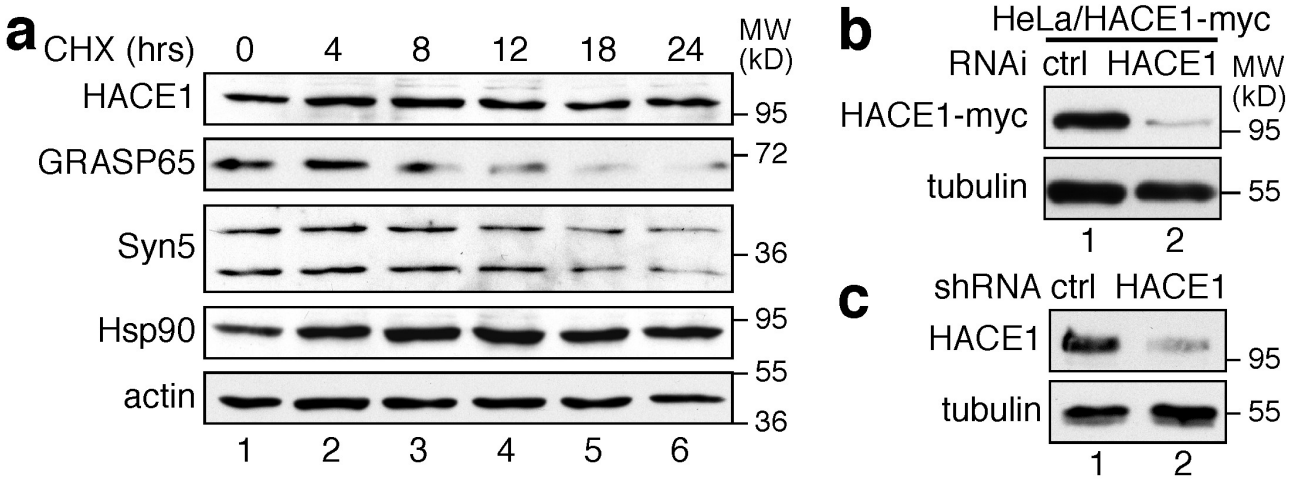
(d) Co-localization of HACE1 with syntaxin 6, a *trans*-Golgi-localized SNARE protein. Scale bars in all panels: 10 µm.



Supplementary Figure S2. Expression of Rab5 and Rab11 does affect HACE1 Golgi localization and Golgi organization.

(a-f) HeLa cells expressing the indicated GFP-Rab11 wild-type and mutant constructs were immunostained for HACE1 (**a-c**) or GRASP65 (**d-f**). Scale bars in all panels: 20 µm.

(g-h) Western blots of cells described in Fig. 3 and Fig. S2**a-f**. Cells were lysed in SDS buffer followed by Western blot to detect indicated proteins. The split of the GFP blot (lane 5 in **g**) from the same gel was due to the different sizes of GFP and GFP-Rab.



Supplementary Figure S3. HACE1 depletion in cells.

(a) HACE1 protein levels do not significantly decrease 24 h after cycloheximide (CHX) treatment. NRK cells were incubated in growth media with 20 µg/ml CHX for the indicated time periods (0-24 hours). Cells were lysed in SDS buffer and analyzed for the indicated proteins by Western blot. Note that HACE1 levels remained relatively unchanged 24 h after treatment, while GRASP65 levels reduced.

(b) Exogenous HACE1 is readily depleted by siRNA treatment. HeLa cells were treated with HACE1 or control siRNA oligos for 3 days followed by transient transfection with a HACE1-myc cDNA. Cells were lysed, and HACE1 and tubulin were detected by Western blot. Note that HACE1-myc expression was reduced in the presence of HACE1 siRNA (lane 2).

(c) Depletion of HACE1 by lentiviral infection. HeLa cells were infected by control or HACE1 shRNA lentiviral particles followed by Western blot for HACE1 and tubulin.

Supplementary Methods

HACE1 cloning

The ILTSLAEVA peptide identified by mass spectrometry was found in a HACE1 EST clone (NCBI accession no. AI130909). This EST clone was sequenced, and the translated sequence contained three other peptides identified by mass spectrometry: VLEHLSQQE, QNEDLR and DTAQILLLR. The full-length HACE1 cDNA clone was isolated by screening the SUPERSCRIPT HeLa cDNA library (Life Technologies) using a primer derived from the 5' end of this EST sequence. The *HACE1* coding sequence was amplified by PCR using primers that contained EcoRI and BamHI sites at the 5'- or 3'-ends and inserted into pcDNA3.1/myc-His(-)A (Invitrogen) for mammalian expression or and a EcoRI and XhoI fragment into pGEX-6p-1 (GE Healthcare) for expression in *E. coli*. The C876A mutation was introduced using the QuikChange mutagenesis kit (Stratagene). The HACE1 ΔC construct was generated by inserting an EcoRI/BamHI fragment (aa 1-553) into pCDNA3.1 or an EcoRI/SalI fragment (aa 1-548) into pGEX-6p-1 for antigen preparation. All of the constructs were confirmed by DNA sequencing.

Preparation of HACE1 fusion proteins

GST-HACE1 proteins were expressed in BL21 (DE3) Gold bacteria and purified on glutathione Sepharose beads (GE Healthcare). For the HACE1-Rab interaction assay, the HACE1 protein on glutathione Sepharose beads was cleaved from the tag by PreScission protease, and the supernatant was further incubated with glutathione beads to remove GST-tagged proteins. Untagged HACE1 protein in the supernatant was used to assay binding to Rab proteins.

Pull-down assay for HACE1-Rab interactions

The interactions between HACE1 and Rab were determined by a pull-down assay following a previously established procedure[28]. For each reaction, GST-tagged Rabs were expressed in BL21 (DE3) bacteria with 125 ml of LB media. The GST-Rabs were immobilized on 25 μl of glutathione beads (bed volume) in a 1.5 ml Eppendorf tube. The beads were washed with 1 ml of NE buffer containing 10 μM GDP or GTPγS and incubated two times with NE buffer containing 1 mM GDP or GTPγS at RT for 30 min. The beads were then washed with 1 ml of NS buffer containing 10 μM GDP or GTPγS and incubated with NS buffer containing 1 mM GDP or GTPγS at room temperature for 30 min.

Five micrograms of HACE1 protein (without a tag) prepared above was incubated with the GDP or GTPγS charged beads in 150 µl of cytosol buffer (20 mM Hepes-KOH pH 7.4, 250 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and protease inhibitors) containing 1 mM GDP or GTPγS at 4ºC for 2 h. The beads were washed three times with cytosol buffer containing 10 µM GDP or GTPγS at 4ºC for 10 min. To elute the bound proteins, the beads were incubated with 12.5 µl of elution buffer (20 mM Hepes-KOH, pH 7.4, 1.5 M NaCl, 20 mM EDTA, 1 mM DTT, 1% Triton X-100 and protease inhibitor cocktail) for 10 min at room temperature. Elution was repeated three times, and the elutions were pooled. Equal volumes were analyzed by Western blot.

To test the interaction between endogenous HACE1 and Rabs, cell lysates were prepared as previously described[12]. Briefly, cells were grown in 10 X 15 cm dishes to 80% confluency, washed with PBS three times, and harvested with 3 ml of homogenization buffer (20 mM Hepes-KOH, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and protease inhibitor cocktail). Cells were cracked with a ball bearing homogenizer to reach 75-80% breakage determined by Trypan blue exclusion. The homogenate was centrifuged for 10 min at 1000 *g* and 4˚C. The post-nuclear supernatant (PNS) was supplemented with 1% Triton X-100 and rotated at 4˚C for 30 min. Solubilized proteins were exchanged into cytosol buffer (20 mM Hepes-KOH pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and protease inhibitors) using the BIO-RAD Econo-Pac® 10DG column following the manufacturer's instructions. Five milligrams of cell lysate was incubated with GDP or GTPγS charged beads in 800 µl of cytosol buffer containing 1 mM GDP or GTPγS at 4ºC overnight. The beads were washed three times with cytosol buffer containing 10 µM GDP or GTPγS at 4ºC for 10 min and once with cytosol buffer containing 250 mM NaCl and eluted with 80 µl of elution buffer for 20 min at RT and subjected to Western blot analysis.

Subcellular fractionation and equilibrium gradients

NRK cells at 80% confluency were scraped and resuspended in 800 µl of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 1 mM Mg acetate, 10 mM Hepes-KOH, pH 7.2 and protease inhibitors). Cells were cracked with a ball-bearing homogenizer to a breakage of 75-80%. The homogenate was centrifuged for 10 min at 1000 *g*, 4°C. The postnuclear supernatant (PNS) was removed and subjected to ultracentrifugation in a TLA55 rotor at 120,000 *g* for 60 min. The supernatant (cytosol) was removed, and the membranes in the pellet were resuspended in homogenization buffer. Equal volumes of the PNS, cytosol and membrane fractions were analyzed by SDS-PAGE and Western blot. The PNS was also fractionated on a sucrose gradient as previously described[6, 7, 12].

Microscopy, quantitation and statistical analyses

Immunofluorescence microscopy and the electron microscopy are performed as described[7, 9,11], collection of mitotic cells were previously reported[12]. For SK-NEP-1 cells, cells were grown on collagen coated glass plate for 24h and processed for immunofluorescence. For antibody displacement, diluted HACE1 antibodies were pre-incubated with 50 µg/ml recombinant HACE1 for 1 h before incubation with the cells. Pictures were taken with a Leica SP5 laser-scanning confocal microscope using a 100X oil lens or a Zeiss Observer Z1 epifluorescence microscope with a 63X oil lens. In interphase cells, fragmented Golgi was defined as scattered dots that were not connected in the perinuclear region. Although some Golgi membranes were concentrated near the nucleus in SK-NEP-1 cells, multiple mini-Golgi (isolated dots detected by fluorescence microscopy) were observed to be dissociated from the major Golgi apparatus. These cells were categorized as containing fragmented Golgi. To quantify the percentage of cells with fragmented Golgi, more than 300 cells transfected with control or HACE1 siRNAs, or with wild type or mutant HACE1 cDNA constructs, were counted, and the results are presented as mean ± SEM (n=3).

For live cell imaging, cells infected with control or HACE1 shRNA lentivirus were co-transfected with cDNAs for ManII-mCherry and H2B-GFP (Addgene) for 16 h. Mitotic cells were analyzed by time-lapse microscopy with 10 min intervals using a Zeiss Axio Observer Z1 epifluorescence microscope with a 63X oil lens and an AxioCam CCD camera using the AV Rel4.8 software (Zeiss). Temperature (37ºC), humidity and CO2 concentration (5%) were maintained using a Tokai Hit Stage Top Incubator (Tokai Hit) during recording.

Electron microscopy

For EM analysis, Golgi stacks and clusters were identified using morphological criteria and quantified using standard stereological techniques[7, 9]. Interphase cells were defined as profiles that contained an intact nuclear envelope. Only stacked structures containing three or more cisternae were measured for cisternal length. Golgi stack images were captured at 11000x magnification to obtain a better view of the stacks. The longest cisterna was measured as the cisternal length of a Golgi stack using the ruler tool in the Photoshop CS3 software or the line tools in ImageJ, both of which gave essentially identical results. At least 20 cells were quantified in each experiment, and the results represent at least three independent experiments. Statistical significance was assessed by Student's *t*-test.