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Supplementary Information for

Regulated Processing and Secretion of a Peptide Precursor in Cilia

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Legend for Dataset S1
SI References

Other supplementary materials for this manuscript include the following:

Dataset S1 (*.xlxs format)

Key Resource Table

Reagent or Resource	Source	Identifier
Antibodies		
PAM luminal (rabbit)	Our laboratory - Ref (1)	CrPHM-PAL-rhod
FMG1 (mouse)	Dr. R. Bloodgood	Ref (2)
ARF1 (rabbit)	Agrisera	Cat #AS08325
Outer arm dynein IC2	Our laboratory - Ref (3)	1869A
Acetylated tubulin (mouse)	Santa Cruz Biotechnology, Inc.	Cat #sc-23950 (6-11B-1)
proGATI N-ter (rabbit)	This study	Affinity-purified CT237(N-ter)
proGATI C-ter (rabbit)	This study	Affinity-purified CT237(C-ter)
Alexa 488-conjugated goat anti-rabbit IgG	Life Technologies, Thermo Fisher	Cat #A110344
Cy3-conjugated donkey anti-mouse IgG	Jackson Immuno-Research Laboratories	Code: 715-166-151
Alkaline phosphatase-conjugated goat anti-rabbit IgG	Bio-Rad	Cat #172-1016
Gold conjugated (10-nm) goat anti-rabbit IgG	Electron Microscopy Sciences	Cat #25108
Peptides		
Amidated peptide, GATI-NH_2 (VLYPNDPAAYAAYAPGTGGGATI-NH_2)	Biomatik	Ref (4)
Control peptide, GATI-OH (VLYPNDPAAYAAYAPGTGGGATI)	Biomatik	Ref (4)
Glycine-extended peptide, GATI-Gly (VLYPNDPAAYAAYAPGTGGGATIG)	Biomatik	Ref (4)
N-ter peptide (CYELGLDIDGKPAHPAAT-NH_2)	Biomatik	This study
C-ter peptide (YAPGTGGGATI-NH_2)	Biomatik	This study
Experimental models		
Chlamydomonas reinhardtii strains	*Chlamydomonas* resource center	CC124 and CC125 mating type *minus* and *plus* wildtype strains

		LMJ.RY0402.131694 VLE1 mutant
HEK-293 cells	American Type Culture Collection	CRL-1573
Software		
Illustrator	Adobe	26.0.3 release
Photoshop	Adobe	23.2.0 release
Image J	NIH	https://imagej.nih.gov/ij/
UNICORN FPLC software	GE Healthcare	ver. 5.2
Prism 5	GraphPad	https://www.graphpad.com
PyMOL	Schrödinger LLC	ver. 2.4.0 https://pymol.org
RoseTTAFold	Baker Laboratory	https://robetta.bakerlab.org/
AlphaFOLD 2	Deep Mind	https://AlphaFold2.ipynb
DALI	Holm Group	https://ekhidna2.biocenter.helsinki.fi/dali/

Materials and Methods

Chlamydomonas cell culture and gametogenesis induction

Wild type *C. reinhardtii* mating type *minus* (CC124) and *plus* (CC125) strains were cultured in R-medium (5) aerated with 95% air and 5% CO_2 under a 12 h light/12 h dark cycle at 22 °C. The strains were obtained from the *Chlamydomonas* Resource Center (https://www.chlamycollection.org/). To induce gametogenesis, vegetative cells of both mating types were washed, and resuspended in nitrogen-deficient minimal medium (M-N medium) for 24-36 h under aeration and a 12 h light/12 h dark cycle. The VLE1 mutant strain (LMJ.RY0402.131694) was grown under similar conditions.

Preparation of ectosomes, cilia and cell lysates from mating gametes

Gametes of both mating types were resuspended in 10 ml of fresh nitrogen-free M-N medium at a density of 5×10^6 cells/ml. An equal number of mating type *minus* and *plus* gametes were mixed for 1 h; after incubation, ectosomes were isolated by differential centrifugation as described previously (4). Ectosome-enriched pellets were resuspended in TMT buffer [20 mM 2-[tris(hydroxymethyl)-methylamino]-ethanesulfonic acid (TES), pH 7.4, 10 mM mannitol, 1% Triton X-100] containing a protease inhibitor cocktail (cOmplete ULTRA Tablets, # 05892791001, Roche, Basel, Switzerland) and 0.3 mg/ml phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO). For electron microscopy (see below), ectosome-rich pellets were resuspended in 10 mM HEPES buffer pH 7.4 containing the same protease inhibitors.

Cell lysates were prepared as described previously (4). *Minus* and *plus* mixed gametic cells were harvested by centrifugation at 1,600 xg and resuspended in TMT buffer containing 0.2 M NaCl, the protease inhibitor cocktail and 0.3 mg/ml PMSF. Where indicated, gametes were deciliated using dibucaine and cilia isolated by standard methods were resuspended in HMS buffer (10 mM HEPES, pH7.4, 5 mM $MgSO_4$ and 4% sucrose) (6, 7); the deciliated cell bodies were resuspended in TMT buffer containing 0.2M NaCl, protease inhibitor cocktail and 0.3 mg/ml PMSF. Protein content was determined using the bicinchoninic acid assay (BCA) (Thermo Fisher Scientific, Rockford, IL, USA).

To assess association of proGATI with the endomembrane system, 150 ml of CC125 gamete cell culture was centrifuged at 1,200 ×g for 5 min and the pellet re-suspended in 10 ml of 1x HMEKN buffer (30 mM Hepes pH 7.4, 5 mM $MgSO_4$, 5 mM EGTA, 5 mM KCL and 0.2 M NaCl) containing protease inhibitor cocktail and 0.3 mg/ml PMSF. Cells were disrupted by three rounds of freeze/thaw using liquid N_2. The lysate was then subject to three sequential rounds of differential centrifugation (370 ×g for 5 min, 6,100 ×g for 15 min and 435,000 ×g for 15 min) as described previously (8) to yield pellets P1-P3 and supernatants S1-S3. The P1 and P2 pellets were solubilized in 1x SDS lysis buffer (50 mM Tris pH 7.6, 1% SDS, 130 mM NaCl, 5 mM EDTA) containing 0.3 mg/ml PMSF and protease inhibitor cocktail. The microsome-rich P3 pellet was solubilized in HMEK buffer containing protease inhibitors. Protease inhibitors were also added to each supernatant fraction, which were then kept frozen at -80°C until further analysis. Protein content was measured using the BCA assay. For immunoblot analysis, 25 μg of each sample was fractionated by SDS-PAGE.

Samples for electrophoresis were prepared by mixing with 2× Laemmli sample buffer (Bio-Rad, Hercules, California) and denatured at 55°C for 5 min; samples were fractionated in Criterion TGX 4–15% SDS-PAGE gradient gels (Bio-Rad) and transferred to PVDF membranes. Proteins were

visualized using Coomassie brilliant blue; the destained blots were blocked using 5% milk dissolved in 1% Tween-20 in Tris-buffered saline. Incubation with primary antibodies was carried out overnight at 4°C; after washing, horseradish peroxidase-tagged second antibody (Thermo Fisher Scientific) was applied for 1 h at room temperature and the signal visualized using SuperSignal enhanced chemiluminescent (ECL) reagent (Thermo Fisher Scientific, #34080).

Ciliary fractionation

Isolated cilia were incubated with TMT buffer for 60 min at 4°C, to solubilize ciliary membrane and matrix proteins. After centrifugation, the remaining axonemes were incubated with 0.6 M NaCl in TM buffer to release axonemal proteins tightly bound *via* ionic interactions. The extracted axonemal pellet was then dissolved in SDS lysis buffer (0.5% (w/v) sodium dodecyl sulfate, 0.05 M Tris.Cl, pH 8.0) containing protease inhibitor cocktail and 0.3 mg/ml PMSF. Soluble samples were desalted and concentrated using Amicon concentrators (10-kDa cut-off; Millipore Sigma, # UFC800308; Merck KGaA, Darmstadt, Germany). Samples (20 µg protein) were fractionated by SDS-PAGE and analyzed by immunoblotting.

Antibody generation

Synthetic peptides (BioMatik, Kitchener, Ontario, Canada) from the N-terminal (CYELGLDIDGKPAHPAAT-NH_2, 1.5 mg) and C-terminal (YAPGTGGGATI-NH_2, 1.5 mg) regions of proGATI were individually conjugated to keyhole limpet hemocyanin (KLH; 3 mg; Sigma H-7017, Lot 110K4833). A Cys residue appended to the N-ter proGATI peptide allowed conjugation to KLH using m-maleimidobenzoyl-N-hydroxysuccinimide ester (Thermo Scientific Pierce #22311). KLH conjugation of the C-ter peptide used glutaraldehyde, facilitating the generation of amide specific antibodies. Three rabbits (CT327, CT330, and CT332) were immunized with a mixture of KLH-conjugated N-ter and C-ter peptides by Covance Immunology Services (Denver, PA). Crude IgG was obtained by ammonium sulfate precipitation from the sera of immunized rabbits and N-ter and C-ter antibodies were purified by peptide affinity chromatography. The N-ter (pI 5.5) and C-ter (pI 9.9) peptides were conjugated to Affi-Gel-10 (Bio-Rad) agarose beads (1 mg peptide for 2 ml beads) and used for affinity-purification. Recoveries during affinity purification and cross-reactivity of purified antibodies were examined using solid phase assays. High-affinity binding 96-well plates coated with N-ter (5 ng) or C-ter (5 ng) peptide were prepared and serial 3-fold dilutions of each sample were tested. Bound rabbit antibodies were quantified using alkaline phosphatase-linked anti-rabbit secondary antibody and 3, 3', 5, 5'-tetramethyl benzidine (Thermo Scientific, Cat# 34028) as substrate.

Immunofluorescence microscopy

Resting gametes were harvested by centrifugation at 1,600 ×g and fixed with 2% paraformaldehyde in buffer containing 30 mM HEPES, 5 mM EGTA, 5 mM $MgSO_4$, 25 mM KCl, 4% sucrose, pH 7.0. Cells were allowed to adhere to 0.1% polyethyleneimine-coated coverslips for 10 min and then treated with methanol for 10 min at -20˚C. Subsequent blocking and antibody incubation were done as described (4). Primary antibodies used were affinity-purified rabbit N-ter and C-ter proGATI antibodies (from CT327; 1:500), mouse FMG1 (1:1,000), rabbit ARF1 (1:1,000), and mouse acetylated tubulin (1:1,000). Alexa 488 goat anti-rabbit (Cat #A110344; Life Technologies, Thermo

Fisher Scientific) (1:500) and Cy3 donkey anti-mouse (code 715-166-151; Jackson ImmunoResearch Laboratories, West Grove, PA) (1:2,000) conjugates were used as secondary antibodies. Images were obtained using a Zeiss 880 confocal microscope with a 63× oil objective.

Electron microscopy analysis

Immuno-gold labeling of ectosomes was performed as described previously (4) with the following modifications. Freshly isolated mating ectosomes were fixed with 1% paraformaldehyde (EM grade; Electron Microscopy Sciences, Hatfield, PA) and incubated on ice for 30 min. Fixed samples were placed on glow-discharged 400-mesh carbon-coated nickel grids (Electron Microscopy Sciences, Hatfield, PA) for 10-20 min and then washed with 1x PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl pH 7.4) and incubated with 50 mM glycine in PBS. Samples were incubated overnight at 4°C with affinity-purified N-ter and C-ter antibodies (1:10), washed and incubated for 1 h at room temperature with gold conjugated (10-nm) goat anti-rabbit-IgG (1:15, Electron Microscopy Sciences).

For thin section EM, freshly isolated ectosomes were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1 h at 4°C. After fixation, ectosomes were centrifuged at 424,000 xg for 30 min and the ectosome pellet was washed with 0.1 M cacodylate buffer, pH 7.4. Pellets were then transferred to 0.5 ml microfuge tubes; after the buffer was carefully removed, ultra-low gelling agarose (100 µl of 4%; Sigma, Cat# A5030) was added and the sample was immediately centrifuged at 1,600 xg for 10 min at room temperature. Tubes were then placed on ice for 10 min to solidify the agarose. Ultra-thin sections of agarose-embedded ectosomes were mounted on 200-mesh copper/rhodium grids. Sections were imaged using an H-7650 transmission EM (Hitachi High Technologies Corporation, Tokyo, Japan) operating at 80 kV. Thin section EM of intact *C. reinhardtii* cells was performed as described previously (1).

PreproGATI in HEK-293 cells

HEK-293 cells were maintained in DMEM/F12 medium containing 10% fetal calf serum (Hyclone), 100 units/ml penicillin-streptomycin and 25 mM HEPES, pH 7.4 at 37°C in a 5% CO_2 incubator. A cDNA (2742 bp) encoding preproGATI was synthesized and cloned into pUC57 (GenScript). This cDNA was then subcloned into pCI-neo (Promega, Madison, WI) and verified by sequencing. Transient transfections were performed using lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific) and stable populations of HEK-293 cells expressing preproGATI were generated by selecting cells in DMEM/F12 medium containing 0.5 mg/ml G418 disulfate (KSE Scientific, Durham, NC). For analyzing spent medium, cells were washed with serum-free medium [DMEM/F-12 medium containing insulin-transferrin-selenium (ITS) (Thermo Fisher Scientific), 25 mM HEPES, pH 7.4, 100 units/ml penicillin-streptomycin, 1 mg/ml BSA] and then incubated in serum-free medium at 37°C with 5% CO_2. Cell lysates were prepared in 1x SDS lysis buffer with 1x protease inhibitor cocktail (Sigma, # P8340) and 0.3 mg/ml PMSF. Soluble fractions (equal protein) were analyzed using standard electrophoretic and immunoblotting techniques.

BCS treatment of HEK-GATI cells

HEK-GATI cells plated into 24 well dishes were washed and incubated for 30 min in serum-free

media, at 37°C with 5% CO_2. Cells were then treated with serum-free media containing 50 µM bathocuproine disulfonic acid (BCS, Sigma) as described by (9). Cells incubated with medium only (untreated) were used as the control. Spent medium was collected and centrifuged at 100 xg to remove cell debris. Cell lysates (15 µg, ~20% of total) and spent media (15 µl, 5% of total) were fractionated in 4-15% SDS-PAGE gels and analyzed by immunoblotting.

HEK-GATI Bioassay

An equal number of Control (non-transfected HEK-293) and HEK-GATI (stably transfected HEK-293) cells were plated into microfluidic channel slides (µ-Slide I0.2 Luer; Catalog #80166; Ibidi GmbH, Gräfelfing, Germany) as described above. Microfluidic slides were placed into Petri dishes humidified with 1x PBS and kept at 37°C in a 5% CO_2 incubator for 24 h. The chambers were then washed with serum-free medium lacking ITS and BSA and incubated in serum-free medium lacking ITS and BSA for 16-18 h at 37°C in a 5% CO_2 incubator. Before introducing *minus* CC124 gametes, the chambers were washed once with PBS, pH 7.4 and twice with *Chlamydomonas* gametic medium (nitrogen-deficient minimal medium (M-N medium), which causes mammalian cells to lyze due to low ionic strength. The microfluidic channel and both reservoirs were then filled with 50 µl gametic medium. An aliquot (10 µl, 1.2×10^6 gametes/ml) of the same suspension of *minus* CC124 gametes was added to one reservoir of slides containing Control or HEK-GATI cells; slides were incubated in the dark at room temperature for the indicated period of time. Images were obtained at the specified times using an inverted microscope (Nikon Eclipse TE300; Nikon Instruments, Melville, NY) with 4× and 10x objective lenses. Phase contrast images of HEK cells (Control or HEK-GATI) were taken. Gametes were identified based on their auto-fluorescence; a red filter was used throughout the imaging process to avoid any phototactic effects. The whole experiment was conducted in a dark room. HEK cell areas were quantified using Image J and the number of *minus* gametes located in these areas were counted manually; data for Control and HEK-GATI cells are presented as *minus* gametes/mm^2 of HEK cell area.

HEK-proGATI purification

Stably transfected HEK-293 cells expressing preproGATI (HEK-GATI cells) were washed and cultured in serum-free media lacking ITS and BSA for 16-18 h. Spent medium was collected and centrifuged at 100 xg to remove cell debris. Protease inhibitor cocktail and 0.3mg/ml PMSF were added to the medium, which was stored at -80°C. Spent medium pooled from multiple sequential collections was used for purification. A weak anion exchange column, HiTrap ANX Sepharose FF (Cytiva # 17-5163-01; Sigma), was used to concentrate the HEK-proGATI (pI = 6.04). Prior to sample loading, the pH of the spent medium was adjusted to 7.5 and the sample centrifuged at 10,000 xg for 15 min to remove any insoluble material. The HiTrap ANX Sepharose FF column was washed with water, and equilibrated with 20 mM Tris, pH 7.5 containing 100 mM NaCl and 5% glycerol. The sample was loaded with a peristaltic pump and the flow-through discarded. The column was washed with 20 mM Tris, pH 7.5 buffer containing 100 mM NaCl and 5% glycerol until the phenol red from the spent medium was no longer visible on the column. Proteins were then eluted using an AKTA Purifier 10 FPLC System (GE Healthcare, Fairfield, CT), with a gradient of 100 mM to 1 M NaCl in 20 mM Tris, pH 7.5 buffer containing 5% glycerol, a flow rate of 1 ml/min and

a total elution volume of 40 ml. The collected fractions were analyzed using 4-15% SDS-PAGE gels, immunoblotted and probed with the C-ter antibody. Peak fractions were pooled and further purified by gel filtration using a Superdex 200 Increase 10/300 GL (GE Healthcare, 28-9909-44) column equilibrated with 20 mM HEPES, pH 7.4 containing 0.5 M NaCl (Fig. S2C). Fractions were pooled based on SDS-PAGE analysis; purified HEK-proGATI (~5 μg) was then analyzed by mass spectrometry (see below). Approximately 5 mg of purified HEK-proGATI was obtained from 500 ml of spent medium.

HEK-proGATI digestion with vegetative ectosomes

Vegetative ectosomes were isolated from *plus* CC125 cells as described (Luxmi et al., 2019). Purified HEK-proGATI (10 μl; approximately 0.1 μg) was incubated with the indicated volume of freshly isolated vegetative ectosomes (0.5 μg/μl) at 37°C for the indicated time. To stop the digestion PMSF (0.3 mg/ml) was added. Purified HEK-proGATI (10 μl) and vegetative ectosomes (5 μg) incubated alone served as controls. Samples were denatured by heating at 55°C for 5 min after addition of 2x Sample Buffer (BioRad) and were then fractionated on 4-15% SDS-PAGE (BioRad). Immunoblots were probed with affinity-purified N-ter or C-ter proGATI antibody.

Deglycosylation assays

The presence of N-linked oligosaccharides was examined using PNGase F (New England Biolabs (NEB), Ipswich, MA, # P0708S) and the presence of O-linked sugars was assessed by combined treatment with O-glycosidase (NEB #P0733S) and α2-3,6,8 neuraminidase (NEB #P0720S). Mating *C. reinhardtii* ectosomes (20 μg) and spent medium (9 μl) from HEK-293 cells expressing proGATI were denatured by heating at 100°C for 10 min with 1x denaturing buffer. Following denaturation, samples were deglycosylated following the manufacturer's protocol. Samples incubated on ice only (-) and treated with buffer only (+B) were used as controls. For mass spectrometry analysis (see below), purified HEK-proGATI (~5 μg) was denatured and deglycosylated using deglycosylation mix II (NEB, #P6044S), which contains the enzymes needed to remove N-linked and many common O-linked glycans. The deglycosylated sample was buffer exchanged using Zeba™ spin desalting columns (40K Mol. Wt. cutoff; Thermo Fisher Scientific, #87768).

Immunoprecipitation

Immunoprecipitation was performed using a slight modifications of previous protocols (10). Cross-reactive proteins were immunoprecipitated from *plus* gametic cell lysates and from mating ectosomes using affinity-purified C-ter antibody. Before immunoprecipitation, samples were denatured. An equal volume of 1× SDS-P buffer (50 mM Tris pH 7.6, 1% SDS, 130 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM $NaPP_i$) containing 0.3 mg/ml PMSF, protease inhibitor cocktail and PhosStop (Roche) was added to TMT cell lysates (1 mg protein) or mating ectosomes (1 mg protein) and samples were heated at 55°C for 5 min. Samples were allowed to cool and incubated with 0.5 volume (for cell lysate) or 1.0 volume (for ectosomes) of 15% NP-40 for 20 min on ice. Samples were then diluted with 5 volumes of TES-mannitol (TM) buffer containing protease inhibitor cocktail, 0.3 mg/ml PMSF and PhosStop. Each sample was centrifuged at 15,000 xg for 15 min at 4°C to remove any insoluble material. For pre-clearing, washed Protein A agarose beads

(50 µl) (Thermo Fisher Scientific, #22810) were added, samples were tumbled for 30 min at 4°C and then centrifuged at 100 xg for 3 min. Affinity-purified C-terminal antibody (100 µl) was then added to the pre-cleared supernatants, followed by Protein A beads (50 µl) that had been washed with 1x TMT buffer containing 1x protease inhibitor cocktail, 0.3 mg/ml PMSF and 1x Phos Stop. After overnight incubation at 4°C, beads were pelleted and the unbound fraction saved; beads were then washed once with TMT buffer containing 0.5M NaCl and twice with TM buffer containing protease inhibitor cocktail, 0.3 mg/ml PMSF and Phos Stop. Bound protein was eluted by boiling in 2x Laemmli sample buffer (Bio-Rad) and particulate material removed by centrifugation at 15,000 xg at room temperature. The input (15 µg) and eluted proteins (~2% of IPT) were fractionated in 4−15 % SDS-PAGE gels (Bio-Rad) and analyzed by immunoblotting. For mass spectrometry samples were fractionated by SDS-PAGE, and visualized using QC colloidal Coomassie stain (Bio-Rad); the 75-kDa band was excised from the *plus* gamete cell lysate and mating ectosome samples.

Mass spectrometry

Excised gel bands were destained using 40% ethanol and 10% acetic acid in water, equilibrated to pH 8 in 100 mM ammonium bicarbonate, reduced by incubation with 10 mM dithiothreitol in 100 mM ammonium bicarbonate (1 hr at 37°C) and alkylated by incubation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate (45 min at 37°C in the dark). Gel bands were dehydrated using acetonitrile, dried, and then rehydrated in a 12.5 ng/µL trypsin solution (Promega porcine sequencing grade trypsin) in 100 mM ammonium bicarbonate. Proteolysis proceeded for 16 hr at 37°C. Tryptic peptides were extracted using alternating washes with 100 mM ammonium bicarbonate and 5% formic acid in 50% acetonitrile and a final wash cycle with 100 mM ammonium bicarbonate and 100% acetonitrile. Peptide solutions were pooled, dried and peptides resuspended in 0.1% formic acid in water prior to mass spectrometry analysis.

Purified HEK-proGATI was diluted with 100 mM ammonium bicarbonate in water and subjected to reduction and alkylation using 5 mM dithiothreitol in 100 mM ammonium bicarbonate (1.5 hr at 37°C) and 10 mM iodoacetamide in 100 mM ammonium bicarbonate (45 min at 37°C in the dark), respectively. Promega sequencing grade trypsin was added (1:20 w/w, enzyme:protein) and proteolysis proceeded for 16 hr at 37˚C. Digestion was quenched by addition of concentrated formic acid. Peptides were desalted using high capacity C_{18} desalting spin columns (Pierce #89851; ThermoFisher). Desalted peptides were dried to completion and resuspended in 0.1% formic acid in water prior to mass spectrometry analysis.

Resuspended peptides were analyzed using nanoflow ultra-high performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) using a Dionex Ultimate 3000 RSLCnano UPLC system and Q Exactive HF mass spectrometer (ThermoFisher Scientific) at the University of Connecticut Proteomics and Metabolomics Facility. Peptides were loaded onto a 75 µm x 25 cm nanoEase m/z Peptide BEH C_{18} analytical column (Waters Corporation, Milford, MA), separated using either a 1 or 2 hr reversed-phase UPLC gradient, and directly ionized into the Q Exactive HF using positive mode electrospray ionization. MS/MS data were acquired using a data-dependent Top15 acquisition method. All raw data were searched against the *C.*

reinhardtii proteome using the following variable modifications: Modification set 1 - Met and Pro oxidation, Ser, Thr, and Tyr phosphorylation, Glu, Asp, peptide C-term amidation, Cys carbamidomethylation, and Asn, Ser, Thr HexNAcylation, or Modification set 2 - Met and Pro oxidation, Glu, Asp and peptide C-term amidation, Cys carbamidomethylation, and the following on Pro residues: 1Hyp1Hex0Pent, 1Hyp2Hex0Pent, 1Hyp3Hex0Pent, 1Hyp4Hex0Pent, 1Hyp0Hex1Pent, 1Hyp0Hex2Pent, 1Hyp0Hex3Pent, 1Hyp0Hex4Pent, 1Hyp1Hex1Pent, 1Hyp1Hex2Pent, 1Hyp1Hex3Pent, 1Hyp1Hex3Pent, 1Hyp2Hex0Pent, 1Hyp2Hex1Pent, 1Hyp2Hex2Pent, 1Hyp3Hex1Pent where Hyp = Hydroxyproline, Hex = hexose, Pent = pentose. Trypsin C-terminal cleavage specificity was set to "semi-specific C-ragged" at "KR" sites to identify C-terminal non-tryptic proteolysis sites and subsequent C-terminal peptide amidation. Peptide output option was set to "automatic score cut" to allow 0-5% peptide level FDR filtering and protein FDR was set to 1%. All other parameters were kept at default settings. Scaffold v4 or v5 (Proteome Software, Inc., Portland, OR) were used for visualization and further analysis.

For comparative proteomics of VLE1 and SUB14, vegetative and gametic cilia were obtained from both mating types by the dibucaine method (see above). Isolated cilia were separated into membrane/matrix and axonemal fractions by extraction with 1% IGEPAL CA-630 and differential centrifugation. Samples were electrophoresed in triplicate using a short SDS-PAGE gel protocol, stained with Coomassie blue and then subject to tryptic digestion. Tandem MS/MS spectra of purified tryptic peptides were obtained at the University of Massachusetts Medical School mass spectrometry facility and analyzed using Mascot with a parent ion tolerance of 10.0 ppm and a fragment tolerance of 0.050 Da. Modifications allowed included carbamidomethyl on Cys, C-terminal minus Gly plus amide, N-terminal pyroglutamylation, methionine oxidation, N-terminal acetylation and phosphorylation (11).

Bioinformatics analysis and structural modeling

The signal peptide was identified using Signal P (www.cbs.dtu.dk/services/SignalP/) and N-glycosylation sites were predicted with NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc/). Glycosylation of hydroxyproline was determined directly by mass spectrometry. The structural models for proGATI and VLE1 were generated using RoseTTAFold (https://robetta.bakerlab.org) (12) and AlphaFOLD 2 (https://AlphaFold2.ipynb) (13) and inter-prediction consistency analyzed using DALI (http://ekhidna2.biocenter.helsinki.fi/dali/) (14). Structures were displayed using PyMOL (Schrödinger LLC). Structural homologues of individual proGATI domains were identified using DALI to search the protein databank.

Statistics and quantification

For each experiment, the number of biological replicates is indicated in the Figure Legend. One-way ANOVAs with Tukey's multiple comparison test and two-way ANOVAs with Bonferroni post-tests were used to compare the means. Results are represented as mean ± SEM or ± range as indicated in the Figure Legend. GraphPad Prism 5 software was used to perform all statistical analyses.

Data Availability

All underlying data are included within the paper and its supporting information.

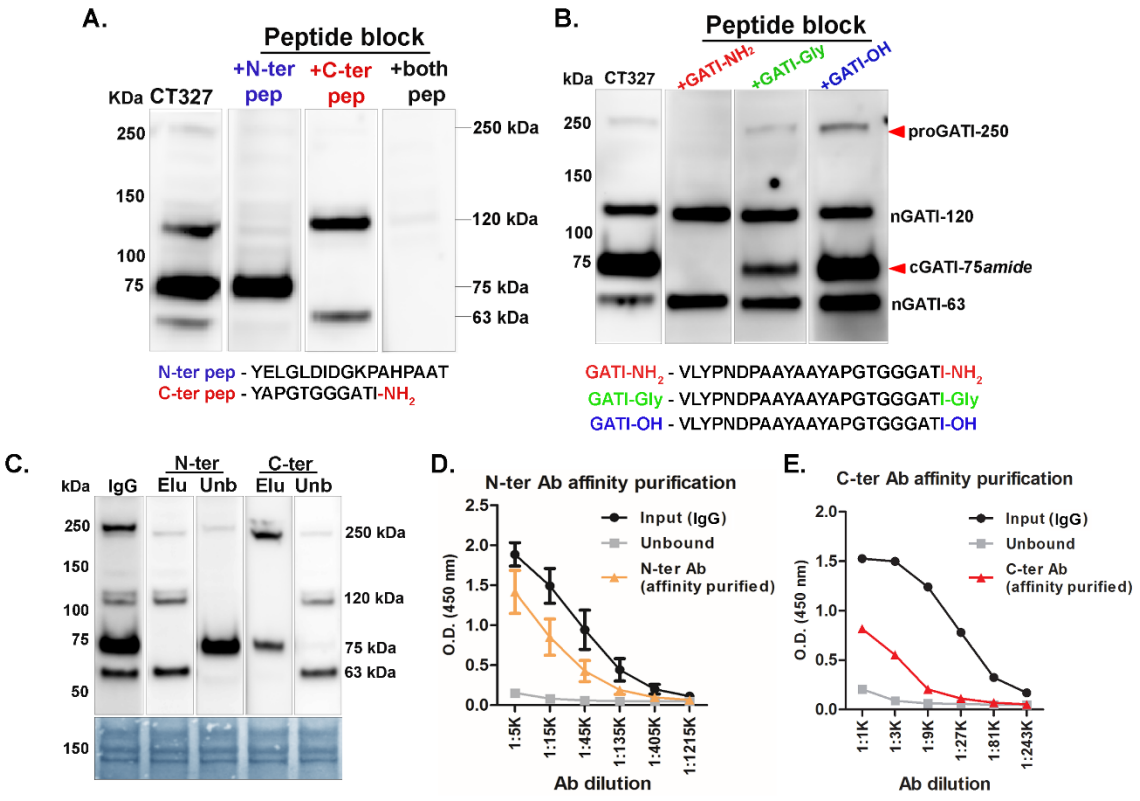

Fig. S1. **Validation of Antibodies against proGATI**

A. Mating ectosomes (10 µg protein) isolated from 1 h mixed gametes were fractionated by SDS-PAGE, blotted and incubated with antiserum (CT327; 1:3,000 dilution) alone or following pre-incubation with the N-ter (blue), C-ter (red) or mixture of both (black) antigenic peptides (0.2 mg/ml). Approximate molecular masses are shown. Data are representative of three independent experiments. **B.** The proGATI antibody generated is amidation specific. Immunoblot of mating ectosomes (10 µg/lane) probed with CT327 antiserum pre-incubated with peptides having -NH_2 (GATI-*amide*), –Gly (GATI-Gly) or –OH (GATI-OH) at the C-terminus. Red arrowheads indicate that the signals for the 250-kDa and 75-kDa bands are almost completely blocked by GATI-NH_2 peptide, attenuated by GATI-Gly and unaffected by GATI-OH. Similar results were obtained in two independent experiments. **C.** Immunoblots showing the results of affinity-purification of N-ter and C-ter proGATI antibodies. Mating ectosomes (15 µg protein) were fractionated by SDS-PAGE and individual PVDF strips incubated with an immunoglobulin-enriched fraction of CT327 rabbit serum (IgG) or aliquots of the material that did not bind (unbound (Unb)) or was eluted from (Elu) columns that contained either the N-ter or C-ter peptide linked to AffiGel beads. The 250-kDa, 120-kDa and 63-kDa bands were detected by the bound and eluted (affinity-purified) fraction of the N-ter antibody and by the unbound fraction of the C-ter antibody. The 250-kDa and 75-kDa bands were detected by the bound and eluted (affinity-purified) fraction of the C-ter antibody and by the unbound fraction of the N-ter antibody. **D.** and **E.** The specificity and yield of the affinity-purified N-ter and C-ter antibodies was determined using a solid phase assay (ELISA) with the N-ter or C-ter peptide, respectively, bound to the plate. The IgG-enriched input (IgG), unbound, and affinity-purified N-ter and C-ter antibodies were tested.

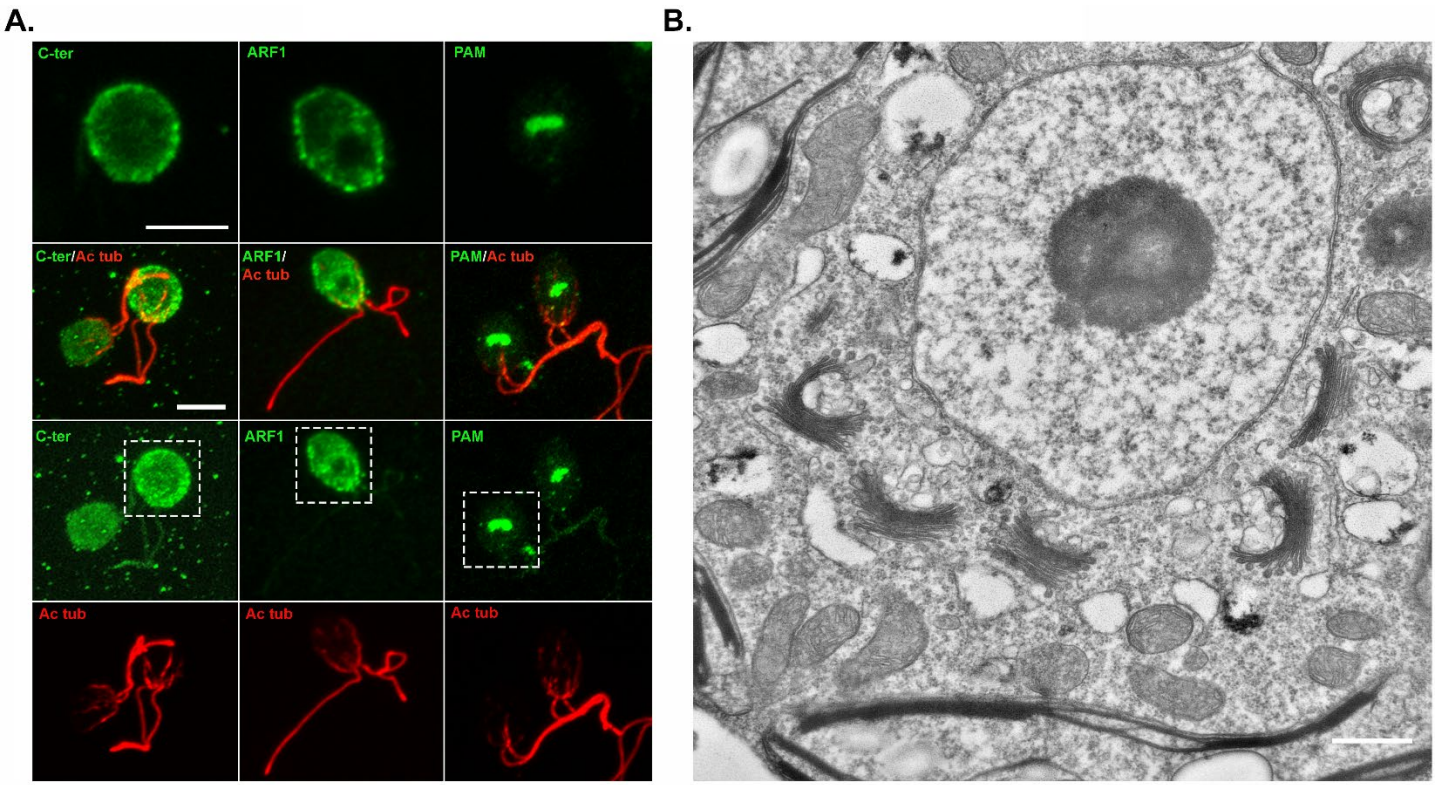

Fig. S2. Localization of Golgi and proGATI within *C. reinhardtii* Cells

A. Immunofluorescence localization of proGATI (using the C-ter antibody), ARF1, PAM (green) and acetylated tubulin (Ac tub; red) within *C. reinhardtii* CC125 *plus* gametes by confocal microscopy. The *upper row* of images show 2-3 slices from the z-stacks of the cell body regions indicated by white boxes to illustrate the presence of proGATI and ARF1 at the plasma membrane compared to PAM. The other images are maximal z-stack projections. Bars = 5 μm. Both proGATI and ARF1 localize to numerous punctate vesicular structures within the cytoplasm and to the plasma membrane. In contrast, PAM is present mainly in the Golgi. **B.** Transmission electron micrograph of a *C. reinhardtii* cell revealing five discrete peri-nuclear Golgi stacks. Numerous vesicles are evident in the cytoplasm. Bar = 500 nm.

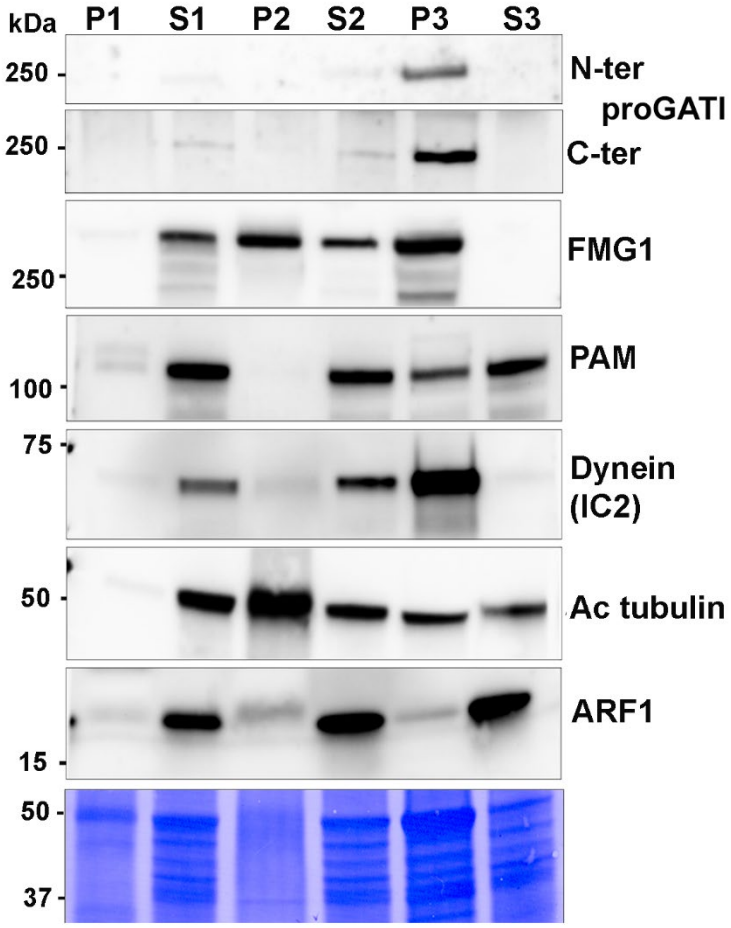

Fig. S3. Presence of proGATI, Endomembrane and Cytosolic Proteins Following Fractionation of Gametic Cell Cytoplasm

C. reinhardtii CC125 *plus* gametes were disrupted by freeze/thaw and subject to three sequential rounds of differential centrifugation that yielded pellets P1-P3 as described previously (8); pellet P3 is enriched in microsomes. These pellets and their corresponding supernatants (S1-S3) were probed with antibodies against proGATI (both N-ter and C-ter), FMG1, PAM, ARF1, outer arm dynein IC2 and acetylated tubulin. Both proGATI and the major ciliary membrane protein FMG1 were present mainly in the microsomal (P3) fraction. In contrast, most ARF1 was present in the S3 supernatant, while PAM was found in both P3 and S3. Note that at the high centrifugal force (435,000 ×g) used to prepare P3, the ~2 MDa outer arm dynein pellets due to its large mass.

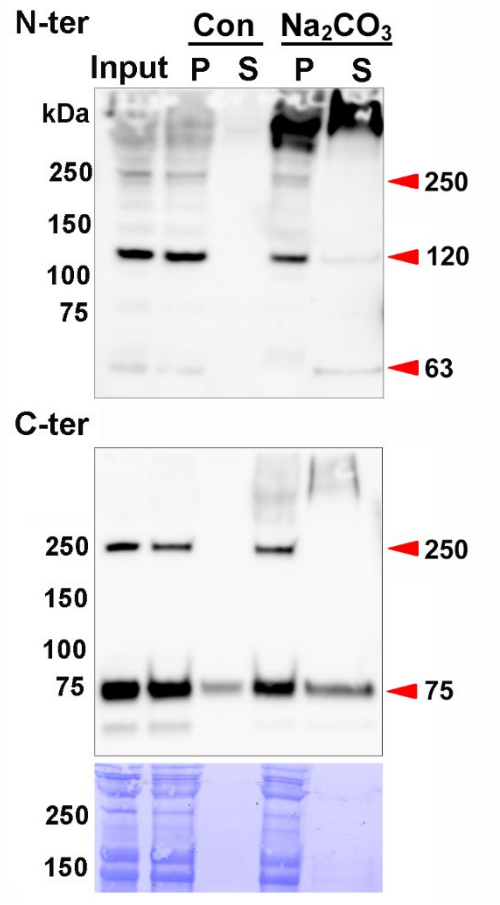

Fig. S4. Effect of Carbonate Treatment of Mating Ectosomes on Solubilization of N-ter and C-ter Fragments of proGATI

Freshly isolated mating ectosomes (Input; 15 µg) were washed with buffer alone (10 mM HEPES, pH 7.4, control (Con)) or with 0.1 M sodium carbonate, pH 11.5 (Na_2CO_3). After centrifugation and SDS-PAGE, the presence of proGATI-derived products in the supernatant (S) *vs*. the pellet (P) was analyzed using the N-ter proGATI antibody (upper panel) or the C-ter proGATI antibody (lower panel). Red arrowheads mark the 250-kDa full-length proGATI, the 120-kDa and 63-kDa N-ter bands and the 75-kDa C-ter band. Quantitation revealed that carbonate treatment released 9% and 79% of the 120-kDa and 63-kDa N-ter bands, respectively, compared to 0% and 4% present in the control supernatant. For the 75-kDa C-ter product, 17% was found in the control supernatant and this was increased to 42% by carbonate. In contrast, intact amidated proGATI remained ectosome-associated, and was not released by carbonate treatment.

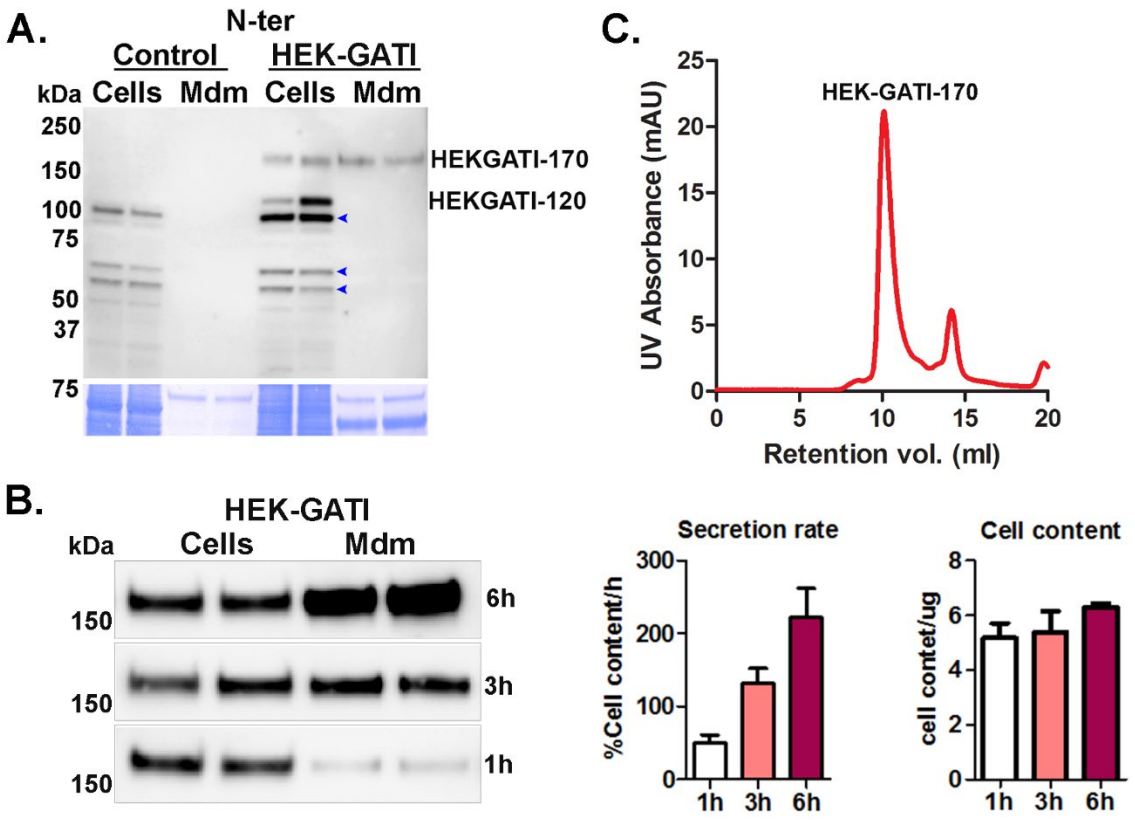

Fig. S5. **Expression of preproGATI in HEK-293 Cells**

A. Cell extracts (Cells) and the spent medium (Mdm) from HEK-GATI cells and Control (non-transfected) cells were probed with the N-ter proGATI antibody. HEK-proGATI protein (HEK-GATI-170) was detected in both cell lysates and spent media, while HEK-GATI-120 was only present in cell extracts. Non-specific bands (present in the Control) are marked with blue arrowheads. Equal amounts of cell lysate (20 µg, 10% of total) and spent medium (1% of total from an 18 h collection) were analyzed. **B.** HEK-GATI cells were incubated in serum-free medium for 1, 3 and 6 h time periods; cell lysates and spent media were analyzed with the C-ter antibody. The HEK-GATI cells secrete the 170-kDa product rapidly; its secretion rate increased over a period of 6 hours (left graph), while the cell content remained constant (right graph). **C.** Gel filtration chromatography step in the purification of HEK-proGATI. UV absorbance, measured at 280 nm (red line), was monitored continuously; immunoblot analysis identified HEK-proGATI-170 in the peak with a retention volume (vol.) just greater than 10 ml.

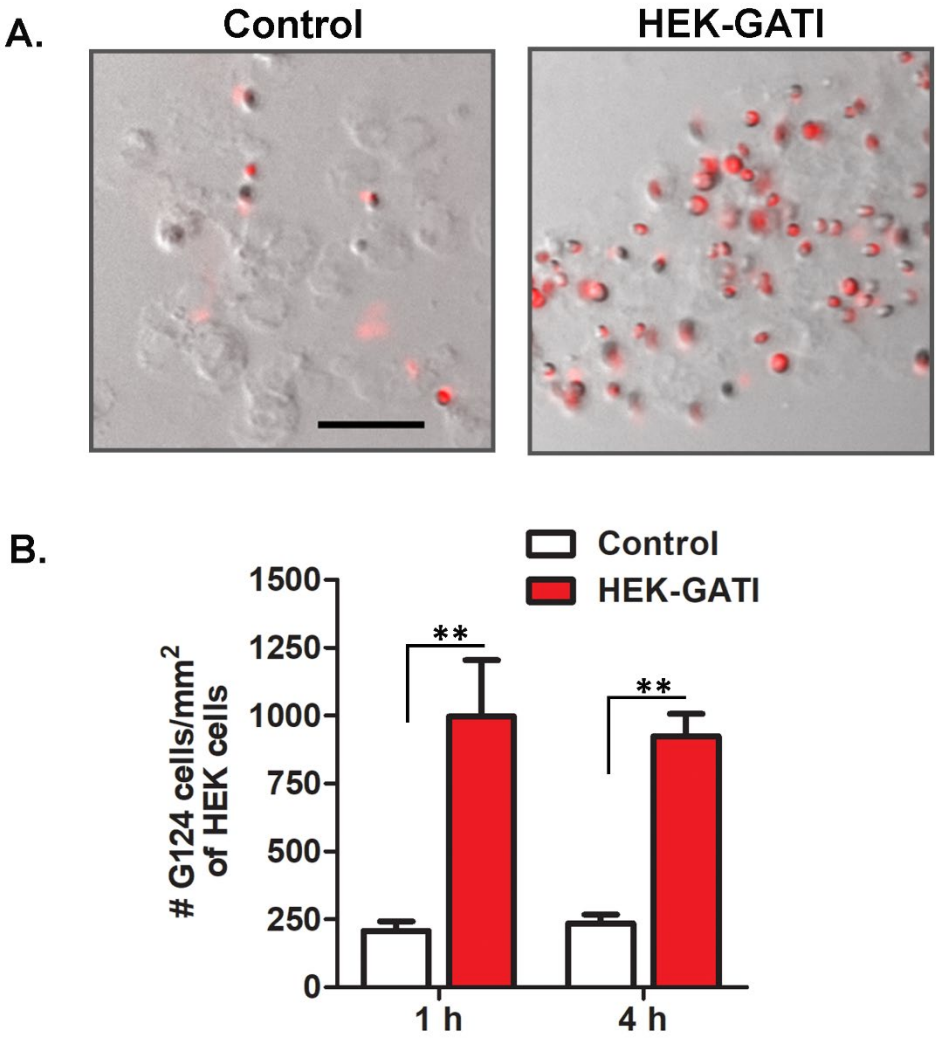

Fig. S6. HEK-proGATI is recognized by *minus* CC124 Gametes

A. Microfluidic chambers containing an equal number of Control or HEK-GATI cells and incubated with an equal number of *minus* CC124 gametes were photographed after 1 h of incubation in the dark. The merged images show a phase contrast micrograph to identify HEK cells (Control or HEK-GATI) and chlorophyll autofluorescence (shown here in red) in the same focal plane to identify *minus* CC124 gametes; each chamber received $1.2x10^4$ *minus* gametes, most of which were swimming in the gametic medium bathing the monolayer of attached HEK cells and are not visible in this focal plane. The area occupied by HEK cells and the number of *minus* gametes in that area were quantified. Scale bar, 50 μm. **B.** Quantification of *minus* CC124 gametes (G124) per mm^2 area of Control or HEK-GATI cells after incubation at room temperature in the dark for the indicated period of time. Results are the average of three independent experiments, data were obtained from n = 10-15 images for each time point. Means were compared ± SEM; two-way ANOVA was used to calculate significance, $^{**}p < 0.01$.

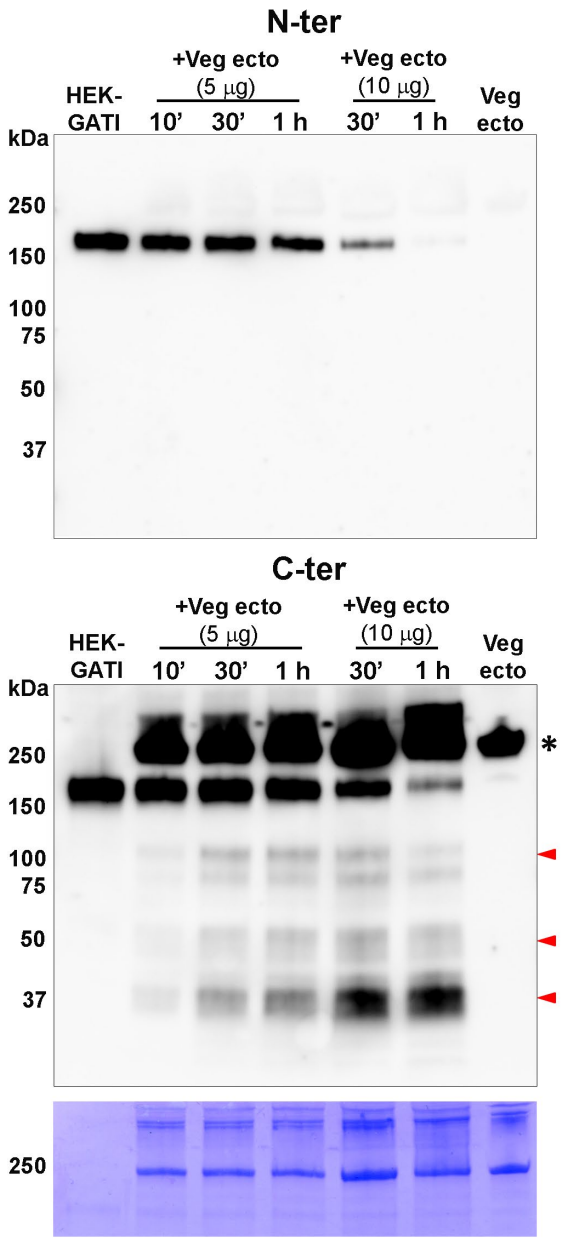

Fig. S7 **HEK-proGATI Digestion with Vegetative Ectosomes**

Aliquots of purified HEK-proGATI were incubated with the indicated amount (5 μg or 10 μg protein) of ectosomes isolated from *plus* CC125 vegetative cells (Veg ecto) for the times indicated. VLE1 is the only subtilisin present in ectosomes from vegetative cell cilia (15); it lacks the pro-domain and thus is active (16). After SDS-PAGE and transfer to a PVDF membrane, cross-reactive material was visualized with affinity-purified N-ter (left panel) or C-ter (right panel) proGATI antibody. Purified HEK-proGATI and vegetative ectosomes alone served as controls. The major C-ter fragments of HEK-proGATI are indicated by red arrowheads and their approximate apparent molecular masses are indicated (~100 kDa, ~50 kDa, ~37 kDa). A non-specific band detected in the vegetative ectosomes by the C-ter proGATI antibody but not by the N-ter proGATI antibody is marked with an asterisk (*). The blot was first probed with C-ter antibody, and then reprobed with N-ter antibody.

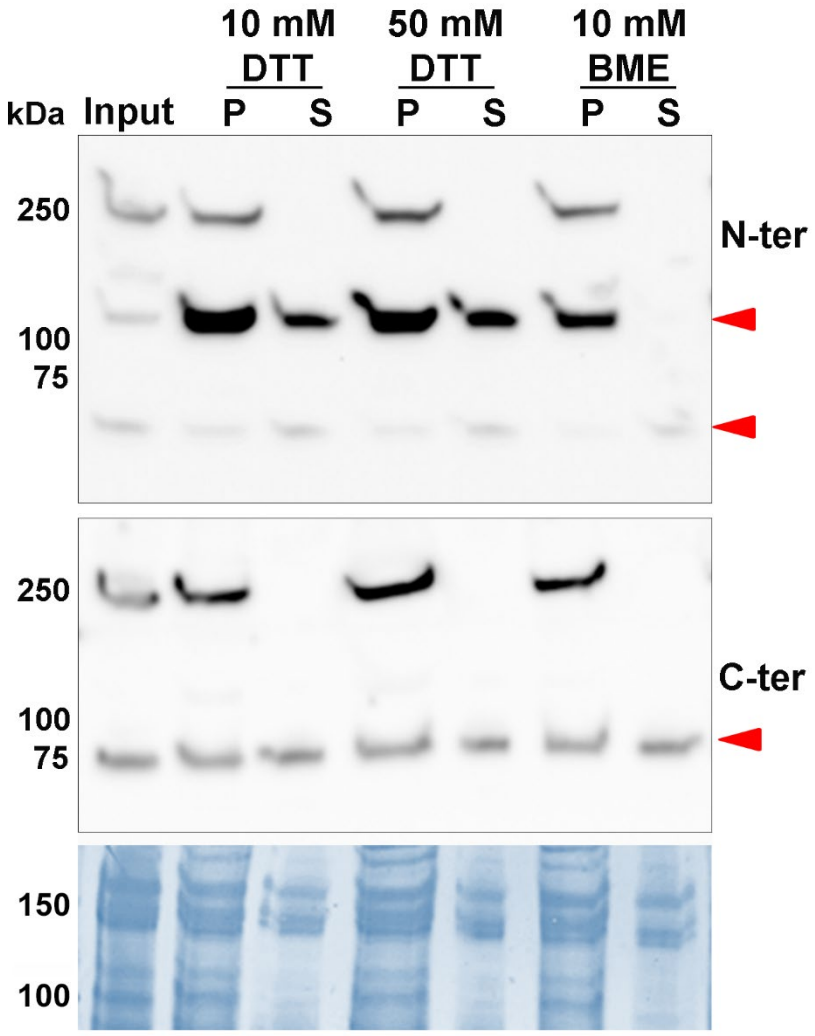

Fig. S8. Release of N-ter and C-ter Fragments of proGATI from Mating Ectosomes by Reducing Agents

Mating ectosomes (input) were washed with buffer containing either 10 or 50 mM dithiothreitol (DTT) or 10 mM β-mercaptoethanol (BME) and the behavior of pro-GATI fragments (red arrowheads) followed. Although the 120-kDa N-ter product was released into the supernatant by DTT, it remained ectosome-associated in the presence of β-mercaptoethanol (*upper panel*). Release of the 63-kDa N-ter fragment (upper panel) and the 75-kDa C-ter fragment (*center panel*) into the supernatant was observed with both DTT and β-mercaptoethanol; both fragments are also released by buffer alone (Fig. 7D). The 250-kDa proGATI band detected by both the N-ter and C-ter antibodies remained in the pellet under all of the conditions tested (and see Fig. 7D).

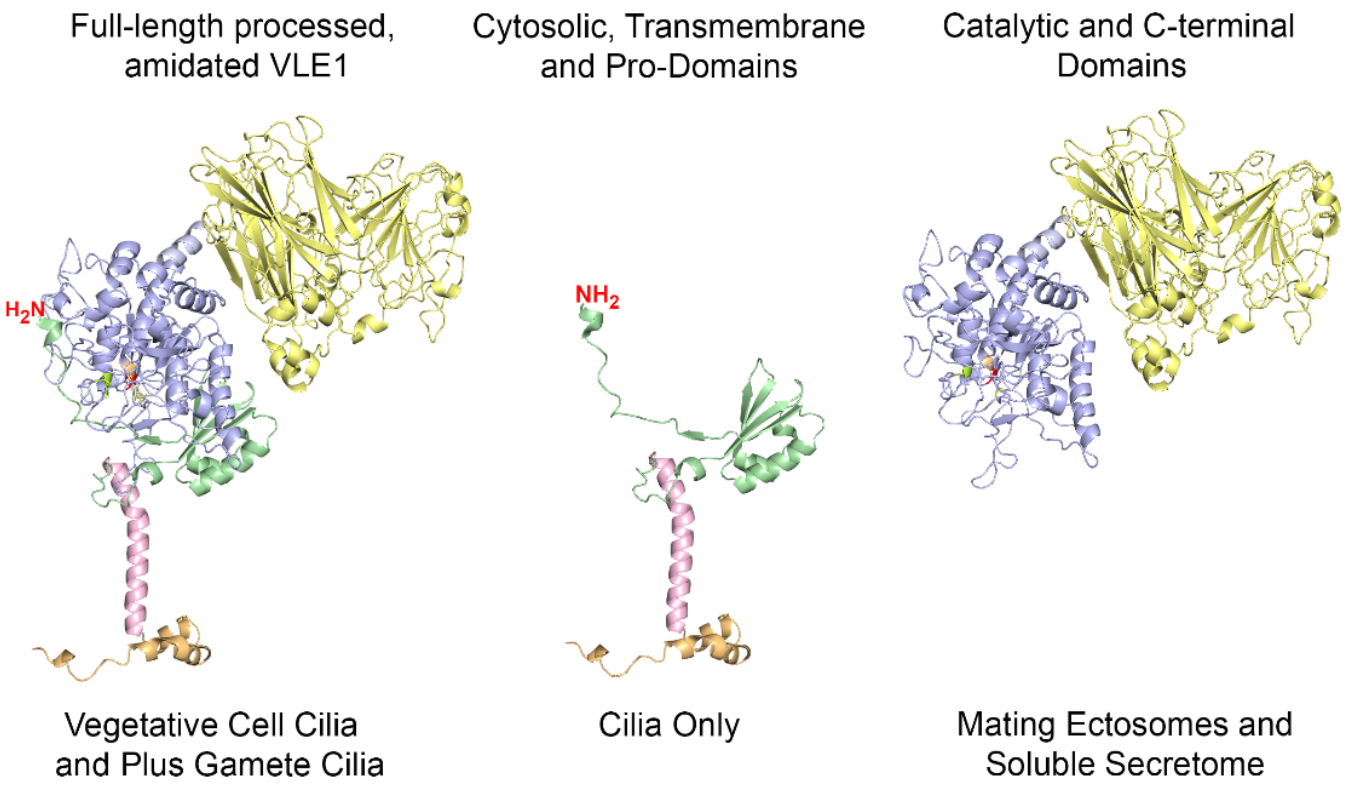

Fig. S9. Changes in VLE1 Domain Organization

Changes in VLE1 domain organization occur during its trafficking from cilia to ectosomes and the soluble secretome. Amidation at a site in the Pro-domain of VLE1 can only occur after an endoproteolytic cleavage and the subsequent exoproteolytic removal of two Arg residues have created a peptidylglycine substrate for PAM. When on the ciliary membrane of both vegetative cells and *plus* gametes, VLE1 has been amidated; although proteolysis has clearly occurred, the S8/C-terminal domains appear to remain associated with the Cytosolic/Transmembrane/Pro-Domain. However, VLE1 recovered from ectosomes and from the secretome, consists of only the S8 and C-terminal domains. The Cytosolic/Transmembrane/Pro-domain fragment is presumably either retained on the ciliary membrane or trafficked back to the cell body for degradation.

Dataset S1 (separate file). Identification of VLE1 and SUB14 in Cilia

Mass spectral data (normalized total spectral counts) for VLE1 and SUB14 in vegetative and gametic cilia of both mating types (data from (11)). Cilia were fractionated into a detergent-soluble membrane plus matrix fraction and an axonemal fraction. All samples were analyzed in triplicate. Also shown are the normalized average spectral counts for the presence of these two proteins in mating ectosomes (data from (4)) and soluble mating secretome (data from (17)) (*.xlsx format).

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