Supplemental Figures

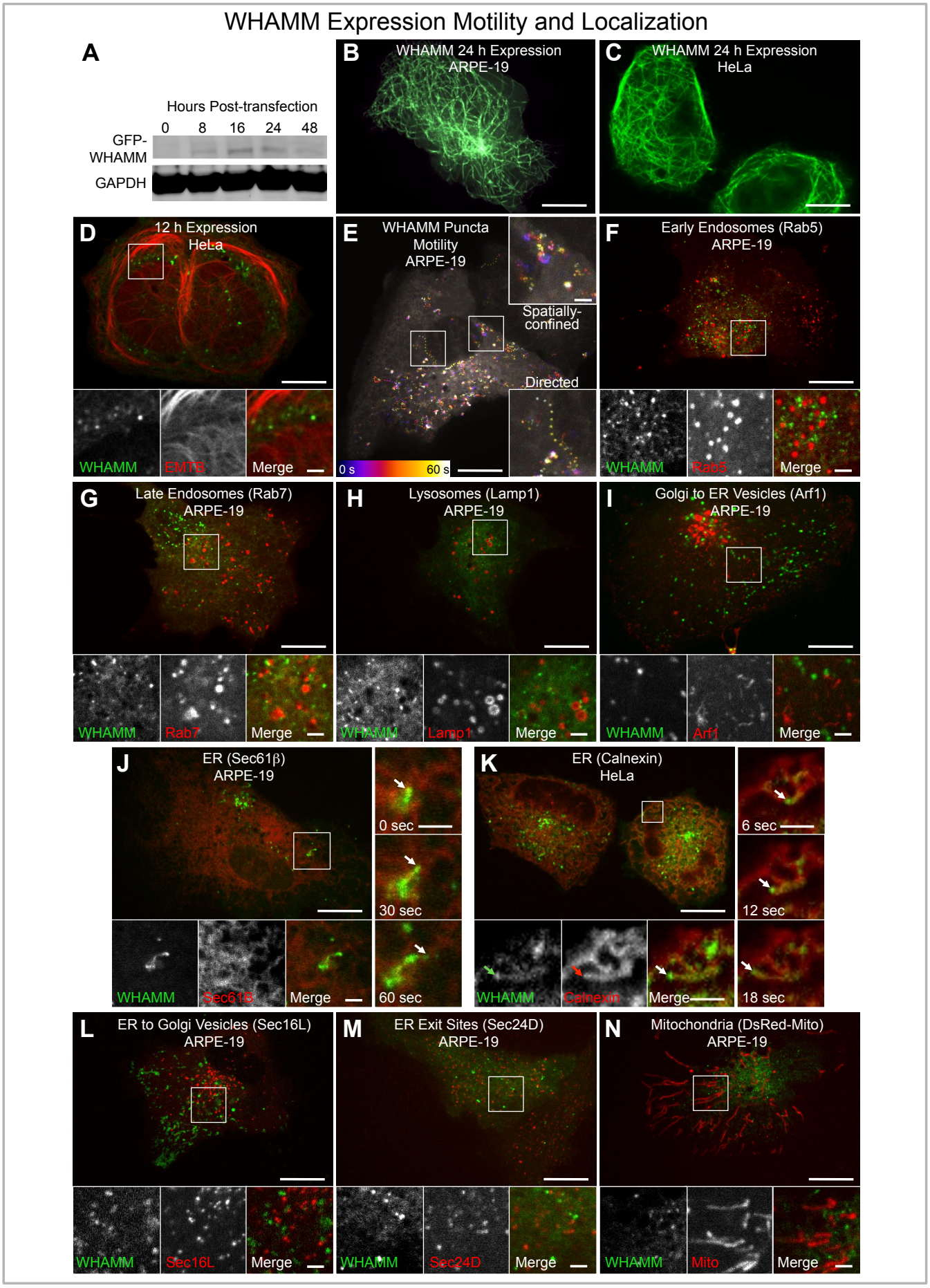


Figure S1. WHAMM Expression, Motility and Localization. Related to Figure 1.

(A) Western blot showing expression of GFP-WHAMM in HeLa cells with time post-transfection.

(B and C) ARPE-19 and HeLa cells transfected with GFP-WHAMM and imaged 24 h post-transfection.

(D) HeLa cells transfected with GFP-WHAMM and mCherry-EMTB and imaged 12 h post-transfection.

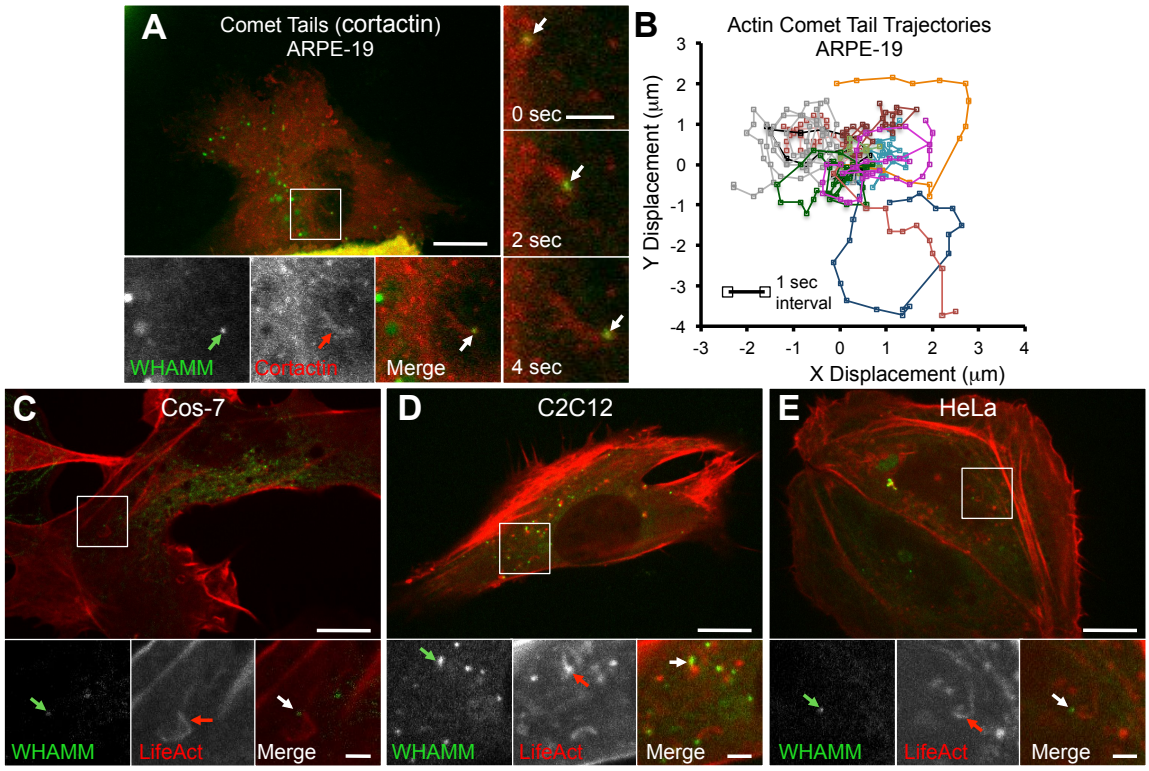
(E) Temporal-color coded projection of GFP-WHAMM puncta movement at 2-s intervals. Colored streaks show puncta movement according to the indicated time scale, whereas white spots correspond to puncta that are mostly stationary. Insets show examples of spatially-confined (spiraling) and directed motions.

(F-I) GFP-WHAMM does not colocalize with the early endosome marker mCherry-Rab5 (F), the late endosome marker mCherry-Rab7 (G), the endosome/lysosome marker Lamp1-RFP (H), or the Golgi-to-ER vesicle marker mCherry-Arf1 (I).

(J-N) ER localization of WHAMM. (J) ARPE-19 cell showing GFP-WHAMM tubules emerging from the ER. These tubules (arrow) elongate with a mean speed of ~0.03 μm s^{-1} (insets). (K) GFP-WHAMM colocalizes with the ER marker mCherry-Calnexin in HeLa cells. Insets show a GFP-WHAMM punctum leading the extension of an ER tubule. (L-M) In ARPE-19 cells, GFP-WHAMM does not colocalize with the ER-to-Golgi (COPII) vesicle and ER exit site markers mCherry-Sec16L (L) and mCherry-Sec 24D (M). (N) GFP-WHAMM does not colocalize with the mitochondrial marker DsRed2-Mito ARPE-19 cells, suggesting that WHAMM is not at ER-mitochondria contact sites.

Scale bars in whole cell and insets images are 10 and 2 μm, respectively.

WHAMM-Dependent Actin Comet Tails



The Motility of WHAMM Puncta Depends on Arp2/3 Complex and Actin Dynamics

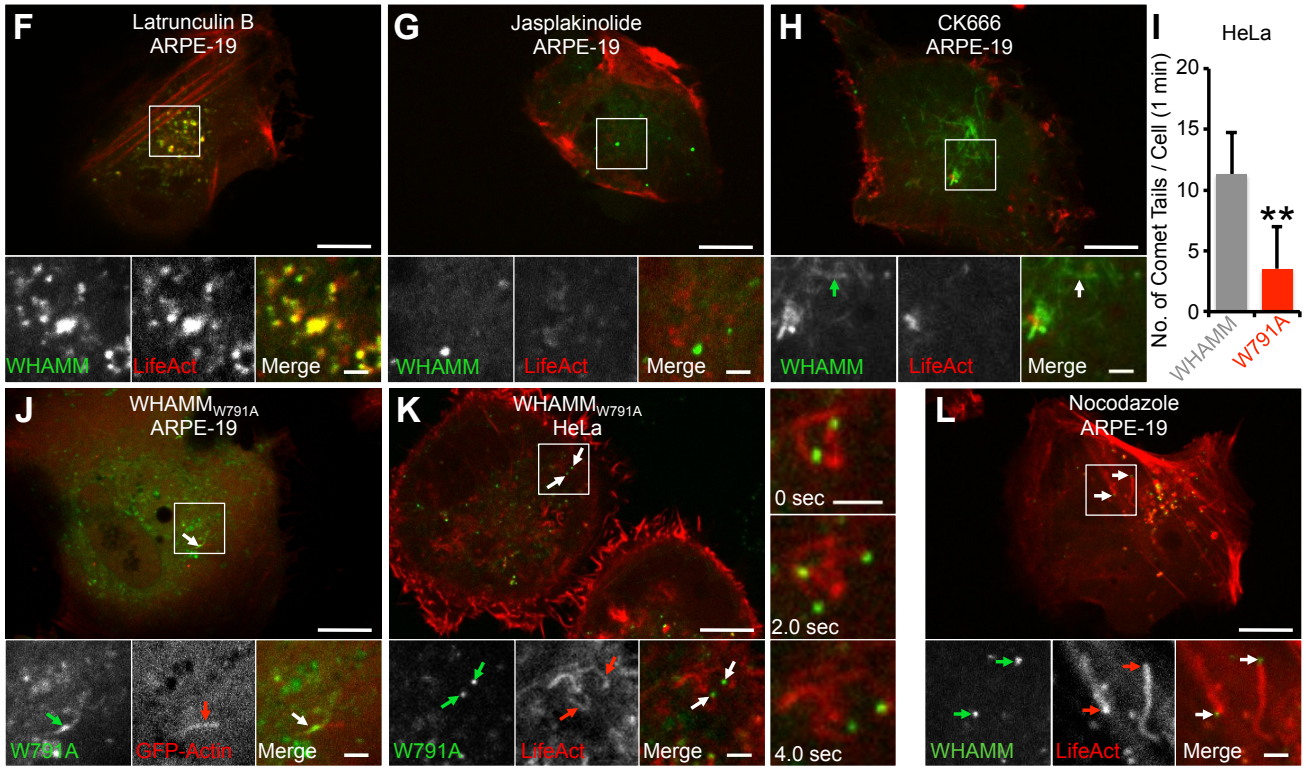


Figure S2. WHAMM-Dependent Actin Comet Tails and their Dependence on Actin Assembly. Related to Figure 1.

(A-E) WHAMM- Dependent Actin Comet Tails. (A) An ARPE-19 cell coexpressing GFP-WHAMM and mCherry-cortactin, showing that the comet tails trailing WHAMM puncta are enriched for cortactin, a protein that stabilizes filament branches formed by the Arp2/3 complex. (B) Trajectories of 10 actin comet tails from Figure 1E (and Movie S1). The trajectories show that WHAMM puncta propelled by comet tails frequently move in circles and spirals, and rarely follow straight paths. (C-E) Formation of WHAMM-dependent actin comet tails in Cos-7, C2C12 and HeLa cells expressing GFP-WHAMM and mCherry-LifeAct.

(F-L) The Motility of WHAMM Puncta Requires Arp2/3 Complex and Actin. (F, G) ARPE-19 cells transfected with GFP-WHAMM and mCherry-LifeAct imaged 5 min after treatment with (F) 1 μM latrunculin B or (G) 1 μM jasplakinolide. (H) An ARPE-19 cell imaged 30 min after treatment with 80 μM CK-666, an Arp2/3 complex inhibitor. Insets show WHAMM tubulation (arrows), which increase with CK666 treatment. (I) The average number of comet tails per cell (n=15 cells from 2 independent experiments) decreases in HeLa cells transfected with mutant $WHAMM_{W791A}$, which cannot activate the Arp 2/3 complex. (J and K) ARPE-19 and HeLa cells transfected with mCherry-$WHAMM_{W791A}$. Insets show that this mutant forms tubular structures (J) that occasionally associate with comet tails (K). (L) ARPE-19 cell, imaged 5 min after treatment with 10 μM nocodazole, showing actin comet tails trailing WHAMM puncta (insets).

Scale bars in whole cell images and insets are 10 and 2 μm, respectively. **$p < 0.001$.

WHAMM and Autophagy

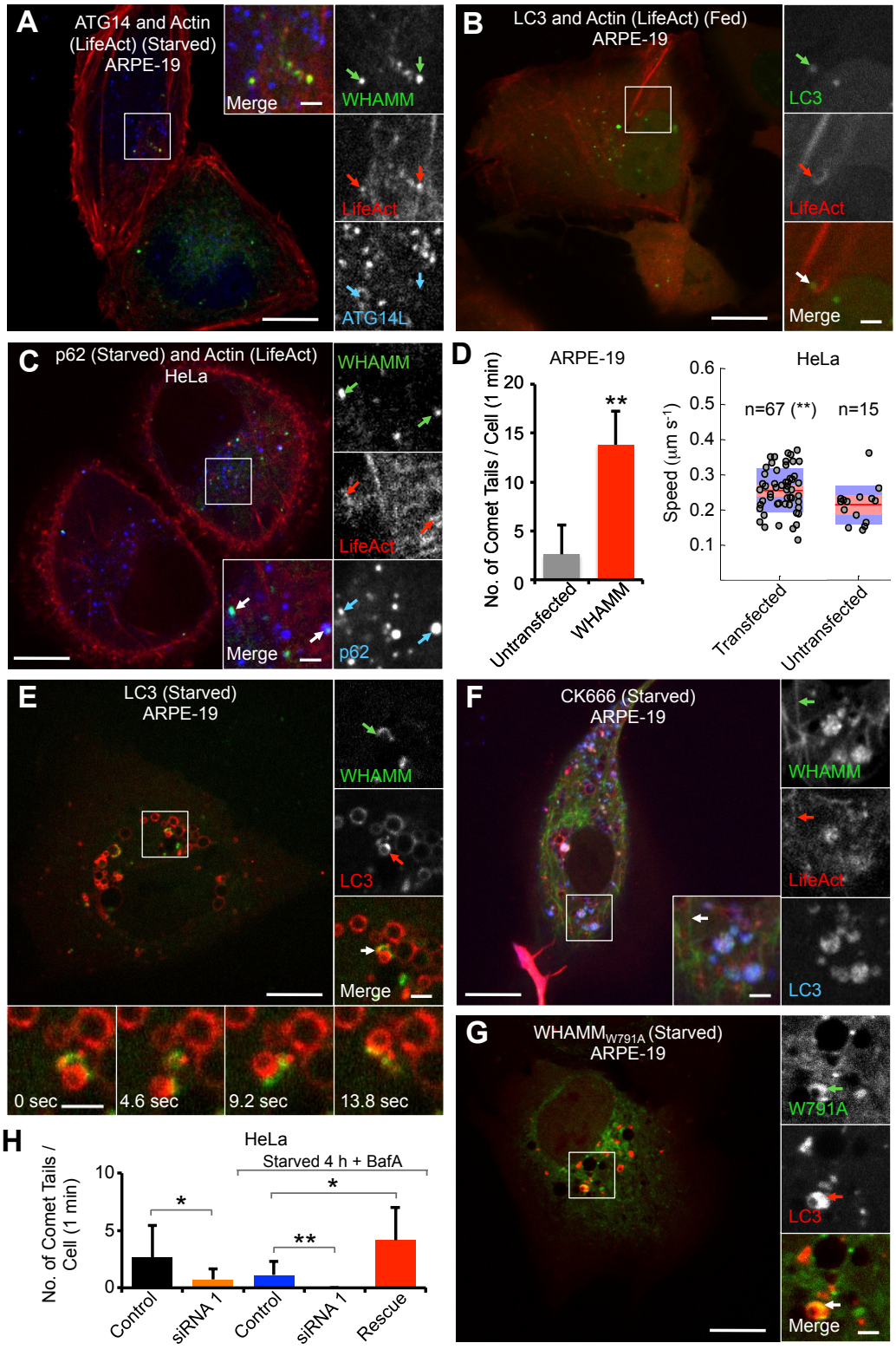


Figure S3. WHAMM and Autophagy. Related to Figures 2 and 3.

(A) GFP-WHAMM does not colocalize with mCherry-ATG14 (blue) in starved HeLa cells (arrows in insets).

(B) Fed ARPE-19 cell expressing untagged WHAMM, GFP-LC3, and mCherry-LifeAct, show LC3 puncta propelled by an actin comet tail (arrows in insets). The speeds of these puncta are quantified in Figure 2E.

(C) GFP-WHAMM colocalizes with mCherry-p62 (blue) coated vesicles near actin (BFP2-LifeAct) patches (red) in starved HeLa cells.

(D) Comparison of the number of comet tails formed in transfected and untransfected ARPE-19 (±SD; n=12 cells from 2 independent experiments) and (n=19 cells from 3 independent experiments) HeLa cells. The speed of actin comet tails in HeLa cells is also shown.

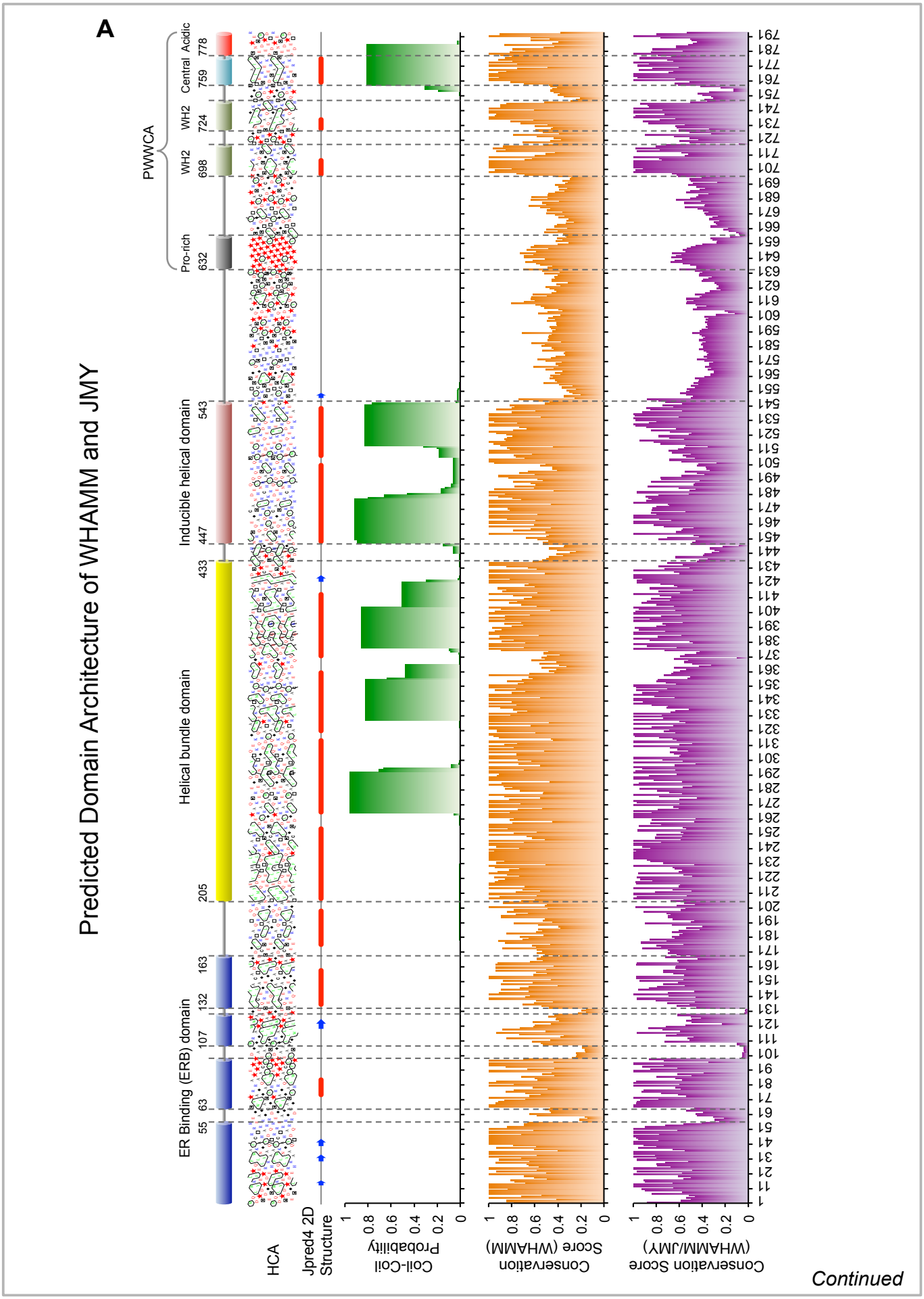
(E) A starved ARPE-19 cell expressing GFP-WHAMM and mCherry-LC3 shows a WHAMM tubule (arrows in insets) tethering neighboring dynamic autophagosomes (time series).

(F) A starved ARPE-19 cell expressing GFP-WHAMM, BFP2-LifeAct (red) and mCherry-LC3 (blue) and treated with CK666 (80 μM for 30 min) shows a reduction in the number of autophagosomes and an increase in WHAMM tubules (arrows in insets) compared to the same cell before treatment (Figure 3A).

(G) A starved ARPE-19 cell expressing mCherry-LC3 and mutant GFP-WHAMM$_{W791A}$ shows a reduction in the number of LC3-positive vesicles as a result of this mutation.

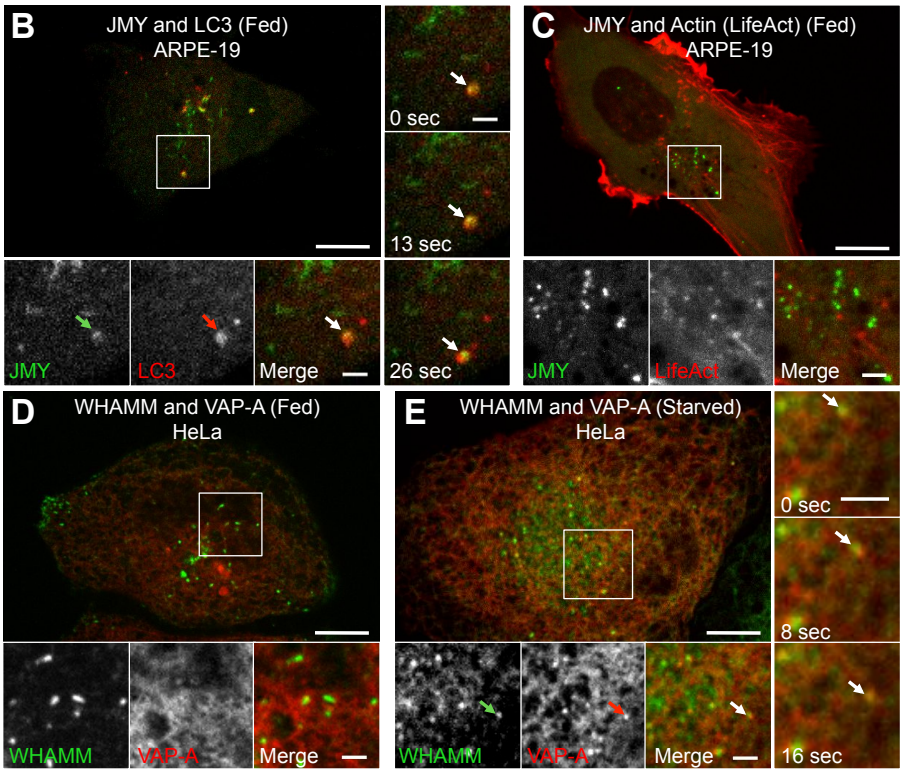
(H) Average number (±SD) of comet tails in untransfected fed HeLa cells (black; n=20 cells from 2 independent experiments), fed knockdown cells treated with siRNA 1 (orange; n=17 cells from 2 independent experiments), untransfected starved cells (blue; n=12 cells from 2 independent experiments), starved knockdown cells (no comet tails; n=17 cells from 2 independent experiments), and rescued cells expressing siRNA-resistant GFP-WHAMM (red; n=9 cells from 1 experiment).

Scale bars in whole cell and inset images are 10 and 2 μm, respectively. *$p < 0.05$, **$p < 0.001$.


A
Predicted Domain Architecture of WHAMM and JMY
PWWCA
Pro-rich
WH2
WH2
Central
Acidic
Inducible helical domain
Helical bundle domain
ER Binding (ERB) domain
55
63
107
132
163
205
433
447
543
632
698
724
759
778
HCA
Jpred4 2D Structure
Coil-Coil Probability
Conservation Score (WHAMM)
Conservation Score (WHAMM/JMY)
Continued

Continued

Similar Localization of WHAMM and JMY



Determinants of WHAMM ER Localization

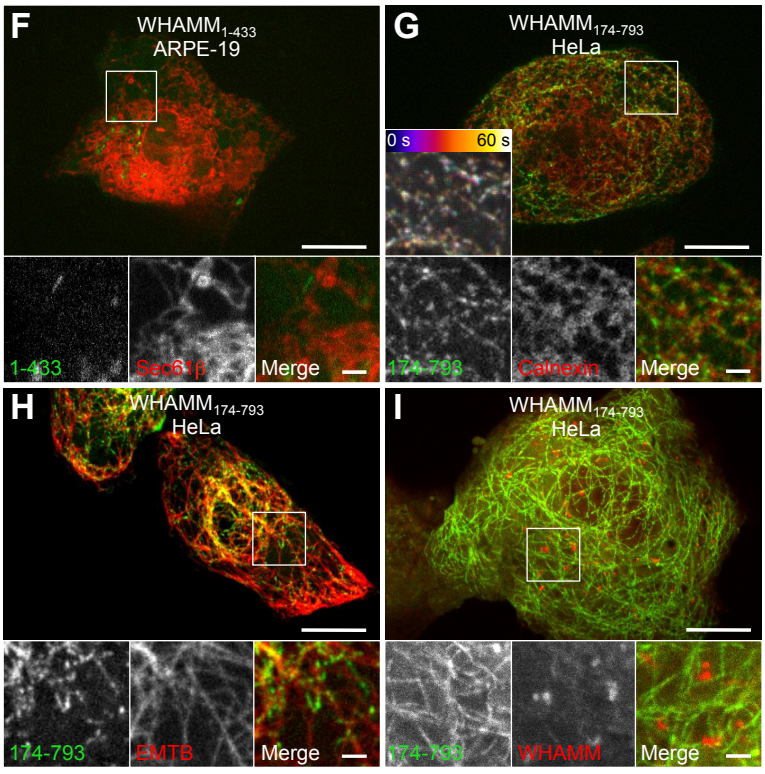


Figure S4. Domain Architecture of WHAMM and JMY. Related to Figure 4.

(A) The domain architecture of WHAMM was analyzed using several bioinformatics approaches: hydrophobic cluster analysis with the program HCA [S2], secondary structure prediction with the program Jpred4 [S3], coil-coil prediction with the program Coils [S4] and amino acid conservation analysis with the program Scorecons [S2]. WHAMM conservation scores were calculated from an alignment of 18 WHAMM sequences with diversity-of-position score > 90%. WHAMM and JMY conservation scores were calculated using 46 WHAMM and JMY sequences with diversity-of-position score > 90%. The resulting per-residue scores were plotted against the mouse WHAMM sequence (UniProt Q571B6-1), i.e. residue insertions (compared to mouse WHAMM) in the remaining 45 sequences are not shown.

(B-E) Similar Localization of WHAMM and JMY. (B) Fed ARPE-19 cell coexpressing GFP-JMY and mCherry-LC3 shows JMY puncta that occasionally colocalize and comigrate with LC3. (C) Fed ARPE-19 cells coexpressing GFP-JMY and mCherry-LifeAct do not display actin comet tails in association with JMY. (D) GFP-WHAMM colocalizes with the ER-resident (and JMY-interacting) protein mCherry-VAP-A in fed HeLa cells. (E). In starved HeLa cells, mCherry-VAP-A puncta on the ER that colocalize and comigrate with GFP-WHAMM puncta.

(F-I) Determinants of WHAMM ER Localization. (F) In an ARPE-19 cells GFP-$WHAMM_{1-433}$ forms tubules that mostly do not colocalize with the ER marker mCherry-Sec61β. (G-H) In HeLa cells, GFP-$WHAMM_{174-793}$ colocalizes poorly with the ER marker mCherry-Calnexin (G), but shows strong colocalization the MT marker mCherry-EMTB (H). (I) ARPE-19 cell coexpressing GFP-$WHAMM_{174-793}$ and full-length mCherry-WHAMM shows that enhanced tubulation by GFP-$WHAMM_{174-793}$ is not due to prolonged expression, because the coexpressed mCherry-WHAMM remains punctate. Furthermore, the two constructs appear mostly segregated, supporting our finding that WHAMM does not form dimers (see Figure 4B).

Scale bars in whole cell and inset images are 10 and 2 μm, respectively.

Supplemental Experimental Procedures

Constructs and Reagents

The cDNA for mouse WHAMM (UniProt Q571B6-4) and human JMY (Uniprot Q8N9B5) were purchased from Open Biosystems. Isoform 4 of WHAMM was converted into isoform 1 (UniProt Q571B6-1) by fusing a synthetic oligonucleotide sequence encoding for the N-terminal 95 amino acids of mouse WHAMM by overlap PCR. The resulting full-length WHAMM sequence (residues 1-793), mutant (W791A) and WHAMM truncations (1-169, 1-541, 1-433, 63-793 and 174-793) were subsequently cloned into vectors pEGFP-C1 (Clonetech), pmCherry-C1 (Clonetech) and/or untagged pSport6 (Open Biosystems). JMY was cloned into pEGFP-C1. mCherry-LifeAct (a gift from Tatyana Svitkina, University of Pennsylvania) was re-cloned into vector mTagBFP2-N1 (Addgene). GFP-LC3, GFP-DFCP1, GFP-Arf1, GFP-p62, and GFP-ATG14L were purchased from Addgene and recloned into vector pmCherry-C1. mCherry-Rab5, Lamp1-RFP, mCherry-Sec24D, mCherry-Sec61β, mCherry-VAP-A and mCherry-calnexin were acquired from Addgene. The 2x mCherry-EMTB (ensconsin MT binding domain) marker was a gift from Janis Burkhardt (The Children's Hospital of Philadelphia). dsRed2-cortactin and mCherry-Rab7 were gifts from Tatyana Svitkina. DsRed2-Mito was a gift from Thomas Schwarz (Harvard Medical School). mCherry-GM130 was a gift from Michael Marks (University of Pennsylvania, PA). The mouse anti-GFP, mouse anti-tubulin and mouse anti-GAPDH antibodies were purchased from Santa Cruz. The rabbit anti-LC3B was purchased from Novus Biologicals. The rabbit anti-WHAMM antibody was purchased from Millipore.

Cell lines, media and inhibitors

ARPE-19 (ATCC) cells were maintained in DMEM/F12 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics/antimycotics (Invitrogen). HeLa cells were cultured in DMEM GlutaMAX media supplemented with 10% FBS and antibiotics/antimycotics. COS-7 cells (ATCC) were cultured in DMEM supplemented with 10% FBS and antibiotics/antimycotics. Mouse C2C12 myoblasts (ATCC) were maintained in DMEM GlutaMAX with 10% fetal bovine serum.

For starvation, cells were incubated for 4 h at 37°C with 5% CO_2 in EBSS (Invitrogen) supplemented with 100 nM Bafilomycin A1 (Santa Cruz). In experiments that test the effect of actin assembly, cells were treated with either 1 μM latrunculin B (ENZO) or 1 μM jasplakinolide (AG Scientific) for 5 min prior to imaging. Experiments that test Arp2/3 complex inhibition were performed with addition of 80 μM CK666 (Sigma) for 30 min prior to imaging. Finally, in experiments that tested the role of the MT network on WHAMM comet tail formation, cells were incubated with 10 μM nocodazole (Sigma) for 5 min prior to imaging.

Transfection of plasmids or siRNA oligonucleotides

Plasmids were transfected immediately after cell seeding, using the transfection reagent FuGENE 6 (Promega). For WHAMM knockdown experiments, 300 pmol of Silencer Select siRNA oligonucleotides (Invitrogen) were delivered to HeLa cells using lipofectamine RNAi max (Invitrogen) as per the manufacturer's protocol. The siRNA sequences are as follows: siRNA 1, 5'-UCAAGAGGGUGUCUGCUGAtt-3'; siRNA 2, 5'-GAAAUCAUCGGUUCAGAUtt-3'; siRNA 3, 5'-AGAUGAAGCAUACCAGGAAtt-3'. Cells were incubated with siRNAs at 37°C in growth media without antibiotics for the first 24 h, and with growth media containing antibiotic for the next 48 h. The extent of WHAMM knockdown was assessed by quantitative real-time PCR (qRT-PCR) using the TaqMan Gene Expression Assays (Applied Biosystems). Total RNA was extracted from 5 x 10^5 HeLa cells using

RNeasy Micro RNA kit (QIAGEN) and gene expression was measured using intron-spanning TaqMan probes (Applied Biosystems) for human WHAMM (Hs00940368_m1) and human GAPDH (Hs03929097_g1). Relative WHAMM expression (normalized to GAPDH expression) was calculated using the $2^{-\Delta\Delta C_T}$ method [S1]. WHAMM rescue was performed in WHAMM knockdown cells (siRNA 1) by transfection of siRNAi resistant GFP-tagged mouse WHAMM.

Cell lysis

Cells were harvested and resuspended in RIPA buffer (Sigma) supplemented with 2% SDS, 1 mM EDTA, 0.2 mM vanadate, 1 mM PMSF and a protease inhibitor cocktail (Roche). Extracts were sonicated for 10 s, with 0.5 s pulses at 50% amplitude, and centrifuged at 14,000 x *g* for 10 min to remove the nuclei and cellular debris. Lysates were analyzed by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad).

Live cell imaging

Two different microscope setups were used for imaging: a 100X (1.40 NA) Plan Apo oil immersion objective lens on a Olympus IX81 inverted microscope equipped with a Yokogawa spinning disk confocal and an Ando iXon3 EMCCD camera; and an Apochromat 100X (1.49 NA) oil immersion objective (Nikon) on a Nikon Eclipse Ti microscope mounted with a UltraView VoX spinning-disk confocal (PerkinElmer) and a C9100 camera (Hamamatsu Photonics). For live cell studies on fed cells, images were acquired at 37^{o}C with or without 5% CO_2 in growth media lacking Phenol Red. Starved cells were imaged in EBSS (Sigma) supplemented with 15 mM HEPES and 160 nM BafA. Images were captured at 0.5-3.0 s intervals at 16-bit resolution using Velocity (PerkinElmer) or Metamorph (Molecular Devices) software. Videos and images were prepared using the software Fiji (NIH). Colocalization, speeds, and diameters were all determined using the Colocalization Threshold or manual-tracking features of Fiji.

Protein expression and purification

Mouse $WHAMM_{1\text{-}541}$ was expressed as a maltose binding protein (MBP) fusion in Rosetta (DE3) competent cells (EMD Millipore). Cells were grown in TB medium supplemented with 2 mg mL^{-1} glucose, 100 mg mL^{-1} ampicillin and 34 mg mL^{-1} chloramphenicol at 37°C to an OD of 2. Protein expression was induced with 1 mM IPTG at 18°C for 6 h. Cells were homogenized in lysis buffer (20 mM HEPES, pH 7.4, 500 mM NaCl, 1 mM EDTA, 4 mM benzamidine hydrochloride and 1 mM PMSF), lysed using a microfluidizer apparatus (Microfluidics), and centrifuged at 20,000 x *g* for 30 min. The protein was first purified through an amylose affinity column and eluted with lysis buffer supplemented with 10 mM maltose, 5% glycerol and 5 mM DTT. The protein was additionally purified by size exclusion chromatography (SEC) using a Superdex 200 gel-filtration column (GE Healthcare) in 20 mM HEPES pH 7.4, 300 mM NaCl, 1 mM EDTA, 4 mM benzamidine hydrochloride, 5% glycerol and 1 mM DTT. Fractions containing $WHAMM_{1\text{-}541}$ were collected, concentrated, and re-purified twice using SEC. $WHAMM_{1\text{-}541}$ was concentrated to ~5 mg mL^{-1} with an Amicon stirred cell (EMD Millipore).

Multi-angle light scattering

MBP-$WHAMM_{1\text{-}541}$ (100 µl at 5 mg mL^{-1}) was fractionated at a flow rate of 0.5 mL min^{-1} on a TSK-gel Super SW2000 column (Tosoh Bioscience) mounted on an Agilent 1100 HPLC system (Agilent Technologies). The molecular species separated by the column were analyzed through a DAWN

HELEOS MALS detector and an Optilab rEX refractive index detector, and their masses calculated with the program Astra (Wyatt Technology).

Statistical Analysis

Quantifications were based on the number of cells and number of independent experiments as indicated. Two-tailed unpaired p-values were calculated using Student's t-test.

Supplemental References

S1. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.
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