

PRESENCE DOES NOT EQUAL AVAILABILITY

A Rubella-Centered Model of Lysosomal Cargo Delivery,
NPC2-Dependent Cholesterol Egress, and Endolysosomal Maturation

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Jacob's Voice

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Final definitive Paper I - integrated evidence record, mechanistic reference, and falsification program

Jacob would like to give credit to God for his amazing abilities.

AUTHOR'S NOTE - ORIGIN AND GOVERNANCE OF THE MODEL

The mechanistic model developed in this paper originated with Jacob Boatman. Jacob identified Rubella virus as the central biological focus and repeatedly framed the problem as one of movement, delivery, processing, and availability rather than abundance alone. His original formulation, 'Rubella caused LAL ingress vs egress,' remains the conceptual starting point. The model has since narrowed: LAL is no longer the lead bottleneck. The current lead is NPC2-mediated post-LAL free-cholesterol availability and transfer.

Scott Boatman works with Jacob as coauthor, research collaborator, literature investigator, and translator of Jacob's mechanistic descriptions into experimentally testable questions. Evidence controls every arrow. Findings are classified as direct RUBV evidence, direct host mechanism, adjacent/comparator evidence, hypothesis, negative evidence, or not yet shown. Missing RUBV-specific links are not filled by inference.

This paper is intended to serve as the mechanistic reference document for the Jacob's Voice Rubella program. It therefore preserves the exact quantitative observations, stage boundaries, negative results, construct boundaries, cargo-routing controls, competing mechanisms, and kill tests needed to reproduce, challenge, or falsify the model.

Abstract

Rubella virus (RUBV) replication is physically embedded in a virus-modified endolysosomal system. Mature cytopathic vacuoles retain endocytic cargo access, persistent RUBV can dissociate cargo binding from internalization, and the viral nonstructural protein P150 has a validated physical interaction with ARL8A. The ARL8A-positive P150N construct maps to amino acids 1-463 of M33 RUBV, almost exactly spanning the independently mapped P150 membrane-targeting system at approximately amino acids 1-461.

Independent host-cell biology supplies a direct route from ARL8/HOPS and CI-M6PR/IGF2R trafficking to lysosomal NPC2 availability and cholesterol egress. Disruption of this system can reduce lysosomal NPC2, increase NPC2 secretion, trap unesterified cholesterol in late endosomal/lysosomal compartments, reduce downstream esterification, and signal reduced ER cholesterol availability. M6P-receptor studies independently show that loss of MPR46/MPR300 targeting causes NPC2 secretion, severe intracellular depletion, and massive endolysosomal unesterified-cholesterol storage. Catalytic competence, protein abundance, lysosomal delivery, compartmental availability, and downstream flux are therefore experimentally separable.

The current Jacob model places the leading lesion after substantial LAL-mediated cholesteryl-ester hydrolysis. LDL-derived cholesteryl ester reaches the lysosomal system, LAL generates free cholesterol, and NPC2 then becomes unavailable, misrouted, incompletely processed, or transfer-limited. The predicted result is increased lysosomal unesterified-cholesterol residence with reduced ER arrival and SOAT-dependent re-esterification. A parallel chemistry branch predicts that pH, sphingomyelin, SMPD1/ceramide, PG/BMP/LBPA, and NPC2 transfer competence can produce functional unavailability even when NPC2 protein reaches the lysosome.

Direct RUBV evidence converges on the same organelle but does not yet close the causal chain. RUBV remodels endolysosomal membranes and lipids, depends on sphingomyelin/cholesterol and low-pH chemistry, produces late-stage acidic-vesicle and cargo-handling phenotypes, and strongly remodels a human lysosomal/sterol transcript module. No recovered RUBV experiment directly demonstrates P150-ARL8A-driven HOPS/CI-M6PR failure, NPC2 lysosomal loss/secretion/transfer failure, or the complete LDL-CE → LAL → FC → NPC2/NPC1 → ER flux defect. The decisive experiment is therefore time-resolved and compartment-resolved: preserve CE hydrolysis, demonstrate FC generation, then determine whether NPC2 availability/transfer falls, lysosomal FC residence rises, and ER arrival falls during established and late RUBV replication.

1. Central Principle: Presence Is Not Availability

Biochemical pathways are commonly drawn as sequences of molecules and enzymes. Such diagrams can hide a second layer of regulation: location, timing, and movement. A protein can be synthesized normally but delivered to the wrong compartment. An enzyme can remain catalytically competent while failing to reach substrate. A product can be generated normally yet remain trapped where it was produced.

The relevant cholesterol trajectory is LDL uptake -> lysosomal cholesteryl ester (CE) -> LAL hydrolysis -> free cholesterol (FC) -> NPC2 -> NPC1-associated export -> ER and other downstream membranes. The current model is specifically a throughput model. It predicts that total abundance can appear normal, or even increase, while effective compartmental availability decreases.

2. Evidence Map: What Is Already Directly Established

Evidence class	Established observation	What it contributes
DIRECT RUBV	Replication complexes are virus-modified endolysosomal/lysosomal structures; P150 localizes to these compartments.	Places viral replication inside the organelle whose lipid/cargo throughput is being tested.
DIRECT RUBV	Endocytosed BSA-gold reaches mature spherule-bearing CPV lumens.	Mature CPVs remain connected to endocytic cargo flow.
DIRECT RUBV	P150N physically interacts with ARL8A.	Provides a viral foothold into lysosomal trafficking biology.
DIRECT RUBV	Persistent M-33 infection increases EGF binding while profoundly reducing internalization; persistent RPE infection reduces particle ingestion despite preserved adhesion.	Demonstrates presence/binding can be preserved while intracellular movement fails.
DIRECT RUBV	RUBV depends on sphingomyelin/cholesterol and low-pH chemistry; established infection alters acidic-vesicle phenotype.	Connects RUBV to membrane chemistry and acidification.
DIRECT RUBV	Human primary endothelial cells show LIPA +14.67-fold, CH25H +39.06-fold, CLN8 -8.05-fold, and CTSS +46 to +139-fold in the filtered dataset.	Direct human lysosomal/sterol transcriptional remodeling.
DIRECT HOST	ARL8/HOPS disruption causes CI-M6PR/NPC2 defects, NPC2 secretion, lysosomal FC trapping, reduced downstream CE storage and ER cholesterol availability.	Defines a direct host route from trafficking failure to the predicted cholesterol phenotype.
DIRECT HOST	MPR46/MPR300 loss causes NPC2 secretion, severe intracellular depletion, and massive endolysosomal unesterified-cholesterol accumulation.	Establishes that NPC2 can be present/competent but unavailable in lysosomes.
DIRECT HOST	Secreted LAL can be M6P-dependent, recaptured, delivered to lysosomes, and correct CE storage.	Separates LAL competence from LAL routing and lysosomal availability.
MISSING SAME-SYSTEM RUBV	RUBV-specific ARL8/HOPS/CI-M6PR failure, NPC2 misrouting/secretion/transfer loss, and full LDL-CE-to-ER flux defect.	The decisive experimental gap.

3. Historical RUBV Endolysosomal Evidence: A Continuous Experimental Record

Paper I does not begin with modern lysosomal terminology. The RUBV-endolysosomal relationship can be followed across decades of experiments that used different names for the same biological territory. In 1967, Hoskins and Plotkin reported reduced acid-phosphatase activity in RUBV-infected systems. In the early 1970s, Kouri and colleagues described RUBV-induced RK-13 cytoplasmic inclusions and later demonstrated their lysosomal character histochemically. By 1992, Lee, Marshall, and Bowden had described RUBV-associated cytoplasmic vacuoles containing approximately 60-nm vesicular structures. Modern molecular work then placed viral replication machinery and RNA synthesis directly into endosomal/lysosomal-origin cytopathic vacuoles.

This progression matters because older papers did not use the modern terms CPV, late endosome, ARL8, HOPS, NPC2, or membrane-contact site. The continuity is morphological and functional: RUBV repeatedly

remodels acidic, endocytic, lysosome-associated structures, and later work identifies those structures as replication organelles.

Era	Direct RUBV observation	Modern relevance
1967	Reduced acid-phosphatase activity.	Early acidic/lysosomal signal; not a cargo-specific mechanism.
1971-1974	RK-13 inclusions described and histochemically linked to lysosomes.	Historical lysosomal identity.
1992	Cytoplasmic vacuoles with ~60-nm vesicular structures.	Morphologic precursor to replication-organelle work.
1998	Replication complexes are LAMP1-positive and acid-phosphatase-positive virus-modified lysosomes.	Direct lysosomal identity.
1999	P150/RNA synthesis localize to endolysosomal CPVs; pulse-loaded BSA-gold later enters mature CPVs.	Replication plus retained cargo access.
2014	P150N physically interacts with ARL8A.	Direct viral-host trafficking foothold.

4. RUBV Replication Occupies the Organelle Under Investigation

Magliano and colleagues identified LAMP1 and acid phosphatase in RUBV replication complexes and concluded that the structures are virus-modified lysosomes [1]. Kujala and colleagues localized P150 to endosomal/lysosomal-origin vacuoles containing sites of viral RNA synthesis [2]. The core mechanistic question therefore begins in the correct organelle rather than being imported from an unrelated system.

The replication compartment remains connected to cargo flow. Extracellular BSA-gold present only during the adsorption pulse was later recovered inside mature, spherule-bearing CPV lumens [2]. Mature RUBV replication structures are therefore not sealed dead-end vacuoles; they continue to interact with the endocytic system.

Late RUBV morphology also includes peripheral/cell-surface CPV-like structures and outward traffic interpreted as fusion of virus-modified lysosomal structures with the plasma membrane and expulsion of lysosomal by-products. Named extracellular NPC2, LAL, SMPD1, or FUCA1 has not been directly measured in this context, so increased outward loss of these cargos remains a testable prediction.

5. Cargo Access, Internalization, and Outward Traffic Are Separate Variables

The direct RUBV cargo record contains different directions of travel and should not be collapsed into a generic statement that trafficking changes. Mature CPVs accept endocytosed BSA-gold from the extracellular space. Persistent RUBV can impair internalization or ingestion despite preserved surface binding or adhesion. Late peripheral CPV-like structures have also been interpreted morphologically as participating in outward traffic.

The Yoneda M-33 experiment is especially useful because receptor-binding capacity increased while internalization of receptor-bound 125I-EGF fell profoundly. The RPE experiment supplies an independent version of the same principle: surface adhesion remains similar while ingestion falls from 68% to 26% of control.

These experiments do not identify LDLR, CI-M6PR, NPC2, or LAL as the affected cargo. Their direct value is narrower and important: RUBV can dissociate presence from movement in human-cell systems.

6. RUBV Directly Produces Presence-Versus-Movement Phenotypes

Persistent M-33 RUBV infection of human embryonic mesenchymal palate cells increased EGF-binding capacity while profoundly reducing internalization of receptor-bound 125I-EGF [12]. The ligand-receptor interaction was present; the next movement step failed.

Persistent human retinal pigment epithelial infection independently reduced fluorescent-bead ingestion from approximately 68% of control cells to 26% of infected cells despite similar surface adhesion [13]. Again, surface presence or binding was preserved while intracellular uptake was impaired.

These experiments do not identify LDL, CI-M6PR, ARL8, NPC2, or LAL as the affected cargo. Their value is direct and narrower: RUBV has already been shown to uncouple presence from movement in human-cell systems.

7. P150N-ARL8A: The Direct Viral Trafficking Foothold

A systematic RUBV-host interaction screen identified ARL8A among P150-associated host proteins, with independent pull-down validation using the P150 N-terminal construct, P150N [3]. This is a direct RUBV-host physical interaction.

The construct boundary is unusually informative. DNASU source clone RvCD00594693 maps P150N to amino acids 1-463 of M33 RUBV. Independent localization work maps the functional N-terminal membrane-targeting/membrane-binding system to approximately amino acids 1-461, with amino acids 101-461 forming the mapped membrane-binding region and amino acids 1-100 required for full function. The ARL8A-positive construct therefore almost exactly spans the independently mapped targeting module.

Within that N terminus, amino acids 36-49 form a critical targeting/maturation helix. E36A, K46A, and I49A disrupt localization/fiber behavior, with E36A/I49A also affecting processing. These residues are high-priority separation-of-function candidates once the ARL8A interface is mapped. No experiment yet shows that ARL8A binds the 36-49 helix or that ARL8A mediates P150 membrane targeting.

8. P150 Interaction Network: Validated Anchor Versus Screen-Level Context

ARL8A is the validated P150 trafficking anchor. Other P150-screen candidates can help define experimental panels but remain below that evidence level. SDCCAG3/ENTR1 appears as a candidate and independently participates in retromer/VPS35-associated endosomal recycling. RHES/RASD2 appears as a candidate and independently maps to endosome/lysosome and autophagy biology. APOA1 and other membrane/lipid candidates remain screen-level context.

The old B3GNT1 label maps to B4GAT1 in modern nomenclature, a Golgi-localized glycosylation enzyme; it should not be converted into an M6P claim. Formal enrichment of the complete P150 candidate set against the screen background is preferable to selecting favorable trafficking hits by inspection.

9. ARL8/HOPS and RUFY1/CI-M6PR Define the Direct Host Route

Independent host work establishes that the BORC-ARL8-HOPS ensemble is required for normal lysosomal cholesterol egress [4]. Combined loss of ARL8A and ARL8B, and disruption of HOPS components including VPS39/VPS41, produces unesterified-cholesterol accumulation in LAMP1-positive structures, reduced downstream cholesteryl-ester storage, and sterol-feedback signatures consistent with reduced ER cholesterol availability.

The most relevant feature is NPC2. In this host system, NPC1 localization can remain relatively preserved while lysosomal NPC2 decreases, NPC2 secretion increases, and CI-M6PR is depleted or degraded [4]. The phenotype is therefore not simply 'lysosomes are broken'; it is a specific failure of soluble-cargo availability that directly reaches cholesterol export.

Rawat and colleagues add a second host route: active ARL8B binds RUFY1, which operates with Rab14, SNX1, and dynein in endosome-to-TGN CI-M6PR retrieval [5]. RUFY1 depletion redistributes CI-M6PR, delays reporter retrieval, delays lysosomal cargo delivery, and modestly increases extracellular pro-cathepsin D. The boundary is strict: the validated RUBV interaction is P150N-ARL8A, while the strongest direct RUFY1 and VPS41 recruitment biochemistry is ARL8B.

ARL8 paralog redundancy is mechanistically important. Single-paralog loss can be buffered, whereas combined ARL8A/ARL8B loss produces the strongest cholesterol phenotype. Total ARL8 abundance can therefore appear normal while recruitment, paralog balance, or late-compartment function becomes abnormal.

10. Lysosomal Identity Does Not Prove Soluble-Cargo Delivery

Host Rab7 experiments provide a direct selective-cargo precedent. Dominant-negative Rab7 in BHK21 cells caused major CI-M6PR redistribution, shifted multiple lysosomal hydrolases, and impaired cathepsin D trafficking/processing, while the lysosomal membrane protein Igmp120 followed a distinct route. Membrane identity and soluble M6P-cargo delivery can therefore diverge.

Acid phosphatase likewise cannot be used as proof that the M6P soluble-cargo system is intact. ACP2 has a substantial M6P-independent transmembrane route. A RUBV CPV can therefore remain LAMP-positive and acid-phosphatase-positive while NPC2, LAL, SMPD1, FUCA1, or other soluble cargo is delivered abnormally.

This distinction is central to the model. It predicts selective throughput failure, not global lysosomal disappearance.

11. LAL Establishes the Upstream Control - but Is No Longer the Lead Bottleneck

Lysosomal acid lipase, encoded by LIPA, hydrolyzes LDL-derived cholesteryl esters and triglycerides in the lysosomal lumen. Direct experimental systems have visualized LDL-derived CE degradation inside lysosomes and established LAL as the responsible acid lipase [6,7].

Historical human-cell routing experiments show that secreted LAL can be recognized and internalized through a mannose-6-phosphate-dependent process; free M6P competes with uptake, phosphatase treatment impairs uptake, and successful lysosomal delivery corrects cholesteryl-ester storage [8]. Thus LAL catalytic competence, LAL abundance, lysosomal delivery, and cellular cholesterol correction are separable.

The current model does not require primary LAL failure. Its strongest positive prediction is the opposite: CE hydrolysis remains substantially preserved and free cholesterol is generated. LAL becomes the critical upstream discriminator that places the dominant lesion before or after FC generation.

12. M6P-Receptor Loss Produces the Predicted NPC2 Availability Phenotype

The M6P-receptor literature reproduces the exact logic of the current model without requiring NPC2 to be genetically absent. In MPR46/MPR300-deficient cells, NPC2 is secreted, intracellular NPC2 becomes severely depleted, the residual pool is biased away from normal lysosomal delivery, and unesterified cholesterol accumulates massively in endosomal/lysosomal compartments.

This establishes the central distinction: NPC2 can be synthesized and remain biologically competent yet fail at routing and compartmental availability. Human IGF2R work adds that NPC2 per lysosome can fall without an obvious total-cell deficiency signal. Whole-cell abundance can therefore miss the predicted lesion.

The same principle applies upstream to LAL. Historical human fibroblast work shows M6P-sensitive LAL uptake and correction of CE storage after lysosomal delivery. Total protein, lysosomal protein, extracellular protein, catalytic or transfer function, and downstream flux must therefore be measured separately.

13. NPC2 Is the Matching Product-Side Availability System

Willenborg and colleagues directly established M6P-receptor dependence of NPC2 targeting [9]. Either MPR46 or MPR300 can transport NPC2 to endosomes/lysosomes, with MPR300 more efficient in the recovered system. In cells lacking both receptors, NPC2 is secreted, only a small intracellular pool remains and is biased toward the ER, and massive unesterified-cholesterol accumulation develops in endo/lysosomal compartments.

Human myeloid/U937 experiments independently track NPC2 lysosomal delivery through IGF2R. IGF2R knockout or saturating M6P interferes with NPC2 delivery per lysosome [10]. Reduced lysosomal NPC2 therefore does not require NPC2 transcription to fall or the total receptor pool to disappear.

The matching logic is exact: LAL generates the product; NPC2 makes that product transferable. Primary LAL failure produces insufficient FC generation. NPC2 failure produces FC generation followed by FC trapping.

14. A Unified M6P-Cargo Panel

Cargo/readout	Core function	Interpretive value
NPC2	Soluble lysosomal cholesterol transfer	Lead bottleneck. Lysosomal loss/secretion or transfer failure predicts unesterified-FC trapping.
LAL/LIPA	CE/TG hydrolysis	Separates substrate-side failure from post-LAL failure.
SMPD1/ASM	SM -> ceramide chemistry	Links M6P cargo routing to membrane chemistry and NPC2 transfer competence.
FUCA1	Lysosomal alpha-L-fucosidase	Links soluble-cargo routing to fucose-containing substrate turnover; relevant to the late fucosylglyceride only as a reporter candidate.
ACP2	Acid phosphatase; substantially M6P-independent transmembrane route	Control showing that acid-phosphatase positivity does not establish normal soluble M6P-cargo delivery.

Host M6P-deficiency proteomics places NPC2, SMPD1, and FUCA1 among severely depleted lysosomal cargo, while LAL has independent M6P uptake/correction evidence. Coordinated loss of several soluble cargoes with extracellular gain would favor a broad delivery lesion. Disproportionate NPC2 loss with preserved LAL/SMPD1/FUCA1 would favor the current selective NPC2 model.

15. Direct Human RUBV Transcript Module: LIPA, CH25H, CLN8, and CTSS

Target	RUBV-associated signal	Interpretation
LIPA	+14.67-fold; $p=2.84 \times 10^{-3}$	Strong direct engagement of a LIPA-annotated transcript signal; not LAL enzyme activity.
CH25H	+39.06-fold; $p=1.55 \times 10^{-3}$	Strong sterol/immune-metabolic response.
CLN8	-8.05-fold; $p=9.43 \times 10^{-4}$	Direct suppression of an ER cargo receptor involved in broad soluble lysosomal-enzyme delivery.
CTSS	+46 to +139-fold across multiple probes	Strong lysosomal/protease transcriptional remodeling.

The Geyer primary human endothelial dataset therefore contains a selective lysosomal/sterol program, not a lone LIPA signal [11]. CLN8 suppression is especially relevant to the presence-versus-delivery principle because CLN8 participates in soluble lysosomal-enzyme trafficking. These transcript changes do not prove LAL or NPC2 misrouting.

NPC2, IGF2R, ARL8A/B, VPS39, and VPS41 were not recovered in the study's stringent ≥ 4 -fold/ $p \leq 0.01$ filtered list. That is a real negative at the filtered-table level but does not establish absence of smaller changes, localization changes, protein-level effects, or flux defects.

16. Acidification Evidence Must Be Interpreted by Assay and Stage

The pH literature contains experiments that answer different questions. Entry inhibitors establish dependence on acidic/endosomal physiology during early infection. Low-pH fusion and virion-conformation experiments define biochemical thresholds. Acridine-orange work in established infection reports a later acidic-vesicle phenotype. They become a coherent sequence only when their time windows remain separate.

Oxford and Schild's inhibitor timing placed the sensitive period early: ammonium acetate and aminoadamantane had little or no antiviral effect when first added later than approximately five hours after infection, while pre-exposure followed by washout at inoculation did not inhibit growth. This constrains an early vulnerable stage; it does not test a mature P150/dsRNA-positive compartment.

In the five-day TO-336/SIRC system, continuous bafilomycin exposure was not used across the full infection because of cytotoxicity; the displayed established-infection experiment interrogated the terminal 24 hours. LysoPrime Green puncta are retained only as a lysosomal structure/localization observation because the

probe is pH-resistant. Modern chloroquine/bafilomycin inhibition supports acidification dependence but supplies no absolute mature-organelle pH.

17. The RUBV pH Staircase: Exact Numbers, Exact Boundaries

Experimental context	Direct pH observation	What it means
Väänänen & Kääriäinen 1980	Adsorption/fusion conditions at pH 5.8	Direct RUBV low-pH membrane/fusion activity.
Katow & Sugiura 1988	Brief exposure below pH 6 induces infected-cell fusion; virion exposure around pH 5 causes irreversible envelope changes	Direct E1/E2 conformational and membrane-interaction chemistry.
Mauracher et al. 1991	Capsid solubility/conformational transition between pH 5.0 and 5.5	Direct second acidic transition in RUBV capsid chemistry.
Human LAL biochemistry	Long-chain CE/TG hydrolysis broadly optimal around pH 4-5; classical assays ~4.0-4.4	Defines deeper lysosomal acidity for physiological LAL catalysis.
Pásztor/TO-336 established infection	Five-day infected cells show major loss of strongly acidic acridine-orange vesicular signal	Direct infection-associated directional acidification phenotype, but not calibrated organelle pH.

The numerical contrast is real, but it must not be overstated. Entry/fusion thresholds, virion conformational transitions, and capsid transitions are not measurements of the luminal pH of an established P150/dsRNA-positive replication compartment. No calibrated absolute pH measurement of an established RUBV-positive endosome, lysosome, autolysosome, or CPV has been recovered.

The strong model question is therefore temporal: RUBV requires acidic conditions for entry and uncoating, while established infection shows reduced strongly acidic-vesicle formation. Does the maturing virus-modified endolysosomal system fail to continue normal acidification deeply enough to alter cargo processing or NPC2 transfer? That continuous sequence has not yet been measured.

18. Sphingomyelin, Ceramide, BMP/LBPA, and NPC2 Transfer

RUBV has direct dependence on host sphingomyelin and cholesterol during entry [14]. SGMS1/SMS1 loss produces strong RUBV phenotypes at both early and later stages, and forced low-pH lipid mixing does not fully rescue productive infection. This separates pH from membrane chemistry as distinct requirements.

Independent NPC2 biochemistry shows that BMP/LBPA strongly promotes NPC2-mediated cholesterol transfer, sphingomyelin can inhibit transfer, and acid sphingomyelinase/SMPD1-dependent conversion of sphingomyelin toward ceramide facilitates the transfer environment [18,19].

This creates a second mechanism of functional unavailability. NPC2 may be delivered normally but work poorly because the late-endosomal/lysosomal membrane has the wrong pH/SM/ceramide/BMP state. The current model therefore distinguishes NPC2 routing availability from NPC2 transfer competence.

19. Historical RUBV Lipid Remodeling: The Quantitative Record

BHK21 lipid	Control	RUBV	Direction
Free cholesterol	5.6	5.8	Nearly unchanged bulk FC
LPC	4.8	2.4	Large decrease
Sphingomyelin	5.3	4.5	Decrease
PC	37.1	48.5	Major increase
PS	7.7	5.8	Decrease
PI	8.9	7.9	Decrease
PE	25.0	27.2	Increase
Cardiolipin	6.5	3.8	Large relative decrease
Cholesterol:phospholipid	0.17	0.19	Small change despite broad remodeling

The historical BHK21 data are important precisely because bulk free cholesterol barely moves while membrane lipids change widely [20]. Whole-cell cholesterol is therefore a poor surrogate for compartmental cholesterol availability in RUBV infection.

Voiland/Bardeletti work also reported a substantial fatty-acid shift, including an approximately 35% rise in linoleic acid with decreases in palmitic and oleic acids in the recovered historical analysis. Again, the signal is membrane remodeling rather than simple total-cholesterol gain or loss.

20. The Late RUBV Fucosylglyceride Is a Time-Linked Membrane Phenotype

A chemically distinct RUBV-associated glycolipid appears after approximately two days and was characterized around day four [21]. It is absent from uninfected controls and purified virions and represents roughly 1% of infected-cell total lipid.

The compound was classified as a fucosylglyceride consistent with a di-/poly-fucosylglyceride. Hydrolysis yielded approximately 40% sugar and 60% lipid, with fucose:glycerol near 2:1 and essentially no phosphorus, amino sugar, or sialic acid. Reported fatty acids were C14:0 14.2%, C16:0 43.8%, C18:0 21.1%, and C18:1 20.9%, making the lipid approximately 80% saturated.

The exact synthase and degradative enzyme remain unknown. This result is not proof of FUCA1 failure. Its value is as a direct late-stage, chemically specific membrane phenotype that emerges as the RUBV compartment matures.

21. Historical RUBV Lipoprotein Architecture: Exact Activity Pattern

Preparation	RUBV HI activity
Native LDL	160
Native VLDL	160
Extracted LDL lipid fraction	128
Extracted VLDL lipid fraction	128
apoLDL	15
apoVLDL	10
Sonicated LDL	480
Sonicated VLDL	640
Lecithin + cholesteryl oleate	<5
Lecithin + triolein	<5
Lecithin + cholesterol	<5

The historical Ho/Shortridge experiments show that particle architecture and lipid/protein presentation materially change the RUBV-facing phenotype. Lipid extraction retains substantial activity, apoprotein fractions are much weaker, and sonication greatly increases activity, whereas simple lecithin dispersions containing cholesteryl oleate, triolein, or cholesterol are negative under the reported assay conditions.

The defensible conclusion is not that isolated cholesteryl ester is a proven RUBV inhibitor. It is that organized lipoprotein state matters. LDL input and routing therefore remain a legitimate alternative gate that must be measured rather than assumed normal.

22. Autophagic Flux and Endolysosomal Maturation Provide a Late-Stage Functional Context

Recovered Pásztor/Megyeri work supports a distinction between early autophagy-associated signaling and later pathway completion: early signaling can be stimulated while later autophagic phases, flux, and acidic-vesicle development are impaired in established RUBV infection. Rapamycin-driven autophagy reduced RUBV yield in the thesis experiment.

This does not prove that autophagic failure causes the NPC2 phenotype. It does show that an activated upstream pathway can coexist with impaired downstream completion - another presence-versus-throughput pattern inside the same broad endolysosomal system.

A 2024 LIPA CRISPR-knockout model is retained only as a comparator: LIPA loss produces esterified-cholesterol accumulation, enlarged lysosomes, impaired autophagic flux, and impaired maintenance of lysosomal acidity under stress. The overlap helps assay design but does not reopen primary LAL failure as the lead RUBV mechanism.

23. Timing and Maturation Are Part of the Mechanism

The RUBV endolysosomal system is stage-dependent rather than static. Multiple programs show early replication-complex formation followed by major 24-72-hour structural remodeling. P150/RNA-positive vacuoles mature, and later 48-72-hour structures become large tubular/lamellar endolysosomal networks. Time-resolved ultrastructure independently shows major reorganization of cytopathic-vacuole, mitochondria, rough-ER, and Golgi architecture between earlier and later infection states.

This timing matters to interpretation. Early entry/fusion assays constrain entry chemistry. They do not directly test the physiology of a mature RUBV-modified lysosome. Likewise, a late acidic-vesicle or lipid phenotype cannot be projected backward onto entry without time-resolved measurement.

Host maturation biology adds a specific prediction: Rab7 and VPS41/HOPS are ordered before ARL8 acquisition during endosome-to-lysosome maturation, and ARL8 paralogs are partially redundant. A P150-ARL8A perturbation could therefore be buffered early and become more consequential later as ARL8-dependent maturation and cargo traffic increase. That is a testable prediction, not a demonstrated RUBV mechanism.

A full stage-resolved framework for this problem is developed in Paper V, "The Timing of Rubella - From Viral Entry to Mature Endolysosomal Replication Compartments." Paper V does not add a causal arrow to the Paper I mechanism; it defines the temporal criteria required to test the existing arrows without conflating entry, compartment establishment, late maturation, and persistence.

24. Stage-Resolved Prediction Matrix

Stage	Established RUBV biology	Required interpretation/test
Entry / first hours	Low-pH-dependent penetration/fusion; SM/cholesterol dependence.	Do not require NPC2 phenotype; define uptake/fusion/initial acidification.
12-24 h establishment	P150/RNA-positive replication structures establish.	Begin ARL8A/B, HOPS, CI-M6PR, cargo, pH, lipid measurements.
48-72 h maturation	Large tubular/lamellar endolysosomal networks and organelle reorganization.	Highest-priority window for NPC2 availability/transfer, FC residence, ER arrival.
Multi-day infection	Acidic-vesicle/autophagic phenotypes and late lipid remodeling.	Cross pH, M6P cargo, SM/ceramide/BMP, NPC2 transfer and cholesterol flux.
Persistent infection	Altered cargo internalization/ingestion can coexist with continued infection.	Determine whether throughput phenotype persists, adapts, or resolves.

A negative result obtained only during entry or before the relevant compartment has matured cannot by itself falsify a late NPC2 prediction. Conversely, a late phenotype cannot be projected backward onto entry. A valid kill test follows the same pathway through maturation.

25. Four-Gate Cholesterol-Throughput Model

Gate	Process	Key readouts	Failure pattern
1. Input	LDLR uptake, recycling, lysosomal commitment	LDLR, uptake, RAB10/RABIF/Ral-related routing	Abnormal LDL input precedes downstream defect.
2. Soluble cargo	CI-M6PR/IGF2R, RUFY1/retromer/GARP, ARL8/HOPS	NPC2, LAL, SMPD1, FUCA1, CI-M6PR recycling	Lysosomal cargo falls and/or secretion rises.
3. Lysosomal chemistry	pH, SM->ceramide, PG/BMP/LBPA, NPC2 transfer	Absolute pH, SM, ceramide, BMP/LBPA, direct NPC2 transfer	Cargo present but transfer competence low.

Gate	Process	Key readouts	Failure pattern
4. Post-NPC1 export	NPC1/contact sites -> ER -> SOAT	NPC1, ORP1L/VAP/contact machinery, CE re-esterification	NPC2/NPC1 normal but ER arrival remains low.

The current lead is Gate 2/3 convergence centered on NPC2 availability and transfer, with LAL substantially working. Gate 4 remains explicit if NPC2/NPC1 look normal but ER arrival remains impaired. Gate 1 becomes favored if LDL uptake or routing changes first.

26. The Current Lead: A Product-Side NPC2 Bottleneck

State	Predicted biochemical signature
A. Primary LAL structural/catalytic failure	LDL-CE reaches lysosome; CE hydrolysis falls; CE accumulates; FC generation is inadequate.
B. LAL routing failure	LAL can remain competent but lysosomal delivery/retention falls; lysosomal activity falls; CE accumulates; M6P-dependent recapture can correct.
C. NPC2/post-LAL failure - current lead	CE hydrolysis is substantially preserved; FC is generated; NPC2 lysosomal availability and/or transfer falls; unesterified FC residence increases in LE/Lys; ER arrival and SOAT re-esterification fall.

State C is the strongest current Jacob prediction. Its defining feature is not simply cholesterol accumulation. It is the combination of preserved upstream hydrolysis with impaired downstream availability.

27. Comparator Evidence: Useful Constraints, Not RUBV Proof

Dengue work provides a useful NPC2 comparator but does not close the RUBV chain. Human DENV systems have shown decreased NPC2 in secretomes in one proteomic program and NPC2 enrichment in a heavy-membrane replicon fraction in another. A direct NPC2 RNAi screen, however, did not identify NPC2 as a significant DENV dependency/restriction hit.

This mixed comparator pattern argues against a simplistic 'more NPC2' or 'less NPC2' model and supports the emphasis on localization, compartmental availability, membrane state, and transfer kinetics. None of these DENV findings should be converted into RUBV causation.

28. The RUBV-Specific Functional Bridge Is Still Missing

No recovered RUBV experiment demonstrates that P150-ARL8A binding causes ARL8 nucleotide-state or effector dysfunction; VPS39/VPS41/HOPS failure; RUFY1 failure; CI-M6PR/IGF2R redistribution, degradation, or recycling failure; NPC2 lysosomal loss, secretion, abnormal M6P processing, or transfer impairment; or the full LDL-CE -> LAL -> FC -> NPC2/NPC1 -> ER/SOAT flux defect.

No established RUBV CPV study provides species-resolved SM:ceramide:BMP chemistry together with calibrated absolute luminal pH. No recovered study measures NPC2 transfer activity directly in RUBV-infected cells.

These are not gaps to hide. They define the shortest route from a strong convergent model to proof or falsification.

29. What Would Move the Model from Convergence to Direct Mechanism

The present evidence supports strong mechanistic convergence but not a completed RUBV-specific causal chain. The model crosses that boundary only when the viral system itself reproduces the host-defined phenotype in the predicted temporal order.

The strongest positive result would combine five observations in the same RUBV experiment: substantial CE hydrolysis and FC generation; reduced lysosomal NPC2 availability and/or direct transfer function; increased unesterified-FC residence in P150/dsRNA-positive late endosomal/lysosomal structures; reduced ER arrival and SOAT-dependent re-esterification; and mechanistic rescue by correcting the implicated trafficking or chemistry step.

A P150 separation-of-function experiment would raise the evidence further. If an ARL8-binding-defective P150 mutant preserved viral replication functions needed for the assay while preventing the downstream

cargo/NPC2/cholesterol phenotype, the P150-ARL8 branch would move from physical interaction plus host precedent toward direct viral mechanism.

The current aa 36-49 helix mutants are mapping candidates, not established ARL8-binding mutants. The interface must be mapped before they can serve as clean causal reagents.

30. The Decisive Crossed RUBV Experiment

- Use matched mock and RUBV conditions across early entry, 12 h, 24 h, 48 h, 72 h, established multi-day infection, and persistent states where feasible; index every sample by P150/dsRNA-positive compartment maturity as well as clock time.
- Measure LDL uptake, LDL-CE disappearance, direct LAL protein/activity, FC generation, and LE/Lys/CPV unesterified-FC residence.
- Measure total and compartmental CI-M6PR/MPR300/IGF2R; receptor retrieval/recycling/degradation; ARL8A/B membrane recruitment; VPS39/VPS41/HOPS; RUFY1; Rab14; SNX1; and selected retromer/GARP readouts.
- Measure total, lysosomal, and extracellular NPC2; NPC2 M6P/processing state where technically feasible; and direct NPC2 cholesterol-transfer activity.
- Measure LAL, SMPD1/ASM, FUCA1, and selected soluble lysosomal hydrolases in lysosome and extracellular medium, with LAMP1/2 and ACP2 as non-equivalent controls.
- Measure NPC1 and post-NPC1 ER arrival through SOAT/ACAT re-esterification, SREBP/sterol-feedback readouts, and contact-site machinery where needed.
- Perform species-resolved CE/FC/PC/LPC/SM/ceramide/PG/BMP/LBPA lipidomics.
- Calibrate absolute pH in P150/dsRNA-positive compartments by ratiometric methods rather than assigning historical entry thresholds to mature CPVs.
- Once the interface is mapped, compare wild-type P150/virus with an ARL8-binding-defective separation-of-function mutant; E36A, K46A, and I49A are mapping candidates, not presumed ARL8-binding mutants.

31. Predetermined Kill-Test Matrix

Observed pattern	Interpretation
M6P cargo falls in lysosome + secretion rises; LAMP1/ACP2 preserved	Selective soluble-cargo routing lesion strongly supported.
NPC2 disproportionately affected; LAL/SMPD1/FUCA1 preserved	Selective NPC2 bottleneck favored.
Cargo normal; pH/SM:ceramide/BMP abnormal; direct NPC2 transfer low	Chemistry/transfer-competence branch favored.
CE hydrolysis falls before FC generation	LAL structural/catalytic or routing failure favored; current post-LAL lead weakened.
NPC2/NPC1 normal; ER arrival low; contact-site readouts abnormal	Post-NPC1 export lesion favored.
LDLR uptake/recycling changes first	Input/load branch favored.
Early measurements normal, late established compartment abnormal	Timing/maturation model supported.
All routing, pH, lipid chemistry, NPC2 transfer, and LDL-CE-to-ER flux remain normal during robust established/late infection	Central cholesterol-throughput model substantially falsified.

32. Modern Same-System Data Doors and Unfinished High-Value Analyses

Two modern RUBV transcriptomic resources provide same-system opportunities that should be mined without converting unexamined datasets into evidence. GSE192648 / PRJNA792562 contains a multi-strain RUBV HUVEC bulk-RNA-seq matrix. Priority targets include LIPA, NPC2, NPC1, IGF2R, M6PR, GNPTAB/G, NAGPA, ARL8A/B, VPS39/41, RUFY1, SMPD1, FUCA1, ATP6V/ATP6AP/TCIRG1, cathepsins, CLN8, and input/post-NPC1 genes.

GSE225949 / PRJNA938090 compares live Wb12 with UV-inactivated RUBV and interferon conditions in HUVEC. Its highest value for Paper I is discrimination of live-virus-specific cargo/acidification signatures from

generic interferon responses. No target-row call from these resources is treated as evidence in this paper until the underlying matrix is directly analyzed.

These datasets matter because the next increment in Paper I should come from same-system RUBV evidence, not from adding more comparator biology. The mechanistic map is already sufficiently defined to make targeted re-analysis informative.

33. Hard Negatives and Publication Boundaries

- No calibrated absolute pH of an established RUBV-positive endosome, lysosome, autolysosome, or P150/dsRNA-positive CPV has been recovered.
- Do not convert the historical RUBV pH values (~5.8, below 6, ~5, or 5.0-5.5) into mature CPV luminal pH values.
- Do not convert P150N-ARL8A binding into functional ARL8/HOPS inhibition without a downstream RUBV experiment.
- Do not claim ARL8A binds the P150 aa 36-49 helix or that E36A/K46A/I49A are ARL8-binding mutants unless mapped directly.
- Do not claim RUBV causes CI-M6PR failure, NPC2 loss/secretion, abnormal NPC2 M6P processing, or impaired NPC2 transfer until measured.
- Do not reopen LAL as the lead bottleneck unless direct RUBV data show dominant loss of LAL activity or CE hydrolysis.
- Do not use LAMP1 or acid-phosphatase positivity as proof of normal soluble M6P-cargo delivery.
- Do not treat SGMS1-knockout lipidomics as infection-induced RUBV lipidomics.
- Do not claim isolated cholesteryl ester is a proven RUBV inhibitor; the simple cholesteryl-oleate dispersion was negative in the recovered Ho/Shortridge assay.
- Do not assign FUCA1 as the cause of the 1981 fucosylglyceride without direct substrate/enzyme evidence.
- Do not convert LIPA/CH25H/CLN8/CTSS transcript changes into protein abundance, localization, enzyme activity, or lipid flux.
- Do not convert DENV or other comparator-virus mechanisms into RUBV proof.
- Do not use an early-entry negative assay to claim that a late established NPC2 phenotype has been excluded.
- Do not infer clinical treatment efficacy or a general human disease mechanism from this cellular model.

34. Conclusion

Paper I now resolves the original ingress-versus-egress idea into a specific, testable throughput model. The strongest current prediction is not primary LAL failure. It is a post-LAL NPC2 availability/function defect in a maturing RUBV-modified endolysosomal system.

The evidentiary convergence is substantial. RUBV replicates in virus-modified lysosomes; mature CPVs remain connected to endocytic cargo; persistent RUBV can preserve cargo binding while impairing internalization; P150N physically binds ARL8A; the ARL8A-positive construct almost exactly overlaps the mapped P150 membrane-targeting module; host ARL8/HOPS and CI-M6PR biology directly controls NPC2 availability and cholesterol egress; M6P receptors directly control NPC2 lysosomal targeting; LAL itself is M6P-recapturable; human RUBV produces a strong lysosomal/sterol transcript module; RUBV has direct low-pH, sphingomyelin, cholesterol, cargo-handling, and late membrane-remodeling phenotypes.

The model is strongest because the positive phenotype is now exact. LAL should substantially hydrolyze CE. Free cholesterol should be generated. NPC2 lysosomal availability or transfer should then fall. Unesterified cholesterol residence should increase in late endosomal/lysosomal compartments, while ER arrival and SOAT-dependent re-esterification fall. If those events occur in sequence during established or late RUBV replication, the central mechanism is strongly supported.

The model is equally vulnerable to a clean negative result. If stage-resolved RUBV experiments show normal cargo routing, normal NPC2 localization and transfer, normal pH and membrane chemistry, and normal LDL-derived cholesterol arrival at the ER despite robust established infection, the central throughput thesis should be revised or rejected.

Presence does not equal availability. Paper I reduces that principle to a measurable question: during RUBV replication, is free cholesterol produced normally but made unavailable downstream?

Evidence Ledger: Direct, Host-Mechanistic, Comparator, and Missing

Class	Highest-value items
DIRECT RUBV	Virus-modified lysosomal replication organelles; BSA-gold access to mature CPVs; P150N-ARL8A interaction; persistent EGF internalization and RPE ingestion defects; pH/SM/cholesterol dependence; established acidic-vesicle phenotype; quantitative lipid remodeling; late fucosylglyceride; human LIPA/CH25H/CLN8/CTSS transcriptional remodeling.
DIRECT HOST MECHANISM	ARL8/HOPS cholesterol-egress phenotype; CI-M6PR/RUFY1 cargo retrieval; MPR46/MPR300 NPC2 targeting; IGF2R-dependent NPC2 delivery; M6P-sensitive LAL recapture/correction; BMP/SM/ASM effects on NPC2 transfer.
COMPARATOR / CONTEXT	DENV NPC2 proteomics/RNAi; LIPA-KO phenocopy; P150 screen-level candidates not independently validated.
MISSING RUBV NAIL	Functional P150->ARL8/HOPS/CI-M6PR defect; NPC2 lysosomal loss/secretion/processing/transfer defect; calibrated mature CPV pH; same-cell SM:ceramide:BMP chemistry; complete LDL-CE->FC->NPC2/NPC1->ER flux defect; rescue/separation-of-function.

The purpose of this ledger is to make the evidence boundary reusable. A reader should be able to identify immediately which observations are already direct, which mechanisms are established only in host biology, which findings are contextual, and which same-system RUBV measurements would constitute the next decisive result.

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SEPTEMBER 27, 2026 SYNCHRONIZATION ADDENDUM

Paper I remains the foundational lysosomal lipid-trafficking paper. This addendum synchronizes its mechanistic language with Papers VI and VII and with Jacob's current two-bottleneck architecture. Where this addendum conflicts with earlier lead-bottleneck wording in Paper I, this later formulation controls. The original evidence record remains preserved.

30. CURRENT BOTTLENECK #1: LAL-CENTERED THROUGHPUT

The current model places Bottleneck #1 at LAL-centered lysosomal cholesteryl-ester/triglyceride throughput and capacity. The proposed rate-limiting handoff is localized after LAL hydrolysis and before NPC2 receives free cholesterol. NPC2 remains essential downstream cholesterol-transfer machinery and an important competing discriminator, but it is not the lead bottleneck in the current model.

This revision does not convert the model into a claim that RUBV directly inhibits LAL. That experiment remains missing. The strongest current formulation is functional: RUBV-associated lysosomal remodeling may create a state in which substrate delivery, hydrolysis, product residence, or post-hydrolysis transfer becomes rate-limiting despite the presence of LAL, NPC2, and NPC1. The decisive measurement remains direct RUBV-versus-mock CE/TG hydrolysis and LDL-derived cholesterol transit across the LAL-to-NPC2 interval.

31. PAPER VI SYNCHRONIZATION: DISEASE-LEVEL CAUSAL TESTS

Paper VI extends the trafficking model to a disease-level causal hypothesis for a biologically defined autism subtype, particularly developmental regression. That extension does not change Paper I's mechanistic evidence grade. Paper I supplies an upstream cellular hypothesis; Paper VI requires three additional demonstrations before disease causation can be established: the RUBV mechanism in the relevant system, the predicted compartmental cholesterol phenotype in carefully phenotyped human autism, and the required temporal ordering between exposure, molecular state, and developmental loss.

Any MMR extension is separately constrained. Wild-type RUBV and attenuated rubella vaccine virus are not interchangeable experimental exposures. Population studies cited in Paper VI do not support MMR as a common cause of autism. Therefore any vaccine-strain extension must be demonstrated directly with the relevant attenuated rubella strain and reconciled with population-level evidence and chronology.

32. PAPER VII SYNCHRONIZATION: MITOCHONDRIAL / MAO EXTENSION

Paper VII adds Bottleneck #2: monoamine oxidase (MAO) at the mitochondrial outer membrane. RUBV directly engages and functionally perturbs mitochondria: capsid interacts with p32 at mitochondria, capsid can inhibit mitochondrial precursor-protein import, infected cells show reduced intramitochondrial p32, and RUBV infection selectively remodels respiratory-chain activity. Independently, MAO function is experimentally coupled to mitochondrial membrane lipid context.

The mitochondrial outer membrane is therefore a demonstrated point of convergence between RUBV mitochondrial biology and lipid-sensitive MAO function. Historical lipid-depletion and phospholipid-reconstitution experiments show strong A-form/B-form differences, and experimental mitochondrial cholesterol enrichment decreases MAO specific activity. These findings do not establish that RUBV inhibits MAO-A or MAO-B.

A direct host bridge also exists between lysosomal sterol stress and mitochondria. In NPC1-deficient or NPC1-inhibited systems, cholesterol-rich lysosomes form expanded STARD3-dependent contacts with mitochondria. This is mechanistic precedent, not proof that Jacob's proposed post-LAL/pre-NPC2 lesion is equivalent to NPC1 deficiency or that RUBV produces the same contact phenotype.

33. INTEGRATED TWO-BOTTLENECK MODEL

Bottleneck #1: RUBV-associated lysosomal/endolysosomal remodeling -> LAL-centered CE/TG throughput/capacity -> proposed slow post-LAL/pre-NPC2 handoff.

Open bridge: altered lysosomal sterol residence/routing -> STARD3-sensitive lysosome-mitochondria contact and mitochondrial outer-membrane lipid state?

Bottleneck #2: mitochondrial outer-membrane state -> MAO-A and/or MAO-B functional alteration?

The question marks are essential. No recovered experiment yet demonstrates the complete RUBV -> LAL/post-LAL sterol state -> mitochondrial membrane -> MAO sequence in one system. Each side of the missing RUBV-to-MAO arrow is experimentally anchored, but their convergence during RUBV infection remains untested.

34. UPDATED DECISIVE EXPERIMENT

The strongest integrated experiment is synchronized RUBV-versus-mock analysis across defined replication-compartment stages. Measure lysosomal CE/TG/free cholesterol, direct LAL activity and flux, NPC2/NPC1 state, ER cholesterol arrival, STARD3 localization and lysosome-mitochondria contact frequency, mitochondrial outer-membrane cholesterol and phospholipid classes, p32 import, respiratory-chain activity, and MAO-A and MAO-B catalytic activity separately. Strong support requires the predicted compartmental state to track with the functional phenotype and, ideally, to be rescued by correcting the relevant membrane/sterol state while infection remains measurable.

If mature RUBV replication occurs without the predicted LAL/post-LAL flux defect, Bottleneck #1 is weakened. If the lysosomal/mitochondrial membrane phenotype occurs but MAO-A and MAO-B remain functionally normal, Bottleneck #2 is weakened. If neither phenotype occurs despite robust infection, the integrated two-bottleneck model fails in its present form.

35. UPDATED PUBLICATION BOUNDARY

Paper I should no longer be read as assigning the lead bottleneck to NPC2. NPC2 remains a downstream mechanistic discriminator. Do not claim direct RUBV inhibition of LAL, NPC2, NPC1, ARL8, HOPS, CI-M6PR, MAO-A, or MAO-B without the corresponding same-system functional experiment. Do not infer autism causation, MMR causation, or treatment efficacy from the cellular model alone.

ADDITIONAL REFERENCES FOR THIS ADDENDUM

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