**Endoplasmic reticulum chaperone Gp96 controls actomyosin dynamics and protects against pore-forming toxins**

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**SUMMARY**

During infection, plasma membrane (PM) blebs protect host cells against bacterial pore forming toxins (PFTs), but were also proposed to promote pathogen dissemination. However, the details and impact of blebbing regulation during infection remained unclear. Here we identify the endoplasmic reticulum chaperone Gp96 as a novel regulator of PFT-induced blebbing. Gp96 interacts with non-muscle myosin heavy chain IIA (NMHCIIA) and controls its activity and remodelling, which is required for appropriate coordination of bleb formation and retraction. This mechanism involves NMHCIIA-Gp96 interaction and their recruitment to PM blebs and strongly resembles retraction of uropod-like structures from polarized migrating cells, a process that also promotes NMHCIIA-Gp96 association. Consistently, Gp96 and NMHCIIA not only protect the PM integrity from listeriolysin-O (LLO) during infection by *Listeria monocytogenes* but also affect cytoskeletal organisation and cell migration. Finally, we validate the association between Gp96 and NMHCIIA *in vivo* and show that Gp96 is required to protect hosts from LLO-dependent killing.

**Keywords:** Actomyosin/ Endoplasmic reticulum chaperone/ Listeria monocytogenes/ Plasma membrane blebbing/ Pore-forming toxins

**INTRODUCTION**

Plasma membrane (PM) blebs are dynamic cell protrusions, which depend on non-muscle myosin II (NMII) activity and have been associated with multiple processes such as apoptosis, cytokinesis, and cell migration [1,2]. Regarding bacterial infections, blebs preserve PM integrity upon damage caused by bacterial pore forming toxins (PFTs) [3]; allow the establishment of an intracellular replicative niche for *Pseudomonas aeruginosa* [4]; are released by infected macrophages promoting *Mycobacterium* *tuberculosis* killing byneighboring macrophages (efferocytosis) [5] or favouring cell-to-cell spreading of *Listeria monocytogenes* (*Lm*)[6].

*Lm* is a facultative intracellular human foodborne pathogen that causes severe infection in susceptible hosts. Virulence mainly depends on the activity of its secreted PFT, the cholesterol-dependent cytolysin listeriolysin O (LLO) [7,8]. LLO targets phagosomal membranes allowing escape of *Lm* to the cytosol and has numerous other roles during infection [7]. In particular, LLO promotes pathogen dissemination by inducing controlled necrosis [9] and release of bacterial-containing blebs [6].

Endoplasmic reticulum (ER) stress pathways are central for host survival against PFTs, including LLO, and ER distribution is often altered during intoxication [10,11]. Concurrently, we have showed that *Lm* infection redistributes the ER chaperone Gp96 to the PM through an uncharacterised mechanism [12]. Gp96 is an ER-resident HSP90 paralogue, which controls the expression and folding of proteins assigned to the secretory pathway and has crucial roles in cellular homeostasis, host development and immunity [13-15]. Gp96 translocates to the PM where it interacts with different bacteria, modulating adherence, internalisation [16-18], survival [19] and endothelial permeability [20]. Cascades involved in these processes may rely on calcium signalling, protein kinase C (PKC) activation and nitric oxide production. However, the molecular outcomes underlying such events remain poorly defined.

Given that PFTs alter ER distribution, we evaluated if LLO affects Gp96 distribution and function, thereby modulating host responses to *Lm*. We found that LLO triggers the interaction between Gp96 and NMHCIIA and the concomitant assembly of unique NMHCIIA cortical bundles, which coordinate the formation and retraction of PM blebs and preserve PM integrity during *Lm* cellularinfection. Strikingly, this process resembles the formation of uropod-like structures required for tail retraction during polarised cell migration [21,22] and is controlled by Gp96 that regulates NMII activity and general cytoskeleton-driven cell properties. Importantly, Gp96 interacts with NMHCIIA during *in vivo* *Lm* infection and promotes host survival. Our data establish a novel ER-cytoskeletal interplay crucial for host protection against PFT-mediated bacterial infection.

**RESULTS**

***Lm* causes LLO-dependent ER redistribution and promotes the interaction between Gp96 and NMHCIIA**

Given that *Lm* infection leads to increased levels of PM-associated Gp96 and to ER expansion and that different PFTs were reported to alter ER distribution, we studied the effect of LLO on ER morphology and Gp96 surface levels. HeLa cells infected with wild type (wt) *Lm,* but not with LLO-deficient bacteria (Δ*hly*),displayedincreased Gp96 PM levels and contained distinct ER vacuoles/structures harbouring proteins with ER retention signal (KDEL), including Gp96 (Fig EV1A-B). Purified LLO was sufficient to induce a dose-dependent ER-Gp96 redistribution with ER vacuoles/structures expanding close to the PM (Fig EV1C-E). Concurrently, surface-exposed Gp96 was also increased upon LLO treatment (Fig EV1F).

We tested whether the LLO-mediated ER-redistribution could alter Gp96 interacting partners. Gp96 immunoprecipitation (IP) fractions from untreated or LLO-treated HeLa cells were resolved by SDS-PAGE. A specific band from LLO-treated sample was identified, by tandem mass spectrometry analysis, as non-muscle myosin heavy chain IIA or NMHCIIA (*p*<0.05). Peptide coverage spanned 23% of the NMHCIIA sequence, covering all functional domains (Fig EV1G). Immunoblot analysis of Gp96 IP confirmed that LLO triggers NMHCIIA-Gp96 co-IP in a dose responsive manner (Fig 1A), yet the interaction decreased upon high concentrations of LLO possibly due to cytotoxicity. LLO increased NMHCIIA-Gp96 co-IP also during *Lm* infection (Fig 1B and EV2A-D). The specificity of the IPs was confirmed using isotype antibodies (Fig EV2A-B). Moreover, the NMHCIIA-Gp96 interaction was amplified throughout the infection time (Fig 1C) and could also be detected in colon epithelial (Caco-2) and macrophage-like (RAW264.7) cells (Fig EV2C-D), showing that *Lm* infection promotes LLO-dependent NMHCIIA-Gp96 co-IP in various cell types.

Analysis of NMHCIIA and Gp96 cellular distribution revealed that, besides NMHCIIA-Gp96 co-IP, LLO induced the formation of distinct NMHCIIA cortical clusters or bundles which associated with cortical Gp96 in HeLa (Fig 1D) and Caco-2 (Fig EV2E). Quantification of NMHCIIA-Gp96 co-localization confirmed that both proteins co-localized within cortical bundles when compared to other cellular regions (control region of interest, ROI) or to the overall NMHCIIA-Gp96 correlation in the entire cell in both untreated and LLO-treated cells (Fig 1E). Infection with wt but not Δ*hly Lm* promoted the formation of similar cortical structures, often associated with intracellular bacteria (Fig 1F). Thus, through LLO, *Lm* redistributes the ER, Gp96 and NMHCIIA into unique cortical bundles and triggers novel association between the ER and the host cytoskeleton.

**NMHCIIA-ER/Gp96 bundles are hallmarks of PFT-induced blebbing**

NMII activity is required for PFT-induced PM blebbing [3]. We thus analysed the dynamics of LLO-induced NMHCIIA remodelling in HeLa cells ectopically expressing GFP-NMHCIIA and/or mcherry-KDEL (mcherry fused to KDEL signature). LLO induced a profound reorganisation of the NMHCIIA network into cortical bundles at sites of PM blebbing (Fig 2A and Movie EV1). Cortical ER structures and vesicles associated with NMHCIIA bundles and localized within PM blebs (Fig 2B and Movie EV2). PM labelling with fluorescently-conjugated wheat germ agglutinin (FITC-WGA) showed NMHCIIA bundles at PM blebs, which occasionally appeared detached from the cell body (Fig 2C). Phosphorylated ezrin (p-ezrin), which connects the PM to cortical actin and is recruited to PM blebs [23], was present at NMHCIIA bundles (Appendix Fig S1A), further validating the link between bundles and PM blebs.

PFT-induced PM blebbing depends on Ca2+ influx from the extracellular milieu caused by PM damage [3]. Accordingly, treatment of HeLa cells with LLO pre-incubated with cholesterol, which blocks its pore-forming activity but allows cell surface binding and signalling [24], did not enhance NMHCIIA-Gp96 interaction or affect their cellular distribution (Fig 2D and 2G). LLO treatment carried in Ca2+-depleted medium disrupted normal NMHCIIA-ER/Gp96 distribution but failed to trigger formation of distinct NMHCIIA bundles and reduced NMHCIIA-Gp96 co-IP (Fig 2E and 2G). In contrast, increasing the extracellular concentration of K+, which prevents the K+ efflux responsible for numerous host responses to PFTs [25], did not affect NMHCIIA bundle formation or NMHCIIA-Gp96 co-IP (Fig 2E and 2G). Using blebbistatin, which inhibits the NMII contractile force required for PM blebbing [26], we showed that NMII activity is also required for both NMHCIIA-Gp96 co-IP and NMHCIIA bundles formation (Fig 2F-G). We then tested other PFTs inducing PM blebbing and showed that Aerolysin (AL) from *Aeromonas hydrophila,* induced formation of NMHCIIA-Gp96 cortical bundles, and AL and streptolysin O (SLO) from *Streptococcus* *pneumonia* promoted NMHCIIA-Gp96 co-IP (Fig 2H-I). Finally, in accordance with the transient nature of PM blebbing, host cells recovered normal NMHCIIA and ER distribution by 8 h after LLO washout (Appendix Fig S1B-D). NMHCIIA-Gp96 association and cortical bundling appear thus as hallmarks of PFT-induced PM blebbing that rely on NMII activity and Ca2+ influx upon PFTs induced PM damage.

**Gp96 and NMHCIIA regulate PM blebbing through modulation of NMII activity**

Next, we evaluated the frequency of cells harbouring NMHCIIA bundles following incubation with LLO, AL or *Lm* infection of HeLa cells expressing control oligonucleotides (shControl) or oligonucleotides targeting the expression of Gp96or NMHCIIA (shGp96 and shNMHCIIA) (Appendix Fig S2). For all conditions, Gp96 depletion significantly reduced the frequency of cells with NMHCIIA bundles when compared to controls (Fig 3A-D). Such effect was observed in response to different LLO concentrations (Fig EV3A). LLO-treated shGp96 cells displayed less bundles per cell (Fig EV3B) and show a disrupted cortical actomyosin network, which appeared unable to bundle. Expectedly, actin co-localised with NMHCIIA bundles (Fig EV3C) and could also hallmark PFT-induced cortical cytoskeletal bundles. Similarly to blebbistatin treatment, depletion of NMHCIIA impaired bundling (Fig 3A-C and Fig EV3C).

Subsequently, we studied GFP-NMHCIIA dynamics during LLO intoxication in control or Gp96-depleted cells. Upon LLO treatment, shControl cells assembled NMHCIIA bundles and displayed organised PM blebbing with PM blebs expanding and retracting (Fig 3G and Movie EV3). In contrast, shGp96 cells failed to stabilize their NMHCIIA network into cortical bundles and displayed uncontrolled PM blebbing, with more blebs per cell and less bleb retraction throughout the experiment (Fig 3E-G and Movie EV4). Blebbistatin inhibited NMHCIIA rearrangements, causing disruption of cell morphology during LLO treatment (Fig 3G and Movie EV5). These cells showed passive blebbing with limited expansion, which agrees with the requirement of NMII activity for PM blebbing [1,3,23].

NMII activity and actomyosin filaments turnover are regulated by phosphorylation of the myosin-II regulatory light chain (MRLC) [27,28]. In resting conditions, when compared to shControl, shGp96 cells showed higher levels of MRLC phosphorylation (pMRLC) (Fig 3H). Moreover, whereas MRLC was rapidly dephosphorylated in shControl cells upon LLO treatment, its phosphorylation was sustained longer in shGp96 cells (Fig 3H). These data demonstrate that Gp96 modulates NMHCIIA activity and dynamics required for actomyosin network remodelling during PM blebbing.

**Coordination of PM blebbing resembles control of cell polarity and trailing edge retraction during polarized cell migration**

To our knowledge, ER-cytoskeletal interactions during PM blebbing have never been described. However, the ER was associated with the assembly of trailing edge uropod-like Wnt-receptor-actin-myosin-polarity (WRAMP) structures during polarised migration [22]. We thus surveyed PFT-induced NMHCIIA bundles for proteins found in WRAMPs. Our co-localization analysis demonstrated that NMHCIIA co-localized with WRAMP markers such as NMHCIIB, lysosomal associated glycoprotein 1 (LAMP1), tubulin, ERK, and Filamin A within the cortical cytoskeletal bundles. In addition, actin and calpain 2 also co-localised at cortical bundles (Fig 4A). Notably, Filamin A, which crosslinks actin filaments and is required for uropod retraction [29], co-IP with Gp96 upon LLO treatment (Fig 4B). Moreover, promoting WRAMP/uropod assembly with Wnt5a [22] induced NMHCIIA-Gp96 co-IP and polarised distribution of NMHCIIA-ER/Gp96 (Fig 4C-D).

Together these data indicate that NMHCIIA and Gp96 stabilize rapid cytoskeletal rearrangements, which coordinate membrane retraction and cytoskeletal organization in different cellular contexts. Consistently, Gp96 depletion increased formation of stress fibers and focal adhesions (Fig 4E-H), which correlated with an elongated and less circular and irregular cell shape (Fig EV4). On the other hand, NMHCIIA-depleted cells expectedly lack stress fibers (Fig 4E-H) and displayed a less circular and irregular but not elongated cell shape (Fig EV4). Conclusively, wound closure assay showed that both Gp96- and NMHCIIA-depleted cells display reduced cell migration compared to control cells (Fig 4I-J and Movie EV6). Thus, Gp96 and NMHCIIA are recruited to polarised cell structures and coordinate cytoskeletal organisation and polarity, affecting cell shape, blebbing and migration.

**Gp96 and NMHCIIA control PM integrity following LLO treatment of host cells**

Given the protective role of PM blebbing during PFT intoxication [3], we investigated whether Gp96 and NMHCIIA depletion affected loss of PM material or compromised PM integrity after LLO treatment. We isolated PM-blebs from culture supernatants of LLO-treated cells and observed that shGp96 and shNMHCIIA cells released more PM material to supernatants when compared to shControl cells (Fig 5A-B). The lack of DAPI staining in our samples confirmed that PM bodies were not originated from dead cell detachment (Fig 5A). Subsequently, we assessed PM integrity by flow cytometry, measuring the incorporation of the impermeable dye, propidium iodide (PI). Depletion of Gp96 and NMHCIIA increased PI uptake throughout the time of LLO treatment and upon increasing LLO concentrations (Fig 5C-D). Nevertheless, cells treated with very high concentrations of LLO displayed equivalent PI uptake, consistent with their inability to recover from exposure to overwhelming PFT concentrations (Fig 5D). Moreover, comparing to shControl, shGp96 or shNMHCIIA cells showed reduced ability to recover PM integrity even after 24 h of LLO washout (Fig 5E). These data show that besides regulating PM blebbing, Gp96 and NMHCIIA control PM integrity in response to LLO intoxication.

**Gp96 and NMHCIIA preserve PM integrity during *Lm* infection**

PM damage caused by PFTs disrupts lipid asymmetry, exposing phosphatidylserine (PS) to the extracellular space [3,6]. Accordingly, exofacial-PS sites labelled with fluorescently-conjugated annexin-V were virtually absent from untreated cells but were abundantly spread throughout the cell and often co-localized with NMHCIIA cortical bundles upon LLO treatment (Fig 6A). Exofacial-PS was also detected in infected HeLa cells and frequently associated with bacteria and/or NMHCIIA cortical bundles (Fig 6B). Notably, the percentage of cells showing exofacial-PS sites was increased in shGp96 or shNMHCIIA cells, 6 h after bacterial invasion (Fig 6B-C). Consistently, depletion of Gp96 and NMHCIIA from both HeLa cells and RAW264.7 macrophages (Appendix Fig S3A) increased PI uptake by host cells following infection with wt *Lm* but not with Δ*hly* bacteria (Fig 6D-E). Equivalent numbers of intracellular bacteria were verified (Appendix Fig S3B-C). These data demonstrate that Gp96 and NMHCIIA, while controlling actomyosin dynamics and PM blebbing, preserve PM integrity from LLO-mediated damage during *Lm* infection.

**Gp96 protects the host against LLO-mediated killing *in vivo***

We used *Lm* infection of zebrafish (*Danio rerio*) larvae to evaluate the protective role of Gp96 during infection *in vivo.* Zebrafish have a single Gp96 ortholog sharing 85% homology with the human Gp96, and Gp96 morphants (i.e., zebrafish embryos depleted of Gp96 by morpholino oligonucleotides) develop normally at 1, 2, and 5 days post fertilization (dpf) [30]. Zebrafish was proposed as a suitable model to study *Lm* pathogenesis and interaction with phagocytes *in vivo* [31]. We confirmed that *Lm* injected in the hindbrain of zebrafish larvae 3 dpf colonize the host and cause a dose dependent lethality that requires LLO expression (Fig EV5A-B). Furthermore, we also verified that *Lm* recruits and interacts with macrophages *in vivo* (Fig EV5C and Mov EV7) and detected bacterial-associated actin clouds and comet tails *in vivo* (Fig EV5D).

We evaluated if *Lm* infection could trigger the formation of Gp96-NMHCIIA complexes *in vivo*. Gp96 IP from infected larvae revealed that *Lm* infection promoted a strong NMHCIIA-Gp96 interaction, which was enhanced by LLO expression (Fig 7A). Furthermore, analysis of infected larvae showed an LLO-dependent ER and cytoskeletal disorganisation (Fig 7C). Infected Gp96 morphants displayed an apparent increase in tissue damage and bacterial spreading compared to controls (Fig 7B-C), which nicely correlated with an increased bacterial load and diminished host survival (Fig 7D-E). Importantly, lethality of Gp96 infected-morphants was dependent on the expression of LLO, since Gp96 morphants recovered from Δ*hly* infection, even when larvae were inoculated with 10 times more bacteria (Fig 7D). Such recovery was equivalent to the one observed in wt zebrafish larvae (Fig 7F). Hence, Gp96 protects against LLO-mediated killing during *Lm* infection *in vivo.*

**DISCUSSION**

The transient blebs displayed by PFT-wounded cells were proposed to confine PM damage and protect the host cytosol from leakage and Ca2+ overload. Despite the evidences that favour such protective role [3,32], blebbing has been mainly studied in the context of cell migration and it remains unclear how host cells maintain cytoskeletal cohesion during PM recovery responses. We show here that the ER chaperone Gp96 plays an important role during this process. Gp96 modulates NMHCIIA rearrangements and NMII activity required for the assembly of cytoskeletal cortical bundles that coordinate formation and retraction of PM blebs. This mechanism preserves PM integrity against PFTs and intracellular bacteria, and involves ER redistribution to cortical sites and interactions between Gp96, NMHCIIA and Filamin A. Notably, we demonstrate that these events also occur during cell migration and assembly of uropod-like WRAMP structures, suggesting a common mechanism to regulate PM retraction and cytoskeletal dynamics during PFT-induced PM blebbing and cell migration [21,22,29]. Consistent with our findings, NM-II has established roles in both processes [28,33] and Gp96 was recently shown to promote cell polarity and migration [34,35].

How the NMHCIIA-Gp96 interplay coordinates cytoskeleton dynamics and PM remodelling remains elusive. Despite the well-known protective role of PM blebbing against PFT-mediated damage, the apparent opposite effects of Gp96 and NMHCIIA activity in PM blebbing indicate that cytoskeletal homeostasis must be fine-tuned in order to respond rapidly to changes in organization and polarity, which can regulate PM blebbing and avoid cell death. Such balance of cytoskeletal activity and membrane plasticity may derive from control of vesicular trafficking and organelle dynamics. Different studies have suggested that polarised secretion of lysosomes and redistribution of ER and/or endosomal compartments regulate uropod dynamics and trailing edge retraction during migration of cancer cells or leukocytes [22,36]. We show that ER and LAMP1-positive compartments localized within NMHCIIA bundles and Gp96 was proposed to modulate polarity *via* vesicular trafficking [35]. Additionally NMHCIIA can control polarised secretion [37], organelle positioning [38] and was recently shown to mediate lysosomal exocytosis in PFT-wounded cells [39]. Thus, it is possible that the link NMHCIIA-Gp96 assists the assembly of actomyosin cortical structures in migrating cells or at wound sites through the control of vesicular compartments. In addition, Gp96 can regulate Wnt signalling, which modulates cytoskeletal dynamics and cell polarity, *via* expression of Wnt co-receptors [14].

We propose that Gp96-mediated actomyosin regulation protects against LLO activity during infection. Pathogenic *E. coli* can target Gp96 to promotenitric oxide production, Ca2+ oscillations and activation of cellular kinases such as PKC-α and FAK, which disrupts endothelial cell junctions and mediates bacterial internalisation [40]. LLO and other PFTs trigger such events, which were associated with increased tissue damage *in vivo* [41,42],and NMHCIIA protects the epithelial barrier *in vivo* [43]. Interestingly *Lm* caninteract with Gp96 *via* its surface protein Vip, which promotes bacterial internalization [18]. Vip is not sufficient to activate host kinases and mediate bacterial invasion of host cells. It is thus possible that LLO and Vip act together to modulate the host cell cytoskeleton and control bacterial internalisation, cell death and/or endothelial damage *in vivo.* Notably, deletion of *vip* affects multiple infection stages besides internalisation, which can also be promoted by LLO or reduced by NMHCIIA [18,44,45].

In the other hand, macrophage-specific deletion of *gp96* renders mice highly susceptible to *Lm*, but this phenotype was attributed to the lack of toll-like receptors (TLRs) [15]. Gp96 did not affect intracellular bacterial replication *in vitro* or the ability of zebrafish larvae to recover from infection with high doses of LLO-deficient bacteria. The susceptibility of Gp96 morphants may result from a combination of the lack of TLRs and the inability of cells to control LLO-mediated damage. LLO and other PFTs can induce programmed necrosis and PM blebbing in infected macrophages. Both process were proposed to favour infection *in vivo* [6,9,46]. In agreement with this, larvae infected with wt bacteria display pronounced tissue damage, disorganised ER distribution, phenotypes that appear aggravated in absence of Gp96.

In conclusion, we describe Gp96 as novel regulator of PM blebbing during PFT-induced PM damage and infection, and further highlight the important crosstalk between the ER and cytoskeleton which impacts fundamental cellular processes [47] and innate responses to bacteria.

**Materials and Methods**

**Plasmids, Antibodies and Dyes**

Plasmid GFPNMHCIIA (#11347) was obtained from Addgene [48] and mCherry-KDEL was a gift from M. Davidson through Addgene (mCherry-ER-3, #55041). The following antibodies were used at 1/200 for immunofluorescence microscopy (IF) or 1/1000 for immunoblotting (IB): rabbit anti-NMHCIIA (Sigma); mouse anti-NMHCIIA (Abcam); rat anti-Gp96 (Enzo); mouse anti-actin, AC-15 (Sigma); rabbit anti-Filamin A (Santa Cruz); rabbit anti-MRLC or anti-phosphoMRLCSer19 (Cell Signaling); rabbit anti-*Lm* (Abcam); mouse anti-α-tubulin (Sigma); goat anti-Calpain 2 (Santa Cruz); rabbit anti NMHCIIB (Sigma), rabbit anti-ERK (Cell Signaling), rabbit anti-Calnexin (Millipore); rabbit anti-GRP78 BiP (Abcam); rabbit anti-FAK (Santa Cruz); rabbit phospho-Ezrin (Thr567) (Cell Signaling); mouse anti-KDEL (Abcam). For IF, F-actin was labeled with Alexa Fluor 647- or 555-conjugated phalloidin ([Invitrogen](http://www.jbc.org/cgi/redirect-inline?ad=Invitrogen)), PM with FITC-conjugated WGA (Sigma) that recognises sialic acid and N-acetylglucosaminyl sugar residues at PM and DNA with 4’,6-Diamidino-2-phenylindole dihydrochloride, DAPI (Sigma). For IF secondary antibodies were used at 1/500: Goat anti-rabbit or anti-mouse Alexa Fluor 488 (Invitrogen), goat anti-rat Alexa Fluor 568 or 647 (Invitogen) goat anti-rabbit or anti-mouse Cy3 (Jackson ImmunoResearch), goat anti-rabbit or anti-mouse Cy5 (Jackson ImmunoResearch), Donkey anti-goat or anti-rabbit Alexa 555 or 488 (Invitrogen). For IB secondary antibodies were used at 1/10000 and obtained from: goat anti-rabbit or anti-mouse HRP (PARIS), goat anti-Rat HRP (Santa Cruz).

**Reagents and Toxins**

Drug and toxin treatments and washes were carried in Hank's Balanced Salt Solution (HBSS) or as indicated in HBSS Ca2+ free medium or supplemented with 140 mM K+. Blebbistatin (Sigma) was used at 25 μM for 30 min prior LLO and maintained throughout treatment. Cholesterol (Sigma) was used as described [24]. LLO was purified as described [49]. Human Active Wnt5a (abcam) was used at 25 μg/ml for the indicated time. For LLO recovery assays cells were washed three times in HBSS and recovered in culture medium supplemented with 10% fetal bovine serum (FBS, Biowest). Purified proaerolysin [50] was used at 0.2 nM for 40 min and SLO [51] at 1.5 μg/μl for 30 min.

**Cell Lines and Zebrafish**

Caco-2 cells (ATCC HTB-37) were cultivated in EMEM medium with L-glutamine, supplemented with nonessential amino acids, sodium pyruvate and 20% FBS. HeLa (ATCC CCL-2) and RAW264.7 (ATCC TIB-71) cells were cultivated in DMEM with glucose and L-glutamine, supplemented with 10% FBS. Cells were maintained at 37 °C in a 5% CO2 atmosphere. Cell culture media and supplements were from Lonza. Wild-type AB zebrafish were from the Zebrafish International Resource Center (Eugene), and the transgenic line Tg(*mpeg1*:G/UmCherry) was previously described [52,53]. Embryos were raised at 28°C in E3 medium with 0.003% 1-phenyl-2-thiourea (Sigma) to prevent pigmentation for microscopy. Larvae were anesthetized with 200 µg/ml tricaine (Sigma) for injection procedure and *in vivo* imaging.

**Transfections, shRNA lentiviral Transductions and Morpholinos**

HeLa cells (5x104) seeded into Ibitreat µ- dishes (Ibidi) were transfected with 0.5 µg of the indicated plasmid DNA using jetPRIME®-Polyplus-transfection reagent according to the manufacturer's instructions. The lentiviral shRNA expression plasmids Mission pLKO.1-puro (control) and Mission shRNA-Gp96 or shRNA-Myh9 (Sigma) were used in combination with the envelope plasmid pMD.G and packaging plasmid pCMVR8.91 and co-transfected into HEK293 cells. Viral supernatants were harvested after 72 h, filtered, and incubated with target HeLa cells for 48 h. Puromycin was used for selection of cell lines. Efficient knockdown was verified by immunoblot. Oligonucleotide sequences (Sigma) used for shRNA knockdown experiments:

shControl-SHC016-CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTTT; shGp96#1.TRCN0000029425. CCGGCCTGTGGATGAATACTGTATTCTCGAGAATACAGTATTCATCCACAGGTTTTT;

shGP96#2.TRCN0000029426 CCGGCGTGGTCTGTTTGACGAATATCTCGAGATATTCGTCAAACAGACCACGTTTTT;

shGp96#4.TRCN0000276247. CCGGCCATGATATGATGCCTAAATACTCGAGTATTTAGGCATCATATCATGGTTTTTG.

shMyh9#1.TRCN0000276055. CCGGCCGCGAAGTCAGCTCCCTAAACTCGAGTTTAGGGAGCTGACTTCGCGGTTTTTG;

shMyh9#3.TRCN0000029467. CCGGCCGCGAAGTCAGCTCCCTAAACTCGAGTTTAGGGAGCTGACTTCGCGGTTTTT;

shMyh9#4.TRCN0000029468. CCGGGCCAAGCTCAAGAACAAGCATCTCGAGATGCTTGTTCTTGAGCTTGGCTTTTT;

Antisense morpholino oligonucleotides, Control (TACCAAAAGCTCTCTTATCGAGGGA, with no known target on the zebrafish genome) and Gp96 specific (GAAGTTCAAGTGCATACAAACCTCT) targeting nuclear processing, were obtained from GeneTool. Thawed morpholinos were heated at 65°C for 10 min to ensure complete dissolution, and diluted to the desired concentration (typically, 250 to 500 µM, for an injected amount of 2-4 ng) in morpholino buffer (120 mM KCl, 10 mM Hepes pH 7.2) containing 0.1% phenol red. Injections were performed (1 nl/embryo) into 1-cell embryos.

**Bacterial Infections**

Wild type (wt), Δ*hly* mutant or GFP-expressing wt or Δ*hly* *Lm* (EGDe), were grown overnight at 37°C, with shaking, in brain-heart infusion (BHI, BD-Difco), sub-cultured at a 1:10 dilution until optical density of 0.8–1, washed and inoculated into cells at a multiplicity of infection of 50. After 1 h infection medium was replaced by complete medium with 20 μg/ml gentamicin (Sigma) and infection was allowed to proceed. For injection of zebrafish larvae, washed bacteria were resuspended in phosphate-buffered saline PBS (Lonza). Anesthetized zebrafish larvae, 3 day post-fertilisation (dpf), were microinjected in the Hindbrain Ventricle or tail muscle with 0.5–2 nl of bacterial suspension as described [52]. Real inoculum was verified by plating at least 3 independent larvae onto BHI agar. Infected larvae were transferred into individual wells (containing 1 ml of E3 in 24-well plates), incubated at 30°C and regularly observed under a stereomicroscope. Infections with a quantified standard dose were performed at least 3 times.

**Immunoprecipitation and Immunoblotting Assays**

HeLa, Caco-2 or RAWS264.7 cells (6×106 cells) treated as indicated, were washed with PBS, lysed in RIPA buffer (Santa Cruz) and lysates were recovered after centrifugation (15,000 × g, 10 min, 4°C). For zebrafish larvae, approximately 10 larvae (4 dpf) left uninfected or infected with low dose (200-1500 cfu/ml) of the indicated *Lm* strains for 24 h were anesthetized, rinsed, lysed in 400-600 μl RIPA buffer and lysates were recovered after 2 rounds of centrifugation (15,000×g, 10 min, 4 °C). Cell lysates (500-700 μg) were pre-cleared with protein G-immobilized Pure Proteome magnetic beads (Millipore) and incubated overnight (4°C) with 3 μg of anti-Gp96 or 5 μg of anti-NMHCIIA antibodies. Immune complexes were captured with 50 μl of protein G-immobilized magnetic beads (Millipore) and processed for Immunobloting (IB). Protein lysates and IP fractions were resuspended in sample buffer (0.25 mM Tris-Cl pH 6.8, 10% SDS, 50% glycerol, 5% β-mercaptoethanol), resolved by SDS-PAGE and transferred onto Nitrocellulose membranes (Hybond ECL, GE Healthcare). Primary and secondary HRP-conjugated antibodies were diluted in TBS-tween (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 0.1% Tween). IB were visualised using ECL (Thermo Scientific). Densitometry was performed on film by high-resolution scanning and analysed using ImageJ64 software (NIH).

**Protein Identification by Mass Spectrometry (MS)**

Protein identification was performed by MALDI TOF/TOF mass spectrometry as described [45]. Protein bands were excised from SDS-polyacrylamide gels, stained using PageBlue™ Protein Staining Solution (Thermo Fisher) and treated to in-gel digestion with trypsin. Peptides were extracted, desalted, concentrated using Ziptips (Millipore), crystallized onto a MALDI sample plate, and analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems). Peptidic mass spectra were acquired in reflector positive mode at a 700–4000 m/z mass window, and proteins were identified by peptide mass fingerprint using Mascot software (Matrix Science, UK) integrated in the GPS Explorer software (ABSCIEX) and searched against the SwissProt/UniProt Homo sapiens protein sequence database. The maximum error tolerance was 35 ppm, and up to two missed cleavages were allowed.

**Immunofluorescence Microscopy**

Cells were fixed in 3% paraformaldehyde (15 min), quenched with 20 mM NH4Cl (1 h), permeabilized with 0.1% Triton X-100 (5 min), and blocked with 10% BSA in PBS (30 min). Antibodies were diluted in PBS containing 1% BSA. Coverslips were incubated for 1 h with primary antibodies washed three times in PBS and incubated 45 min with secondary antibodies and phalloidin Alexa 555 or 647. DNA was counterstained with DAPI (Sigma). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (Polysciences). Images were collected with an epifluorescent Olympus BX53 microscope or a confocal laser-scanning microscope (Leica SP5II) and processed using ImageJ64 or Adobe Photoshop software. Anesthetised zebrafish larvae were fixed overnight at 4°C in 4% paraformaldehyde with 0.4% Triton X-100, then washed with PBS 0.1% Tween, and processed for IF. Larvae were incubated in PBS 1% Triton X-100 (20 min) followed by 3x 5 min washes in PBS 0.4% Triton X-100, and incubation in blocking solution (PBS supplemented with 10% BSA, 1% DMSO, and 0.1% Tween), 1 h. Primary antibodies were diluted in blocking solution (1/300) and incubated with larvae overnight at 4°C. Larvae were washed 4x 15 min in PBS 0.1% Tween and incubated with secondary antibodies diluted in block solution, overnight at 4°C followed by 4x 15 min washes in PBS 0.1% Tween. Fluorescently labelled larvae were cleared by progressive transfer to 80% glycerol and imaged using a Leica SP5II confocal microscope.

**Immunofluorescence quantifications**

Unless indicated, for all IF quantifications, at least 200 cells were analysed for each sample in at least three independent experiments. For NMHCIIA bundles cells were scored positive when displaying at least one compact NMHCIIA or actin bundle. For ER vacuoles cells were scored positive when displaying at least one KDEL-positive ER distinct vacuole. Individual focal adhesions were detected by FAK labelling. Stress-fibers cells were scored positive when displaying distinct NHMCIIA-actin fibers. For exofacial-PS sites, infected cells were scored positive when displaying at least one distinct puncta of exofacial-PS. DAPI nuclear staining was used for the quantification of the number of cells per field. For co-localization analysis, confocal microscopy images were used to define Individual regions of interest (ROI): entire cell, cortical bundles or equivalent size cellular regions outside cortical bundles (Control ROI). Pearson’s correlation coefficient was obtained using Coloc 2 for FIJI-Image J. At least 30 individual cells and 50 bundles or Ctrl ROIs were quantified in 6 independent experiments for NMHCIIA-Gp96 co-localization analysis and 3 independent experiments for co-localization analysis of WRAMP components. PM blebs from supernatants of HeLa cells seeded into six-well culture plates (5x106 cells), left untreated or treated with 0.5 nM of LLO were collected through mild centrifugation (2000 *g*, 10 min) into glass coverslips previously coated with Poly-L-Lysine solution (Sigma) according to the manufacturer’s instructions. Coverslips were then processed for immunofluorescence microscopic analysis using DAPI and FITCWGA. The levels of PM blebs released to the supernatant upon LLO treatment represent the quantification of total fluorescence (FITCWGA) area per field of view (in arbitrary units) quantified for at least 5 different fields for each sample from three independent experiments using ImajeJ64. Values were normalised to the basal levels obtained for untreated cells.

**Ultrastructure analysis**

The samples were collected and fixed in 2,5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4, postfixed in 2% OsO4, dehydrated and embedded in epon (Electron MicroscopySciences). The ultrathin-sections (60 nm) were counterstained with aqueous uranyl acetate solution and lead citrate. Sections were examined under a JEOL JEM 1400 TEM 120kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan).

**Analysis of cell shape parameters**

Different live HeLa cell shRNA lines were imaged in three independent days by transmission microscopy using a CKX41 Olympus microscope. Individual cells (shControl n=227, shGp96 n=207, shNMHCIIA n=192) were selected manually and standard shape indicators calculated using ImajeJ64. Circularity: 4π\*area/perimeter^2. A value of 1.0 indicates a perfect circle. Elongation or aspect ratio: major axis/minor axis. Solidity: measurement of the overall concavity or surface irregularity: area/convex area. A value of 1.0 indicates a solid or regular shape where the real area coincides with convex hull area of the same particle. Values < 1.0 indicate surface irregularity.

**Live imaging and quantification of PM blebbing of LLO-treated cells**

Cells seeded into Ibitreat μ-dishes (Ibidi), expressing GFPNMHCIIA alone or combined with mCherryKDEL, maintained in HBSS or HBSS supplemented with the indicated drug treatments at 37°C with 5% CO2 were imaged using an Andor Revolution XD Spinning-disk confocal system with an EMCCD iXonEM+ camera, 488 nm lase lines, and a Yokogawa CSU-22 unit on an inverted microscope (IX81; Olympus), driven by Andor IQ live-cell imaging software. LLO (0.5 nM) was added 10 min after initial image acquisition. Differential interference contrast (DIC) images and GFP fluorescent datasets with 0.5 μm z-steps were acquired using a UPLSAPO 100x/1.40 objective lens every 10 sec for cells expressing GFPNMHCIIA alone or every 15 sec for cells expressing both GFPNMHCIIA and mcherryKDEL. ImageJ64 was used for image sequence analysis and video assembly. For the quantification of the number of blebs per cell or retracting blebs per cell, 32 shControl cells and 40 shGp96 cells were analysed during an average of 1501±103.1 sec (shControl) and 1636±229.0 sec for shGp96 samples. Blebs were detected by analysis of DIC or transmission images.

**Wound closure assay**

Confluent HeLa cell monolayers seeded into Ibitreat culture-insert μ-dishes (Ibidi) were imaged using the Axiovert 200M microscope (Zeiss) equipped with a NanoScan Piezo Z stage (Prior Scientific Instruments), driven by Micro-Manager 1.4 software and transmission images acquired every 10 min with A-Plan 20x/0.30 Phase objective after removal of silicon stopper. Samples were maintained at 37°C with 5% CO2 and for each sample at least 3 independent areas were imaged for 3 independent wounds. Images were compiled and analysed using imageJ64.

**Flow cytometry analysis of LLO-treated and infected cells**

For flow cytometry, 5x106 cells seeded in 6-well plates 24 h before use and treated as indicatedwere washed twice in ice-cold PBS, trypsinized and resuspended in 0.5 ml of cold PBS containing 2 μg/ml PI. For detection of Gp96 surface levels, cells were immunolabelled with PE-conjugated rat anti-Gp96 9G10 (Enzo) and PI was used to discriminate live cells. At least 20000 cells were analysed for each sample of LLO-treated cells or 5000 to 10000 of GFP-positive for infected samples. Analysis was carried using a FACS calibur cytometer or FACS Canto II flow cytometer (BD Biosciences) and data were analyzed using FlowJo (TreeStar).

***In vivo* imaging of infected larvae**

Anaesthetized zebrafish larvae were oriented and immobilized in 1% low melting point agarose in 60 mm plastic bottom Petri dishes, then covered with 2 ml E3 medium containing tricaine. Transmission and fluorescence widefield imaging was done using a Leica M205FA at 30°C with a 10x Planapo objective. Multiple-field *Z*-stacks with a 10 µm *Z*-step were acquired every 15 minutes.

**Determination of bacterial burden *in vivo***

Animals were anesthetized, rinsed, and collected in 30 µl of sterile water. Lysis was carried in 200 µl of 0.4% Triton X-100 and serial dilutions of the homogenates were plated onto BHI for CFU counts.

**Statistical Analyses**

Statistical analyses were carried with Prism 6 software (GraphPad) using one-way-ANOVA with Dunnett post hoc analyses to compare different means in relation to a control sample, Tukey’s post hoc analyses for pairwise comparisons of more than 2 different means or using two-tailed un-paired Student's t-test for comparison of means between 2 samples. Survival curves were analysed using Log-rank (Mantel-Cox) test.

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**AUTHOR CONTRIBUTION STATEMENT**

FSM, DC and SS conceived and designed the experiments. FSM, CB, MJMM performed the experiments. FSM, CB, MJMM, SM, DC and SS analyzed the data. JCP engineered bacterial strains. SM contributed to discussions. FSM, DC and SS wrote the paper.

**CONFLIT OF INTEREST STATEMENT**

The authors declare that they have no conflict of interest.

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**FIGURE LEGENDS**

**Figure 1 - *Lm* causes LLO-dependent ER and NMHCIIA redistribution and promotes the interaction between Gp96 and NMHCIIA**

(**A-C**) Immunoblots of Gp96 and NMHCIIA levels from whole cell lysates (WCL) and Gp96 IP fractions (IP Gp96) of HeLa cells: (**A**) left untreated or treated with increasing concentrations of LLO for 15 min; (**B**) left uninfected (U) or infected with wt or Δ*hly* *Lm* for 1 h; or **(C)** infected with wt *Lm* for the indicated time points. (**A, C**) Quantifications of NMHCIIA in IP Gp96 are the mean±SEM (n≥3) (a.u. - arbitrary units). (**D**) Confocal microscopy images of HeLa cells: (**D**) left untreated or treated with LLO (0.5 nM, 15 min), immunolabelled for NMHCIIA (green), Gp96 (red) and stained with DAPI (blue). Arrows point to various NMHCIIA-Gp96 positive cortical bundles in different cell. Insets show high magnification image (**E**) Quantification of Pearson’s correlation coefficient (PCC) within: uninfected cells (U), LLO-treated cells, cortical NMHCIIA bundles or equivalent size control cellular ROI. (Ctrl ROI) Data are mean±SEM (n=6); *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 (**F**)Confocal microscopy images of HeLa cells infected with wt or Δ*hly Lm* for 6 h immunolabelled for NMHCIIA (green), Gp96 (red) *Lm* (blue) and stained with DAPI (white). Arrows indicate NMHCIIA-Gp96 cortical bundles at cortical sites close to wt *Lm*. (**D** and **F**) Scale bar - 10 μm. See also Figs EV1 and EV2.

**Figure 2 - NMHCIIA-Gp96 bundles are hallmarks of PFT-induced PM blebbing**

(**A-B**) Sequential frames of time-lapse confocal microscopy sequence of LLO-treated HeLa cells expressing (**A**) GFPNMHCIIA or (**B**) simultaneously GFPNMHCIIA and mcherryKDEL. LLO was added to culture medium 10 seconds before t0. DIC – differential interference contrast. (**A**) Arrows indicate NMHCIIA bundles at PM blebbing sites. (**B**) Highlights depicting ER structures within NMHCIIA bundles and PM blebs. Arrows indicate cortical ER surrounding NMHCIIA accumulations, arrowheads indicate contact points between ER vesicles and NMHCIIA cables and asterisks point to ER vacuoles within PM blebs (**C**) Confocal microscopy images of HeLa cells left untreated (Control) or treated with LLO (0.5 nM 15 min). Cells were stained with FITCWGA (Plasma membrane, PM-red) and immunolabelled for NMHCIIA (green). Insets show PM blebs and arrows indicate recruitment of NMHCIIA bundles to PM blebs associated (1) or detached (2) from the cell body. (**D-F**) Immunoblots of Gp96 and NMHCIIA levels from Gp96 IP of HeLa cells left untreated (U) or treated with: LLO (0.5 nM, 15 min) (LLO) and (**D**) LLO pre-incubated with cholesterol (LLOCHT); (**E**)LLO in medium supplemented with 140 mM K+(HighK+) and LLO in Ca2+-free medium Ca2+ (Ca2+free); **(F)** LLO in the presence of 25 μM blebbistatin (BB). (**G**) Confocal microscopy images of HeLa cells treated as indicated and immunolabelled for ER-KDEL (blue), NMHCIIA (green) and Gp96 (red). (**H**) Confocal microscopy images of HeLa cells treated with aerolysin and immunolabelled for NMHCIIA (green) and Gp96 (red). Insets show NMHCIIA bundles and arrows indicate association between Gp96 and NMHCIIA. (**I**) Immunoblots of NMHCIIA and Gp96 levels from Gp96 IP of HeLa cells left untreated (U) or treated with LLO (0.5 nM, 15 min), SLO (1.5 μg/ml, 30 min) (SLO) or aerolysin (0.2 nM, 40 min) (AL). (**A-C, G-H**) All scale bars are 10 μm. See also Appendix Fig S1.

**Figure 3 - Gp96 controls NMHCIIA remodelling and PM blebbing in response to PFTs**

(**A**) Confocal microscopy images of shCtrl, shGp96 or shNMHCIIA HeLa cells treated with LLO (0.5 nM, 15 min) and immunolabelled for KDEL-proteins (blue), NMHCIIA (green) and Gp96(red). Insets show compact NMHCIIA bundles in shCtrl cells and dispersed NMHCIIA bundles in shGp96 cells. Scale bar - 10 μm. (**B-D**) Quantification of the % of cells harbouring NMHCIIA bundles after incubation with (**B**) LLO (0.5 nM, 15 min), (**C**) aerolysin (0.2 nM, 40 min) or (**D**) with wt *Lm*. Values are the mean ±SEM (n≥3), *p-*values were calculated using (**B-C**) one-way-ANOVA with Dunnett post hoc analyses and (**D**) two-tailed un-paired Student's t-test,\*p<0.5, \*\*\*p<0.001. For shNMHCIIA, bundles were detected following actin staining (Fig EV3C). (**E-F**) Number of (**E**) blebs per cell or (**F**) retracting blebs per cell evaluated by time-lapse microscopy analysis of LLO-treated shCtrl or shGp96 cells. shCtrl n=32 cells and shGp96 n=40 cells, *p-*values were calculated using two-tailed un-paired Student's t-test \*p<0.5. (**G**) Sequential frames of time-lapse microscopy analysis of LLO-treated HeLa cells expressing GFPNMHCIIA (shCtrl, shGp96 and shCtrl with 25 μM Blebbistatin - shCtrl-BB). LLO was added to culture medium 10 seconds before t0. (**H**) Immunoblots of Ser19-phosphorylated MRLC (pMRLC), MRLC and actin levels from shCtrl or shGp96 cells left untreated (U) or treated with 0.1 nM LLO for the indicated time points. Quantification of pMRLC levels corresponds to the mean ± SEM (n≥3), *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*\*p<0.01, \*\*\*p<0.001. See also Appendix Fig S2 and Fig EV3.

**Figure 4 - NMHCIIA bundles resemble tail-retraction structures and Gp96 regulates cytoskeleton remodelling**

(**Ai**) Confocal microscopy images of LLO-treated HeLa cells immunolabelled for the indicated proteins. Insets show colocalization between NMHCIIA and WRAMP components at cortical bundles. (**Aii**) Quantification of Pearson’s correlation coefficient (PCC) between NMHCIIA and the indicated proteins within entire LLO-treated cells, cortical bundles or equivalent size control cellular ROI (Ctrl ROI). For Calpain 2 cortical bundles were defined by actin staining. Data are representative of one experiment repeated 3 independent times with similar results. *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*\* p<0.01; \*\*\* p<0.001, ns – non significant (**B**) Immunoblots of NMHCIIA, Gp96 and Filamin A levels from WCL and Gp96 IP of HeLa cells left untreated (U) or treated with LLO (0.5 nM, 15 min) (LLO). **(C)** Immunoblots of NMHCIIA and Gp96 levels from WCL and Gp96 IP of HeLa cells left untreated (U) or treated with 25 μg/ml of Wnt5a for the indicated time. (**D**)Confocal microscopy images of HeLa cells untreated (Mock) or treated with 25 μg/ml of Wnt5a for 30 min. Cells were immunolabelled for ER-KDEL (blue), Gp96 (red) and NMHCIIA (green). Arrows indicate polarised localization of NMHCIIA, ER-KDEL and Gp96 in Wnt5a-treated cells. (**E-F**) Confocal microscopy images of shCtlr, shGp96 or shNMHCIIA HeLa cells stained for actin (red) and DAPI (blue) and immunolabelled for (**E**) NMHCIIA (green) or (**F**) focal adhesion kinase (FAK) (green). **(A, D-F**) Scale bar - 10 μm. Insets show sites with focal adhesion points and arrows indicate stress fibers or distinct focal adhesion points. Quantification of the % of cells with (**G**) stress fibers or (**H**) focal adhesion points labelled by FAK. (**I-J**) Wound closure assay. (**I**) Sequential frames of time-lapse microscopy of HeLa cells grown to confluence separated by a stopper. Stopper was removed at t0 and cellular migration was imaged for the indicated times. (**J**) Quantification of the % of wound area occupied by migrating cells over time stopper removal (upper panel) and respective rate of closure (lower panel). (**G-J**) Data are the mean ±SEM (n≥3). *p-*values were calculated using one-way-ANOVA with Dunnet post hoc analyses \*p<0.05, \*\*\*p<0.001 \*\*\*p<0.001. See also Fig EV4.

**Figure 5 - Gp96 and NMHCIIA reduce release of PM blebs and protect PM integrity upon LLO** (**A**) Epifluorescence microscopy images of supernatants from LLO-treated (0.5 nM 15 min) shCtlr, shGp96 or shNMHCIIA HeLa cells collected into poly-lysine-coated cover slips, fixed and stained with FITCWGA (green) and DAPI (blue). Insets show PM blebs of variable sizes and DAPI staining confirms the absence of cell nuclei. Scale bar - 10 μm. (**B**) Quantification of released PM blebs (total FITCWGA/field). Values are the means ± SEM (n≥3). \*\*\*p<0.001. (**C-E**) Flow cytometry analysis of the % of PI-positive shCtlr, shGp96 or shNMHCIIA HeLa cells. Cells were treated with (**C**) 0.5 nM LLO for the indicated times; (**D**) 1.5 or 3 nM LLO for 15 min; or (**E**) 0.1 nM LLO for 10 min followed by LLO washed out and recovery for indicated times. Levels of PI incorporation by untreated cells were subtracted from all LLO-treated samples. Values are the mean ± SEM (n≥4) and *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*p<0.5, \*\*p<0.01, \*\*\*p<0.001.

**Figure 6 - Gp96 and NMHCIIA preserve PM integrity during *Lm* infection**

(**A-B**) Epifluorecence microscopy images of HeLa cells (**A**) left untreated or treated with LLO (0.5 nM, 15 min) and (**B**) infected with GFP-expressing wt *Lm* for 6 h. Cells were incubated with exofacial-PS probe (Alexa-568 annexin A5) (red) 30 min prior fixation, immunolabelled for NMHCIIA (green) and stained with DAPI (blue)*.* Insets show PM damage marked by exofacial-PS sites.Arrows indicate exofacial-PS associated with intracellular bacteria and in shCtrl cells with NMHCIIA bundles; arrowheads indicate NMHCIIA bundles associated with exofacial-PS sites without detectable bacteria. (**C**) Quantification of the % of infected cells with exofacial-PS sites in HeLa cells infected with wt *Lm* for 6 h. Data are the mean ±SEM (n=3) and *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*p<0.5. (**D-E**) Flow cytometry analysis of the % of PI-positive shCtlr, shGp96 or shNMHCIIA HeLa and RAW264.7 cells, infected with GFP-expressing wt or Δ*hly* *Lm* for 6 h. The levels of PI incorporation by uninfected cells were subtracted from infected samples. Values are the mean ± SEM (n≥4) and *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*p<0.5, \*\*p<0.01, \*\*\*p<0.001. PM permeability was assessed in cells harbouring equivalent numbers of intracellular bacteria (Fig Appendix Fig S3).

**Figure 7 - Gp96 interacts with NMHCIIA and promotes host survival during *in vivo* *Lm* infection**

(**A**) Immunoblots of NMHCIIA and Gp96 levels from whole cell lysates (WCL) and IP fractions (IP Gp96) of extracts of zebrafish larvae 3 dpf uninfected (U) or infected with wt or ∆*hly* *Lm* (low dose 200-1500 CFU) for 24h. (**B**) Immunoblots of Gp96 and tubulin levels from zebrafish 3 dpf larvae injected with control (Ctrl-mo) or Gp96 morpholino oligonucleotides (Gp96 mo). (**C**) Confocal microscopy images of zebrafish larvae (Ctrl mo or Gp96 mo) infected (low dose) in the tail muscle, with the GFP-expressing wt or ∆*hly* *Lm* for 24 h, immunolabelled for KDEL-proteins (green), and stained with phalloidin (actin, red) and DAPI (white). Images show actin rich structures at the cell cortex (arrows) and ER-KDEL puncta (arrowheads) in larvae infected with wt strain. (**D**) CFU counts per zebrafish larvae (Ctrl or Gp96 mo) infected with wt (low dose) or ∆*hly* (high dose > 10,000 CFU) *Lm* and analysed at 0, 24, 48 hpi. Results are mean ± SEM (n≥3) (horizontal bars) and each circle represents 1 larvae. *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*\*p<0.01. (**E**) Survival curves of zebrafish larvae (Ctrl-mo or Gp96-mo) infected with a wt (low dose) or ∆*hly* (high dose > 10,000 CFU) *Lm.* Results are the mean ± SEM (n≥3). wt infection of Ctrl and Gp96 mo, n=28 larvae; Δ*hly* infection of Gp96 mo n=13. *p-*values were calculated using Log Rank test, \*\*p<0.01. (**F**) CFU counts per zebrafish larvae infected with a high dose of ∆*hly* *Lm* at different times post-infection. Results are mean ± SEM (n≥3) (horizontal bars); each point represents 1 larvae. See also Fig EV5.

**EXPANDED VIEW FIGURE LEGENDS**

**Figure EV1 - *Lm* infection induces LLO-dependent ER-redistribution and NMHCIIA-Gp96 interaction**

(**A**) Flow cytometry analysis of surface-exposed Gp96 levels in HeLa cells left uninfected (U) or infected with wt or ∆*hly* *Lm* for 1 h. Values are mean ±SEM (n≥3) and *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses, \*\*p<0.01 (**B**) Confocal microscopy images of HeLa cells left uninfected or infected with wt or ∆*hly* *Lm* for 1 h, fixed and immunolabelled for ER-KDEL (green), Gp96 (red) and stained with DAPI (blue). Insets show ER-Gp96 vacuoles. (**C**) Confocal microscopy images of HeLa cells left untreated or treated with the indicated concentrations of LLO for 15 min, fixed and immunolabelled for ER-KDEL (green) and stained with DAPI. Arrows indicate ER-KDEL vacuoles. Scale bar - 10 μm. (**D**) Confocal microscopy images of HeLa cells left untreated or treated with 0.5 nM LLO for 15 min immunolabelled for Gp96 (red) and ER-KDEL (green). Arrows indicate ER-Gp96 vacuoles shown enlarged in the insets 1 and 2. (**E**) TEM images of HeLa cells left untreated or treated with 0.5 nM LLO for 15 min. Arrowheads indicate normal ER cisternae, arrows show ER vacuoles at the proximity of PM. (**F)** Flow cytometry analysis of surface-exposed Gp96 levels in HeLa cells left untreated (U) or treated with 0.5 nM LLO for 15 min, values are mean ±SEM (n≥3) and *p-*values were calculated using two-tailed un-paired Student's t-test \*p<0.05 (**G**) Amino acid sequence of the human NMHCIIA with functional domains indicated. Peptide sequences recovered from MS/MS are highlighted in red. (**B-D**) Scale bars are 10 μm.

**Figure EV2 - NMHCIIA-Gp96 interaction and association at cortical structures in HeLa, Caco-2 and RAW 264.7 cells infected with *Lm* or treated with LLO.**

(**A-D**) Immunoblots of NMHCIIA and Gp96 levels from whole cell lysates (WCL) and IP fractions, (**A**) NMHCIIA IP or (**B-D**) Gp96 IP fractions from (**A, B**) HeLa cells, (**C**) Caco-2 cells or (**D**) RAW264.7 macrophages left uninfected (U) or infected with wt *Lm* for 1 h, probed with anti-Gp96 and anti-NMHCIIA antibodies. (**A-B**) Control IP was carried using unspecific isotype antibodies (IgGiso). (**E**) Confocal microscopy images of Caco-2 cells left untreated or treated with 0.5 nM LLO 15 min, immunolabelled for NMHCIIA (green), Gp96 (red) and stained with DAPI (blue). Arrows indicate Gp96-positive NMHCIIA cortical structures. Scale bar - 10 μm.

**Figure EV3 - Gp96 and NMHCIIA controls actomyosin remodelling during LLO treatment**

(**A**) Quantification of the % of shControl and shGp96 HeLa cells harbouring NMHCIIA bundles in response to increasing concentrations of LLO. (**B**) Quantification of % of shCtrl and shGp96 HeLa cells harbouring at least 1 to 5 or >5 NMHCIIA bundles per cell in response to 0.5 nM LLO (15 min). For all quantifications, values are mean ±SEM (n≥3) and *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. (**C**) Confocal images of shCtrl, shGp96 or shNMHCIIA HeLa cells treated with 0.5 nM LLO for 15 min and immunolabelled NMHCIIA (green) and stained with phalloidin (actin, red) and DAPI (blue). Arrow indicates compact actomyosin cortical bundle. Scale bar - 10 μm.

**Figure EV4 - Gp96 and NMHCIIA modulate cell shape**

(**A**) Brightfield images of the indicated unfixed HeLa cell lines used for analysis of shape indicators. Scale bar - 100 μm and 10 μm, top and bottom panels, respectively. (**B**) Quantification of cellular shape indicators. Each point represents one individual cell imaged and analysed for each parameter in each sample. At least 190 cells were analysed. *p-*values were calculated using one-way-ANOVA with Tukey post hoc analyses \*p<0.05, \*\*\*p<0.001

**Figure EV5 - Zebrafish is a valuable model to study the cell biology of *Lm* infection**

(**A**) CFU counts per zebrafish larvae infected with various doses (low, medium and high) of wt *Lm* at different times post-infection. Results are mean ± SEM (n≥3) (horizontal bars); each point represents 1 larvae. (**B**) Survival curves of zebrafish larvae infected with different doses (low, medium and high) of wt or ∆*hly* *Lm*. Values are mean ± SEM (n≥3) and include a total of n=28 larvae per condition. (**C**) Interaction between *Lm* (GFP-Green) and macrophages (Mpeg1-mCherry; Red). Sequential frames of time-lapse microscopy analysis of transgenic zebrafish larvae infected in the hindbrain ventricle with a low dose GFP expressing *Lm* for 12 h (t0). Scale bar - 20 μm. (**D**) Confocal microscopy images of wt zebrafish larvae infected (low dose) in the tail muscle, with the GFP-expressing wt *Lm* for 24 h, stained with phalloidin (actin, red) and DAPI (blue). Insets show*in vivo* actin comet tails.

**Movie EV1 - NMHCIIA rearrangements during PFT-induced PM blebbing**

Time-lapse confocal microscopy analysis of LLO-treated HeLa cells expressing GFPNMHCIIA used in Figure 2A. Sequential frames were acquired every 10 seconds (10 frames per second display rate). LLO was added to culture medium 10 seconds before t0. DIC – differential interference contrast. GFPNMHIIA fluorescence image corresponds to a z-stack projection. Scale bar - 10 μm. Movie depicts NMHCIIA rearrangements and enrichment at sites of PM blebbing.

**Movie EV2 - NMHCIIA and ER rearrangements during PFT-induced PM blebbing**

Time-lapse confocal microscopy analysis of LLO-treated HeLa cells expressing simultaneously GFPNMHCIIA and mcherryKDEL used in Figure 2B. Sequential frames were acquired every 15 seconds (10 frames per second display rate). LLO was added to culture medium 10 seconds before t0. Scale bar -10 μm. DIC – differential interference contrast. GFPNMHIIA fluorescence image corresponds to a z-stack projection. Inset highlight depict sites where NMHCIIA bundles occur where it is possible to observe association between ER structures and NMHCIIA bundles and movement of ER vesicles within PM blebs.

**Movie EV3 - NMHCIIA rearrangements during PFT-induced PM blebbing in control HeLa cells**

Time-lapse confocal microscopy analysis of LLO-treated HeLa cells expressing GFPNMHCIIA and control shRNA oligos used in Figure 3G left panel. Sequential frames were acquired every 10 seconds (10 frames per second display rate). LLO was added to culture medium 10 seconds before t0. DIC – differential interference contrast. GFPNMHIIA fluorescence image corresponds to a z-stack projection. Scale bar -10 μm.

**Movie EV4 - NMHCIIA rearrangements during PFT-induced PM blebbing in Gp96-depleted HeLa cells**

Time-lapse confocal microscopy analysis of LLO-treated HeLa cells expressing GFPNMHCIIA and shRNA oligonucleotides targeting the expression of Gp96, used in Figure 3G middle panel. Sequential frames were acquired every 10 seconds (10 frames per second display rate). LLO was added to culture medium 10 seconds before t0. DIC – differential interference contrast. GFPNMHIIA fluorescence image corresponds to a z-stack projection. Scale bar -10 μm.

**Movie EV5 - NMHCIIA rearrangements during PFT-induced PM blebbing in control HeLa cells treated with NMII inhibitor blebbistatin**

Time-lapse confocal microscopy analysis of LLO-treated HeLa cells expressing GFPNMHCIIA, control shRNA oligonucleotides and treated with 25 μM blebbistatin used in Figure 3G right panel. Sequential frames were acquired every 10 seconds (10 frames per second display rate). LLO was added to culture medium 10 seconds before t0. DIC – differential interference contrast. GFPNMHIIA fluorescence image corresponds to a z-stack projection. Scale bar -10 μm.

**Movie EV6 - Wound closure assay**

Time-lapse transmission microscopy of Control- (Ctlr), Gp96- (shGp96) or NMHCIIA-depleted (shNMHCIIA) HeLa cells grown to confluence in the presence of stopper. Stopper was removed at t0 and cellular migration was imaged every 10 min for the indicated times (15 frames per second display rate) and is represented in Figure 4I.

**Movie EV7 - Interactions between macrophages and *Lm in vivo***

Time-lapse microscopy analysis of transgenic zebrafish larvae with fluorescent macrophages (Meg1-mCherry) infected in the hindbrain ventricle with a low dose GFP expressing *Lm* (Green) for 12 h (t0), represented in Figure EV5C. Sequential frames were acquired every 15 min (7 frames per second display rate).