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Inhibition of autophagy limits vertical transmission of Zika virus in pregnant mice

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Zika virus (ZIKV) infection during pregnancy leads to devastating fetal outcomes, including intrauterine growth restriction and microcephaly. Greater understanding of mechanisms underlying ZIKV maternal–fetal transmission is needed to develop new therapeutic interventions. Here, we define an important role for the autophagy pathway in ZIKV vertical transmission. ZIKV infection induced autophagic activity in human trophoblasts and pharmacological inhibition limited ZIKV infectivity. Furthermore, deficiency in an essential autophagy gene, *Atg16l1*, in mice limited ZIKV vertical transmission and placental and fetal damage and overall improved placental and fetal outcomes. This protection was due to a placental trophoblast cell–autonomous effect of autophagic activity, not to alterations in systemic maternal ZIKV infection. Finally, an autophagy inhibitor, hydroxychloroquine, approved for use in pregnant women, attenuated placental and fetal ZIKV infection and ameliorated adverse placental and fetal outcomes. Our study reveals new insights into the mechanism of ZIKV vertical transmission and suggests that an autophagy-based therapeutic warrants possible evaluation in humans to diminish the risks of ZIKV maternal–fetal transmission.

INTRODUCTION

Zika virus (ZIKV) is an emerging mosquito-transmitted flavivirus and global public health threat. Hitherto, ZIKV was known to cause a self-limiting febrile illness characterized by rash, myalgia, conjunctivitis, and headache. However, recent epidemics have linked ZIKV infection to Guillain-Barré syndrome in adults (Cao-Lormeau et al., 2016) and placental insufficiency, fetal demise, microcephaly, and other congenital malformations in fetuses and newborn infants in the setting of maternal infection during pregnancy (Chibueze et al., 2017). In the human placenta, ZIKV has a broad cell tropism, including villous placental trophoblasts, endothelial cells, fibroblasts, and fetal Hofbauer macrophages in the intervillous space (El Costa et al., 2016; Jurado et al., 2016; Miner et al., 2016; Quicke et al., 2016; Tabata et al., 2016; Aagaard et al., 2017). This evidence suggests that ZIKV can disseminate into the intrauterine space and infect the fetus through a transplacental route.

Recent studies have demonstrated that inoculation of ZIKV through a subcutaneous or intravaginal route in pregnant mice with compromised type I interferon signaling results in severe placental and fetal damage, including intrauterine growth restriction and fetal demise (Miner et al., 2016; Yockey et al., 2016), which recapitulates many features of congenital ZIKV syndrome in humans (Mysorekar and Diamond, 2016; Cao et al., 2017). Furthermore, in situ hybridization (ISH) and viral antigen staining has established the

presence of ZIKV infection in multiple mouse and human trophoblast cells (Miner et al., 2016; Tabata et al., 2016; Cao et al., 2017; Vermillion et al., 2017). The preferential replication of ZIKV in trophoblasts and fetal endothelial cells in the placenta suggests that ZIKV enters into the fetal circulation in mice in part by compromising the placental barrier. Bayer et al. (2016) showed that type III interferon (IFN- λ) secreted by primary cultured human trophoblast cells was able to protect trophoblasts from ZIKV infection in an autocrine or paracrine manner. TIM1, a member of the T cell immunoglobulin and mucin domain protein family, has been shown to play a critical role in ZIKV infection in primary trophoblasts (Tabata et al., 2016). However, the pathogenesis and underlying mechanisms of ZIKV-induced maternal–fetal transmission in vivo across the placental trophoblast barrier remain unknown.

The placenta normally uses physical and immunological strategies to protect the fetus from maternal–fetal transmission of pathogens. One important mechanism the placenta uses in its defense is autophagy (Delorme-Axford et al., 2013; Cao et al., 2016), which targets intracellular components for lysosomal degradation and is important for host defense against many pathogens (Levine et al., 2011). Notwithstanding this, some pathogens have evolved mechanisms to evade, inhibit, or even hijack the host autophagy machinery to facilitate infection and survival (Cemma and Brumell, 2012). Recent studies have indicated that autophagy has as an important function in placental defense against microbial agents (Delorme-Axford et

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Abbreviations used: 3-MA, 3-methyladenine; Baf, bafilomycin; CQ, chloroquine; CTB, cytotrophoblast; FFU, focus-forming unit; HCQ, hydroxychloroquine; HM, hypomorphic; hpi, hours postinfection; ISH, in situ hybridization; ZIKV, Zika virus.

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al., 2013; Cao et al., 2016), as autophagy pathway activity is decreased in human placentas with clinically diagnosed infections (Cao et al., 2016). Placenta-specific microRNAs, packaged in exosomes secreted from human syncytium, can attenuate viral replication of vesicular stomatitis virus through induction of autophagy (Delorme-Axford et al., 2013). Although autophagy occurs in trophoblasts from term placental villi (Signorelli et al., 2011), several pathogens that are transmitted to the fetus by a placental route, including *Brucella abortus* (Khan et al., 2001) and *Listeria monocytogenes* (Lamont et al., 2011), can evade autophagic cellular machinery to survive or replicate in other cell types (Cemna and Brumell, 2012).

At present, the effects of autophagy on ZIKV infection in trophoblasts and its impact on vertical transmission remains unknown. Here, we performed ZIKV infection studies in mice and cultured trophoblasts to address the impact of autophagy on vertical transmission of ZIKV. We show that ZIKV infection activates autophagic activity in human trophoblast cells and in the mouse placenta. Inhibition of the autophagy pathway in both human trophoblast and autophagy gene-deficient mice reduced ZIKV infection in placentas and fetuses and resulted in improved fetal outcomes. Consistent with these findings, we demonstrate that inhibiting autophagy by treating pregnant mice with hydroxychloroquine (HCQ) reduced ZIKV vertical transmission and limited placental damage and fetal death. Our findings demonstrate that autophagy promotes ZIKV pathogenesis during pregnancy and provide a foundation for developing therapeutics to reduce maternal-fetal transmission of ZIKV.

RESULTS

ZIKV infection induces autophagic activity in human trophoblasts

Autophagy is a vital part of the host response to many microbial infections. Thus, we reasoned that ZIKV may regulate autophagic activity in trophoblasts cells to facilitate its replication in the placenta. We infected a human cytotrophoblast (CTB) cell line, JEG-3, with a Brazilian strain of ZIKV (Pariba 2015) at a multiplicity of infection of 0.1 and collected samples 6, 12, 24, or 48 h later for virus titration as described previously (Miner et al., 2016). We examined the level of autophagy markers in CTBs after ZIKV infection by Western blot for the microtubule-binding protein light chain 3 (LC3) protein, which converts from the soluble form LC3-I to the lipidated form LC3-II and serves as an indicator of autophagic activity or flux. We found that LC3-II was significantly increased at 6 and 12 h postinfection (hpi; Fig. 1 A and Fig. S1 A). Because this could indicate either increased autophagy or inhibition of autophagosome maturation, we next treated cells with bafilomycin (Baf) A1, which inhibits autophagosomal and lysosomal fusion (Klionsky et al., 2016). In this case, LC3-II further accumulated in ZIKV-infected cells, indicating that ZIKV activated autophagy (Fig. 1 A and Fig. S1 A).

ZIKV infection has been associated with LC3-II-positive autophagosome formation in skin fibroblasts (Hamel et al.,

2015) and neural stem cells (Liang et al., 2016). To identify whether LC3⁺ autophagosome formation was affected in trophoblasts infected with ZIKV, we transfected CTBs with a plasmid carrying EGFP-LC3 and subsequently exposed them to ZIKV. Immunofluorescence staining for GFP revealed diffuse LC3 staining in uninfected CTBs and punctate staining in ZIKV-infected CTBs (Fig. 1 B). The number of EGFP-LC3⁺ punctae remained higher in ZIKV-infected than in uninfected cells upon BafA1 treatment (Fig. 1, B and C), indicating that the increase in LC3 punctae was due to enhanced autophagosomal formation. These results demonstrate that ZIKV infection induces canonical autophagy response in human trophoblasts.

Suppression of autophagic activity impairs ZIKV infection in human trophoblasts

Manipulation of autophagy pathway activity can alter microbial infection and pathogenesis in other cells and tissues (Levine et al., 2011). We next determined whether increasing or decreasing baseline autophagy in trophoblasts altered ZIKV persistence in these placental cells. We infected JEG-3 CTBs with ZIKV and then treated infected and control cells with chemical modulators of autophagy pathways: rapamycin and Torin 1 induce autophagy, whereas 3-methyladenine (3-MA), chloroquine (CQ), and Baf A1 inhibit autophagy at initiation (3-MA and CQ) and autophagolysosome formation (Baf A1) stages. Importantly, these autophagy modulators did not induce changes in CTB viability, with the exception of Torin 1 treatment, which was associated with a small reduction in cell viability (Fig. S1 B). Treatment of CTBs with the autophagy inhibitors 3-MA, CQ, and Baf A1 resulted in a significant decrease in ZIKV replication at 48 hpi (Fig. 1 D). Reciprocally, administration of the autophagy inducers rapamycin and Torin 1 resulted in an increase of viral infection in CTBs (Fig. 1 D). Immunofluorescence staining in infected trophoblasts confirmed fewer ZIKV antigen-positive cells (Fig. 1, E and F) after treatment with autophagy inhibitors but greater numbers of antigen-positive cells after incubation with autophagy inducers (Fig. 1, E and F). Collectively, these data show that inhibition of autophagy limits ZIKV infection in trophoblasts and suggests that modulation of this pathway can be harnessed to combat this infection.

ZIKV infection induces autophagy in vivo, and loss of ATG16L1 expression impairs in utero transmission of ZIKV

To investigate the physiological role for autophagy in the context of an in vivo model of maternal-fetal ZIKV transmission, we assessed whether ZIKV induced autophagy in the placenta. Similar to the human trophoblast response to ZIKV infection, immunoblotting for LC3-II in ZIKV-infected whole placentas (5 d postinfection) showed increased levels compared with uninfected placentas (Fig. S2 A). We also detected a concomitant reduction in levels of p62 (also called sequestrome-1 [Sqstm1]), a substrate degraded by autophagy pathway (Klionsky et al., 2016; Fig. S2 A). Immunohistochem-

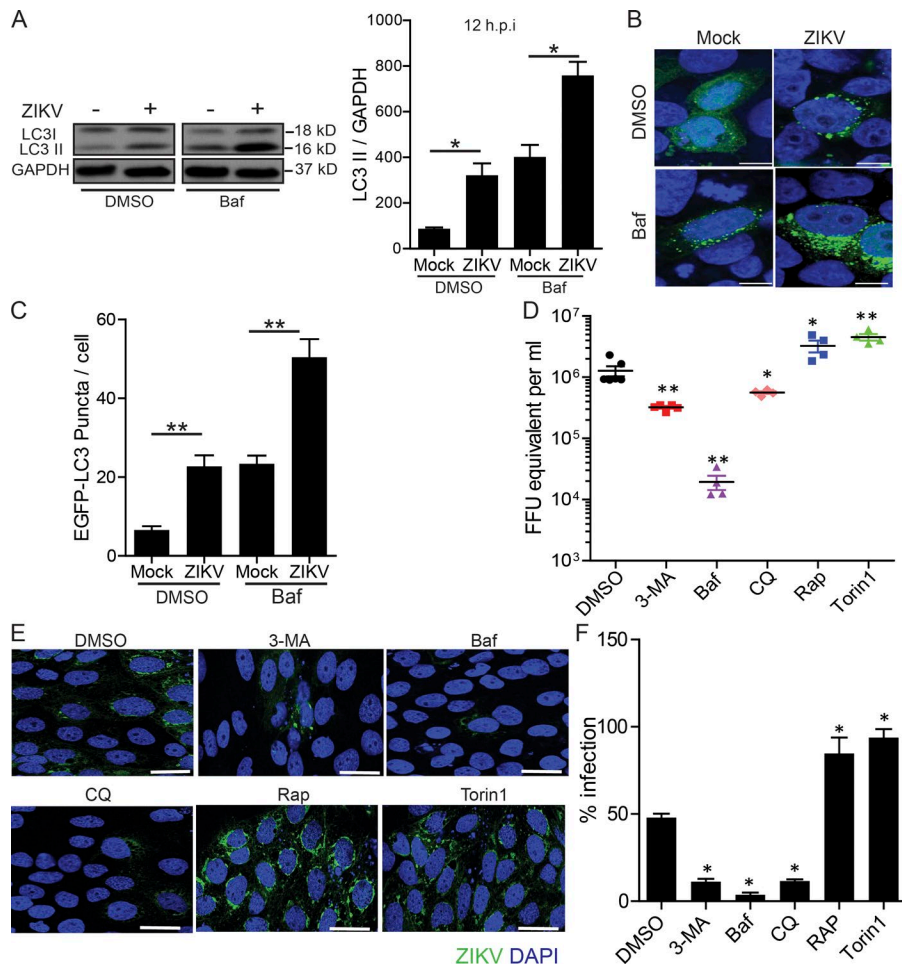


Figure 1. **Modulation of autophagy pathway limits ZIKV infection in human trophoblasts.** A CTB cell line, JEG-3, was infected with the ZIKV Brazil (Paraiba 2015) at a multiplicity of infection of 0.1 for 2 h. Cells were washed and cultured in fresh medium with either Baf A (50 nM) or DMSO for 30 min before harvesting for monitoring autophagic flux. (A) Western blot and quantification of LC3-II levels 12 hpi. Results represent the mean \pm SEM of four independent experiments. *, $P < 0.05$, Mann-Whitney test. (B and C) Representative images (B) and quantification (C) of EGFP-LC3⁺ punctae in ZIKV-infected JEG-3 at 12 hpi. JEG-3 cells transiently overexpressed with EGFP-LC3 were infected by ZIKV for 2 h and fixed for imaging at 12 hpi; $n = 20$ –23 per group from three independent experiments. **, $P < 0.01$, Mann-Whitney test. (D) Titers of ZIKV-infected JEG-3 cells with indicated treatments at 48 hpi: DMSO, 3-MA (2 μ M), Baf (50 nM), CQ (5 μ M), rapamycin (Rap; 1 μ M), and Torin 1 (1 μ M). Results represent the mean \pm SEM of at least four independent experiments. (E) Representative immunofluorescence microscopy for ZIKV-E protein-positive (green) cells following indicated treatments. Nuclei are stained blue. (F) Quantification of ZIKV-positive cells in each indicated group. Values represent data from four independent experiments. In D and F, *, $P < 0.05$; **, $P < 0.01$ (ANOVA with a multiple-comparison test). Bars: 10 μ m (B); 25 μ m (E).

ical staining revealed that the ZIKV infection-associated reduction in p62 levels, thereby supporting autophagy pathway activation, was localized primarily to the trophoblast-rich region of the placenta (Fig. S2 B). Thus, ZIKV infection in vivo can activate autophagic activity in the placenta.

On the basis of these findings, we hypothesized that genetic manipulation of autophagic activity would alter ZIKV pathogenesis in pregnancy. To test this hypothesis, we used mice hypomorphic (HM) for a key autophagy gene, *Atg16L1* (*Atg16L1^{HM}*), because (1) ATG16L1 has a key role in autophagosome maturation as part of the protein complex that directs LC3 to autophagosomes en route to fusion with lysosomes, (2) *Atg16L1^{HM}* mice exhibit stalled autophagosome formation and reduced autophagy in multiple tissues, and (3) *Atg16L1^{HM}* mice exhibit normal fertility, and their placentas are histologically indistinguishable from wild-type (WT) mice in the absence of infection (Cao et al., 2016). We inoculated pregnant WT and *Atg16L1* HM mice (*Atg16L1^{HM}*) with 10^3 focus-forming units (FFU) of ZIKV (Paraiba 2015) through a subcutaneous route at day E9.5, 1 d after pretreatment with a single dose of anti-*Ifnar1* to facilitate dissemination (Fig. 2 A), as reported previously (Miner et al., 2016). Pregnant dams were followed longitudinally for

morbidity, levels of maternal viremia, and viral burden in the placenta and fetal heads at E14.5. Notably, ZIKV infection in *Atg16L1*-deficient placentas was ~ 10 -fold lower compared with WT controls (Fig. 2 B). ISH revealed that the overall abundance of ZIKV RNA-positive placental trophoblasts was decreased in *Atg16L1^{HM}* placentas (Fig. 2 C). The reduced viral burden in *Atg16L1^{HM}* placentas was associated with decreased placental damage: pathological phenotypes observed in ZIKV-infected WT placentas, such as apoptotic trophoblasts and increased number of nucleated fetal erythrocytes, were reduced in *Atg16L1^{HM}* placentas (Fig. 2 D). Further histological analysis showed that ZIKV-infected *Atg16L1^{HM}* placentas had larger and thicker placental layers than WT placentas (Fig. 2 E), and the labyrinth zone thickness was greater as well (Fig. 2 F).

Finally, ZIKV titer in *Atg16L1*-deficient fetus was significantly lower (~ 15 -fold) compared with WT controls (Fig. 3 A). Moreover, fetuses in the *Atg16L1^{HM}* group at E14.5 were larger than WT counterparts, indicating that the fetal growth restriction induced by ZIKV infection was limited by the HM *Atg16L1* allele (Fig. 3 B). These data together indicate that a deficiency in *Atg16L1* function results in reduced placental and fetal infection of ZIKV, thus suggesting that

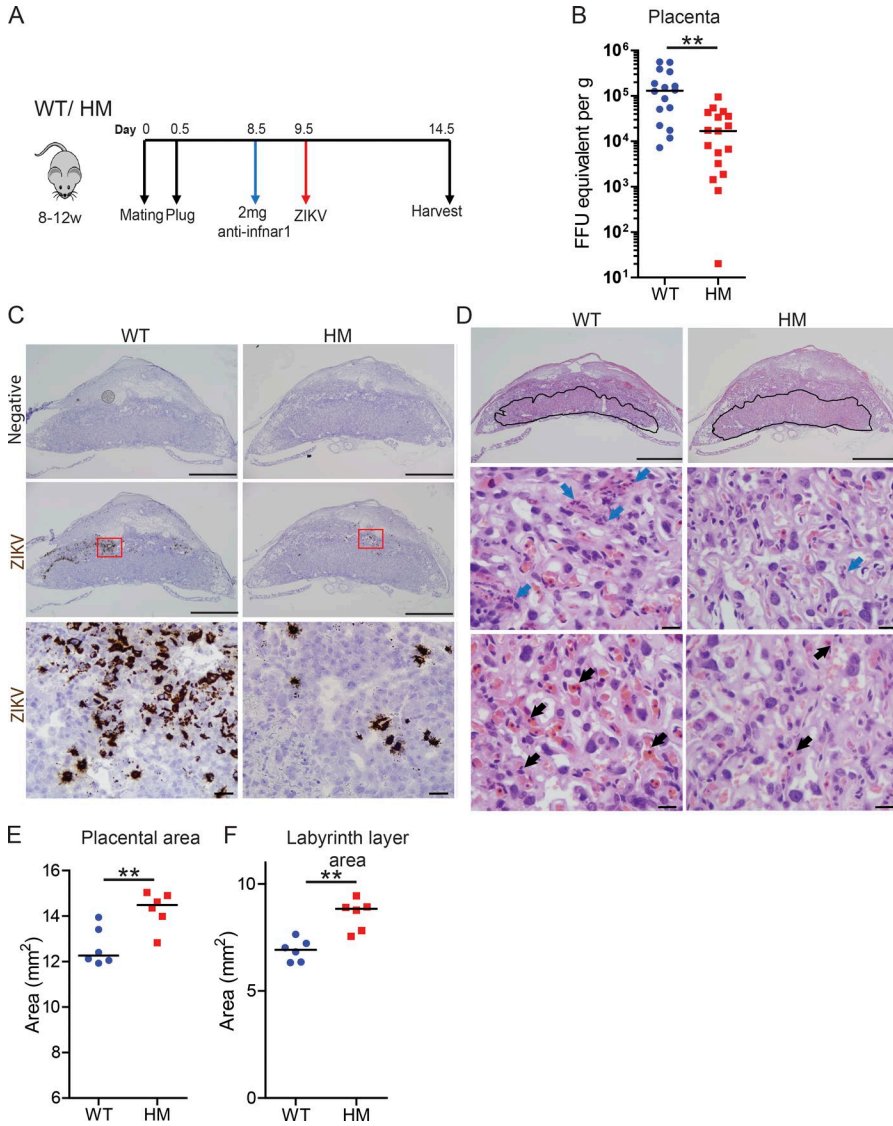


Figure 2. Loss of ATG16L1 limits ZIKV infection in placenta and fetus and improves fetal outcomes. (A) Schematic depiction of experiment. Female mice, 8–12 wk old, with indicated genotypes were cohoused with male mice of the same genotype. Day 0.5 is defined as the first observation of the vaginal plug. Pregnant dams treated with 2 mg of an anti-Ifnar1 antibody on day 8.5 were infected with 10³ FFU of ZIKV via subcutaneous route. Mice were sacrificed at E14.5. (B) Viral titer of placentas at E14.5 measured by quantitative RT-PCR. Values represent data from 12–17 samples per group (from 5–7 different litters). Bars indicate the median values of samples. **, P < 0.01, Mann-Whitney test. (C) Representative ISH images of mouse placentas (E14.5). Negative, negative probe. Lower panels show magnified ZIKV ISH staining in the junctional zone of placentas (red box) in the middle panels. Similar results were observed in five to eight placentas from five independent litters of each genotype. (D) Histopathological analysis of ZIKV-infected placentas at E14.5 by hematoxylin and eosin (H&E) staining. Labyrinth layers are outlined in black. Blue arrows indicate apoptotic trophoblasts, and black arrows indicate increased number of nucleated fetal erythrocytes in the labyrinth layer. (E and F) Size of placental area and corresponding labyrinth area are measured on the basis of H&E staining on cross sections of mouse placentas. Each dot represents one placental sample from at least four independent litters. **, P < 0.01, Mann-Whitney test. (C and D) Bars: low magnification, 2 mm; high magnification, 100 μm.

ATG16L1 plays an important role in governing placental susceptibility to ZIKV infection and maternal–fetal transmission.

Atg16l1 deficiency in trophoblasts limits ZIKV infection in the placenta

To assess whether the observed virological phenotype in *Atg16l1*^{HM} mice was due in part to changes in maternal infection, we measured viral burden in maternal serum and spleen but observed no significant differences in between the pregnant *Atg16l1*^{HM} and WT mice (Fig. 4, A and B). Moreover, the HM *Atg16l1* allele had no effect on ZIKV RNA localization or abundance within the maternal decidua (Fig. 4 C). Consistent with these data, no difference in viral titers were noted in nonpregnant *Atg16l1*^{HM} mice infected with ZIKV (Fig. 4, D and E) or in infected male *Atg16l1*^{HM} mice (not depicted). These findings indicate that a loss of *Atg16l1* function has no little or no effect on systemic or maternal decidua in-

fection but preferentially affects infection of the fetal-derived placental compartments.

To confirm these findings, we assessed whether loss of *Atg16l1* exclusively in cells of the trophoblast lineage was sufficient to limit ZIKV maternal–fetal transmission. We generated mice that lack *Atg16l1* only in trophoblast lineage cells by crossing the *Cyp19*-promoter-driven *Cre*⁻ recombinase mice (Wenzel and Leone, 2007) with *Atg16l1*^{fl/fl} mice. Pregnant *Cyp 19 Cre*⁺ *Atg16l1*^{fl/fl} mice and *Cre*⁻ control mice were treated with anti-Ifnar1 as described above and subsequently inoculated subcutaneously at E.9.5 with 10³ FFU of ZIKV (Paraiba 2015; Fig. 5 A). Analysis of the placentas from *Cyp 19 Cre*⁺ *Atg16l1*^{fl/fl} mice revealed that loss of this gene in trophoblasts resulted in reduced ZIKV infection in the placenta, similar to that seen with the *Atg16l1*^{HM} mice (Fig. 5, B and C). Unexpectedly, these differences in placental infection did not translate into reductions in fetal head titers

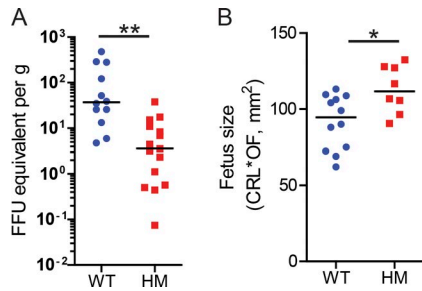


Figure 3. Deficiency of ATG16L1 impairs ZIKV infection in fetus and improves fetal outcomes. (A) ZIKV titers of fetal heads at E14.5 detected by quantitative RT-PCR; $n = 12-14$. (B) Measurements of fetal size with indicated genotypes by crown-rump length (CRL) and occipital-frontal (OF) diameter and expressed as square millimeters. In A and B, bars indicate median values; $n = 8-12$. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test).

(Fig. 5 D) or increases in fetus size (not depicted), suggesting that ATG16L1 may be important for viral survival or replication in other cell types (e.g., fetal brain neurons, Hofbauer cells at the maternal-fetal interface).

Pharmacological inhibition of autophagy results in decreased ZIKV infection at the maternal-fetal interface

Given that a reduction of autophagic activity in human trophoblasts and genetic loss of function of *Atg16l1* in mice limited ZIKV infection, we reasoned that an existing drug that inhibited autophagy and could be administered in pregnancy might have immediate therapeutic utility. HCQ is a Food and Drug Administration–approved class C drug that is used clinically to treat pregnant patients with malaria or autoimmune diseases (Kaplan et al., 2016). As systemic administration of HCQ has been successfully used to dampen autophagic activity in vivo (Rosenfeldt et al., 2013), we tested its efficacy against ZIKV infection during pregnancy. WT pregnant mice were administered HCQ (40 mg/kg/d) via intraperitoneal injection beginning at day +1 (E10.5) after ZIKV infection (Fig. 6 A); this dosage has known in vivo inhibitory effects on autophagy (Rosenfeldt et al., 2013) and was not associated with any differences in litter size compared with PBS-treated mice (Fig. S3 A). HCQ treatment significantly increased the levels of p62 (indicative of reduced autophagic activity) in trophoblasts, which validates the inhibitory effects of HCQ on autophagy in the placenta (Fig. 6 B). We next evaluated the effect of HCQ treatment during ZIKV infection in pregnancy. Notably, HCQ-treated mouse placentas sustained lower levels of ZIKV infection compared with PBS-treated controls (Fig. 6 C).

Although several groups have observed that treatment with HCQ or the related CQ can reduce flavivirus infection in cell culture, possibly through effects on viral fusion or maturation through inhibition of acidification of intracellular vesicles (Farias et al., 2014; Delvecchio et al., 2016), its function in vivo is less clear, as a randomized clinical trial of CQ treatment of Dengue virus infection showed no benefit

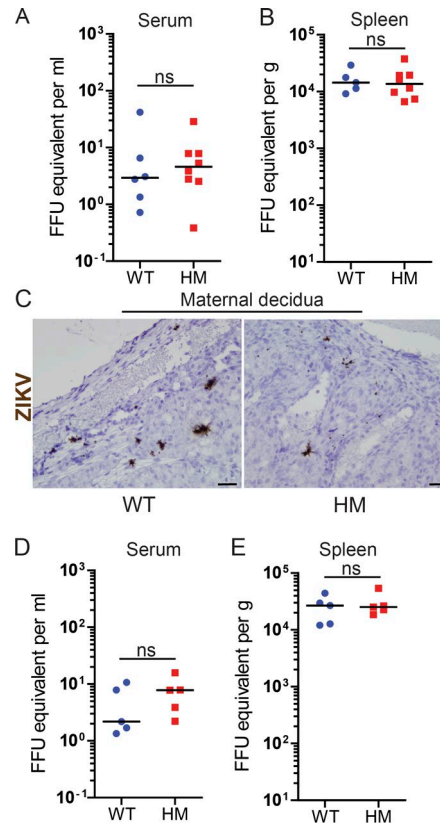


Figure 4. ATG16L1 does not affect systemic ZIKV infection in pregnant or nonpregnant mice. (A and B) Viral burden in maternal serum (A) and spleen (B) in ZIKV-infected WT and *Atg16l1* HM dams; $n = 6-8$ individuals in the indicated groups from three independent experiments. (C) Representative ISH images showing ZIKV infection in WT and HM decidua (maternal compartment). The images in panels are representative of several samples from at least five independent dams. Bars, 100 μm . (D and E) ZIKV titers in serum and spleen samples from ZIKV-infected nonpregnant female mice ($n = 5$ from three independent experiments). ns, not significant, Mann-Whitney test.

in adults from Vietnam (Savarino et al., 2003; Tricou et al., 2010). To ensure that the reduction in viral burden in the placenta was not due to reduced infection titers in the peripheral maternal tissues, we analyzed viral titers in the pregnant dams. No differences in viral burden in the serum, spleen, or maternal decidua were observed between the HCQ- and PBS-treated groups (Fig. 6, D and E; and Fig. S3 B). However, ISH staining for ZIKV RNA showed a marked reduction of ZIKV RNA in the placentas of HCQ-treated mice compared with controls (Fig. 6 F). Thus, the reduction in ZIKV infection by HCQ treatment in the context of pregnancy was specific to the placenta.

Histological analysis of HCQ-treated ZIKV-infected mice showed improvement in placental areas with reduced placental damage, as indicated by decreased numbers of apoptotic trophoblasts and immature fetal erythrocytes (Fig. 6, G and H). Similar to the results noted in *Atg16l1*^{HM} mice,

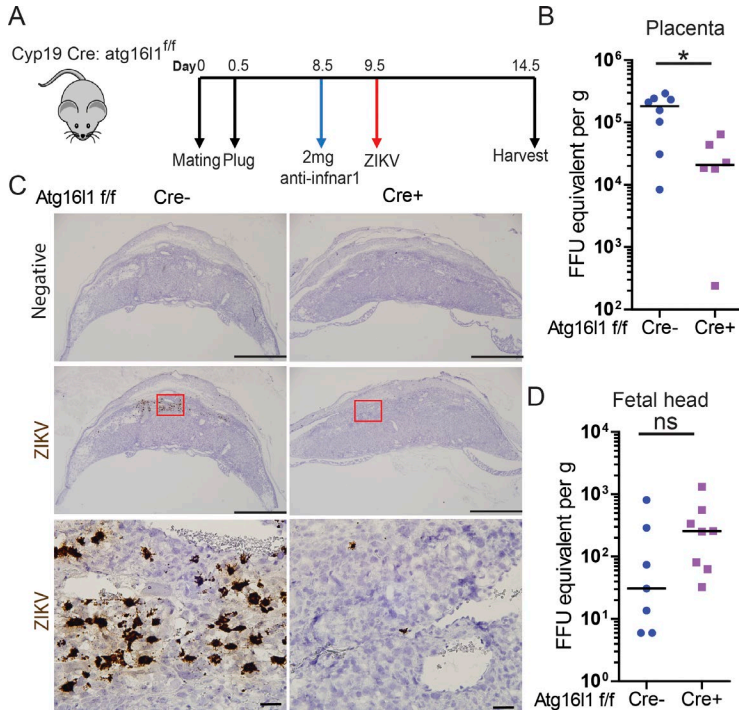


Figure 5. ATG16L1 deficiency in trophoblasts is sufficient to restrict ZIKV infection in the placenta. (A) Schematic diagram of experiment design. (B) Viral burden of ZIKV in placentas from Cyp19 cre⁺-Atg16l1^{fl/fl/fl} mice compared with Atg16l1^{fl/fl/fl} mice as a control; n = 6–8. (C) ISH images showing ZIKV infection in placentas from placenta-specific deficient ATG16L1 mice compared with Atg16l1^{fl/fl/fl} mice as a control. Higher power images of area within red boxes are depicted in the bottom panels. Bars, low magnification, 2 mm; high magnification, 100 μm. (D) ZIKV titer of fetal heads with indicated genotypes; n = 7–8. In B and D, *, P < 0.05; ns, not significant (Mann-Whitney test).

HCQ treatment reversed the reduction in area of the labyrinth (Fig. 6 I). Thus, HCQ treatment rescues the placental insufficiency that occurs after ZIKV infection. Consistent with a reduction in placental infection and improved tissue disease, HCQ treatment reduced ZIKV infection in the fetal head, which was associated with larger fetal body size (Fig. 6, J and K). Finally, we sought to confirm that this improvement in fetal status was due to inhibition of autophagy by treating ZIKV-infected *Atg16l1*^{HM} mice with HCQ. As expected, there was no difference in placental or fetal ZIKV titers in *Atg16l1*^{HM} mice with or without HCQ treatment (Fig. S3, C and D). Thus, our findings indicate that HCQ administration in pregnant dams can reverse the ZIKV associated autophagy induction in the placenta and reduce placental insufficiency, thus limiting vertical transmission of ZIKV infection and fetal growth defects.

DISCUSSION

The placenta acts as barrier to protect the developing fetus from the invading pathogens. In previous studies, we showed that autophagy protects against bacterial infection of the placenta (Cao et al., 2016). Here, in the context of ZIKV, we demonstrate the opposite: blocking the autophagy pathway in the placenta restricted ZIKV infection in the placenta and fetus during pregnancy. Most important, we demonstrated that placental trophoblast specific knockout of a key autophagy gene, *Atg16l1*, was sufficient to limit ZIKV placental infectivity. To our knowledge, our work is the first to show a placental cell-derived mechanism governing susceptibility to maternal-fetal transmission of ZIKV. These findings enhance our understanding of the cellular mechanisms of ZIKV ma-

ternal-fetal transmission and a possible therapy for enhancing the placental barrier function and controlling ZIKV infection.

Flaviviruses have been known to modulate autophagy in the context of infection (Pirooz et al., 2014). Different flaviviruses appear to have evolved distinct abilities to avoid or use autophagy to promote survival or infection at different stages of the viral life cycle, including viral replication, assembly, and release (Chiramel et al., 2013). For example, West Nile virus infection does not induce LC3 lipidation, and depletion of ATG5, a binding partner of ATG16L1, does not affect its replication (Vandergaast and Fredericksen, 2012; Martín-Acebes et al., 2015). However, administration of Tat-beclin-1, an autophagy-inducing peptide, reduces neuronal infection, cell death, and mortality associated with West Nile virus intracranial inoculation (Shoji-Kawata et al., 2013). In contrast, a deficiency in ATG5 in mouse neuroblastoma cells resulted in higher viral replication of the Japanese encephalitis virus (Sharma et al., 2014), suggesting that autophagy restricts infection. Others have shown that inducers of autophagy can lead to increased Dengue virus burden and pathogenicity in mice (Mateo et al., 2013), with a requirement of autophagy for optimal viral RNA replication and virion maturation (Mateo et al., 2013).

In vitro evidence has suggested that the autophagy pathway can modulate ZIKV infection: ZIKV was shown to induce autophagy in skin fibroblasts (Hamel et al., 2015) and human fetal neural stem cells (Liang et al., 2016), and this was associated with enhanced replication. However, in vivo evidence for such effects, especially on maternal-fetal transmission of ZIKV, has been lacking. We show that a genetic or pharmacologically induced deficiency in autophagy impairs

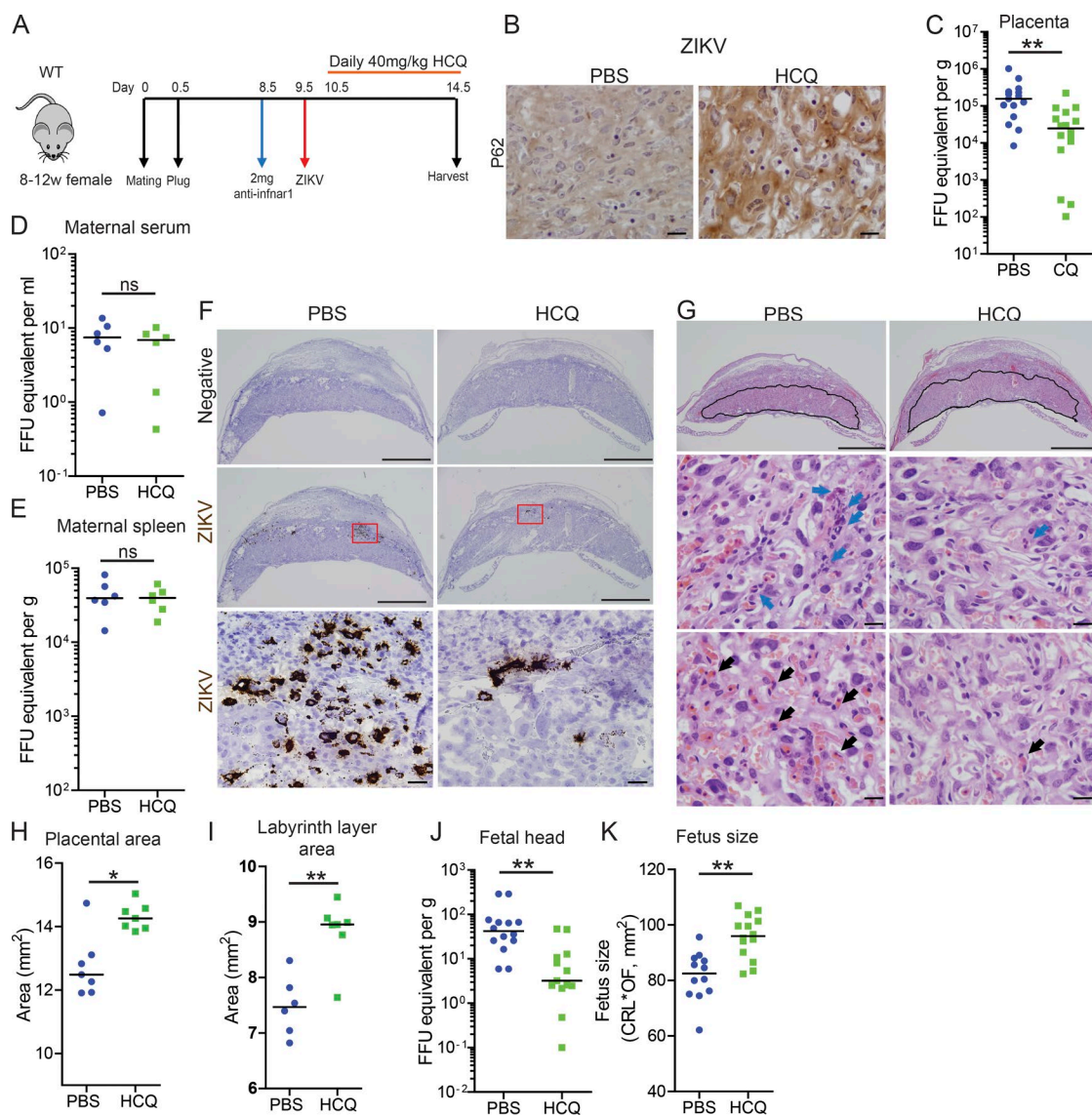


Figure 6. HCQ treatment during pregnancy reduces maternal-fetal transmission of ZIKV. (A) Schematic depiction of experiment of administration of HCQ (40 mg/kg/day via intraperitoneal route) as a treatment for ZIKV infection during pregnancy. Pregnant WT female mice were treated with HCQ or PBS as a mock control from day +1 post-ZIKV infection (E10.5) to E14.5. (B) Immunohistochemical staining for autophagy protein, p62, in mouse placentas. Image represents four to six placentas from four to six independent litters of each treatment group. Bars, 100 μ m. (C) ZIKV RNA from infected placentas treated with either HCQ or PBS and detected by quantitative RT-PCR (qRT-PCR); $n = 14$ –15 placental samples from four or five litters. Bars represent medians. (D and E) Viral burden assayed by qRT-PCR from maternal serum (D) and spleen (E) shows no difference. Symbols represent individual pregnant mice ($n = 6$ per group) from three independent experiments. (F) ISH images showing ZIKV infection in mock- or HCQ-treated placentas. Higher power images of area within red box are depicted in the lower panels. The images are representative of five to seven placentas from three independent dams. (G) Representative hematoxylin and eosin staining shows histopathological features of placentas from indicated treatment groups; solid lines mark the labyrinth. Blue arrows, apoptotic trophoblasts. Black arrows, increased number of nucleated fetal erythrocytes in the labyrinth. (H and I) Measurement of placental and labyrinth area; $n = 6$ –7 samples from at least three independent dams. (J) ZIKV RNA in fetal heads measured by qRT-PCR. Mock, $n = 14$; HCQ, $n = 13$. Bars represent the median values of 13 or 14 samples from four independent experiments. (K) Fetus size was measured as crown-rump length (CRL) times occipital-frontal (OF) diameter expressed as mm². Each symbol represents data from individual fetuses; $n = 12$ –13. In all dot plot panels, *, $P < 0.05$; **, $P < 0.01$; ns, not significant (Mann-Whitney test). (F and G) Bars: low magnification, 2 mm; high magnification, 100 μ m.

ZIKV infection in the placenta and fetus without affecting systemic infection of other maternal organs. Thus, inhibition of autophagy is beneficial for the host to limit ZIKV mater-

nal-fetal infection, which contrasts with its host defense roles in other placental infection models (Delorme-Axford et al., 2013; Cao et al., 2016). This finding suggests that ZIKV has

evolved strategies to usurp autophagy pathway for its own replicative advantage, but that this effect is restricted to certain cell types. It remains unclear why the impact of autophagy on ZIKV infection is more apparent in placental cells compared with other cell types. Our current work provides *in vivo* evidence that decreased autophagic activity is beneficial for the host in fighting ZIKV maternal-fetal transmission and indicates why ZIKV would induce autophagy. Recent studies have addressed the mechanisms of how ZIKV may have this effect. A study investigating ZIKV pathogenesis in human neurospheres showed that two nonstructural proteins of ZIKV, NS4A and NS4B, induced autophagy by suppressing the Akt-mTOR pathway (Liang et al., 2016). Moreover, ZIKV NS3 protease can cleave FAM134, an ER-localized protein required for reticulophagy, a selective form of autophagy that leads to ER degradation (Lennemann and Coyne, 2017).

Devastating fetal and neonatal outcomes caused by the recent ZIKV epidemic highlight the importance of studying mechanisms underlying maternal-fetal transmission of ZIKV. Placental trophoblast barriers contribute to defense responses to ZIKV vertical transmission. We showed that HCQ can successfully reduce viral burden both in human trophoblast cells and in mouse placentas. Whereas HCQ treatment leads to an increase in pH in intracellular vesicles, including lysosomes, and autophagosomes, and CQ has been shown to have antiviral activity in cell culture by inhibiting viruses including ZIKV at different stages of the viral life cycle (Savarino et al., 2003; Delvecchio et al., 2016), our p62 expression data suggest that the anti-ZIKV activity after HCQ treatment *in vivo* is due to modulation of autophagy. Consistent with this idea, other autophagy inhibitors, (e.g., 3-MA), which target different stages of autophagy, showed anti-ZIKV activity, whereas treatment with the autophagy activators rapamycin and Torin 1 resulted in increased viral infection in cultured trophoblasts. Moreover, HCQ treatment in mice phenocopied the reduced viral burden phenotype observed in mice deficient in *Atg16l1*, with decreased infection in the placenta and fetus but not in maternal tissues, which might be expected by a drug that was affecting a key stage in the viral life cycle. In addition, HCQ-induced inhibition of autophagy had no discernible impact on ZIKV infection of *Atg16l1*-deficient mice. These findings suggest that inhibition of autophagy specifically impairs ZIKV infection of the placenta, which results in reduced infection and disease in the fetuses.

Given the epidemic nature of ZIKV infection and its potential for congenital malformations, immediate interventions to prevent or treat ZIKV infection, especially at the maternal-fetal interface, are urgently required. Although several studies have shown promising anti-ZIKV activity with clinically approved drugs (Barrows et al., 2016; Delvecchio et al., 2016; Elfiky, 2016; Eyer et al., 2016; Xu et al., 2016; Zmurko et al., 2016; Sacramento et al., 2017), most of these experiments were performed in cell culture, and of the few showing *in vivo* efficacy, none of the studies were performed in the setting of pregnancy. Although mouse placentas are

anatomically different from human and nonhuman primate placentas (Rossant and Cross, 2001), the placental trophoblast barrier functions analogously. Our experiments on ZIKV infection inhibition in a mouse model of pregnancy are applicable to the understanding of ZIKV infection pathogenesis and may provide the foundation for experiments in nonhuman primates and clinical trials in humans to further define the therapeutic effects of modulating autophagic activity on ZIKV congenital disease.

Additionally, a limitation of many of the drugs (e.g., PHA-690509, a cyclin-dependent kinase inhibitor) is that they may not be suitable for administration during pregnancy because of their own potential teratogenic effects. HCQ is a Food and Drug Administration-approved class C drug that has been used to treat malaria and autoimmune diseases during pregnancy without apparent injury to the fetus, although it has not been evaluated in a randomized controlled trial. The current recommendation is that the potential benefits may warrant HCQ use in pregnant women despite potential risks, depending on the target disease. Our results suggest that a pharmacological inhibitor of autophagy warrants possible evaluation in nonhuman primates and humans during pregnancy to diminish the risks of ZIKV infection and disease in developing fetuses.

MATERIALS AND METHODS

Ethics statement

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine. Inoculations and dissections were performed under anesthesia to minimize animal suffering.

Viruses and titration

The Brazilian strain of ZIKV (Paraíba 2015) was provided by S. Whitehead (Bethesda, MD) and originally obtained from P.F.C. Vasconcelos (Instituto Evandro Chagas, Levilândia, Brazil). Virus stocks were propagated in Vero cells. The titers of ZIKV stocks were determined by focus-forming assay (FF on Vero cells as described previously; Miner et al., 2016). Studies with ZIKV were conducted under biosafety level 2 and animal biosafety level 3 containment.

Cell culture and infection

JEG-3 cells were obtained from ATCC (HB-36) and cultured in F12/DMEM media supplemented with 10% FBS (Thermo Fisher Scientific) at 37°C with 5% CO₂. JEG-3 cells were infected with ZIKV at a multiplicity of infection of 0.1 for 2 h, washed twice with warm PBS, and cultured in fresh medium with indicated treatments. At indicated time points, supernatants were harvested for virus titration or fixed for immunofluorescence staining of ZIKV using a ZIKV-specific mAb, ZIKV-2, as previously described (Miner et al., 2016). For monitoring autophagic flux, JEG-3 cells were transiently transfected with an EGFP-LC3 plasmid (11546; Addgene);

deposited by K. Kirkegaard) using the TransIT-X2 reagent (MIR 6000; Mirus) for 24 h. EGFP-LC3⁺ JEG-3 cells were infected and fixed for fluorescence microscopy.

Mouse experiments

Atg1611^{HM} and *Atg1611^{flox/flox}* mice were provided by H. Virgin (Washington University, St. Louis, MO; Cadwell et al., 2008). Cyp19-Cre transgenic mice were a kind gift from Gustavo Leone (The Ohio State University, Columbus, OH) and were generated as previously described (Wenzel and Leone, 2007). All mice were on a C57BL6 background and housed under a 12-h light/12-h dark cycle in a specific pathogen-free mouse breeding facility. For mating, 8- to 10-wk-old male and nulliparous female mice were cohoused from 5 p.m. to 8 a.m., and day 0.5 of pregnancy was defined as the first observation of a vaginal plug. Pregnant mice were inoculated by subcutaneous route in the footpad with 10³ FFU of ZIKV in a volume of 50 μ l. One day before infection, mice were treated with a single dose of 2 mg of an Ifnar1-blocking mouse antibody (MAR1-5A3, purchased from Leinco, Inc.) by intraperitoneal injection. Fetal size were measured by crown-rump length and occipital-frontal diameter at 4 d postfixation.

Measurement of viral burden

ZIKV-infected pregnant mice were euthanized on E14.5, and fetoplacental units were dissected. Placentas and fetal heads were weighed and homogenized in 250 or 500 μ l PBS. All homogenized tissues from infected animals were stored at -80°C until virus titration. RNA from tissue samples was extracted with the RNeasy Mini kit (QIAGEN). Serum from ZIKV-infected mice and culture medium from ZIKV-infected JEG-3 cells were extracted with Viral RNA Mini kit (QIAGEN). ZIKV RNA copy number was determined by one-step quantitative RT-PCR on an ABI 7500 Fast Instrument using standard cycling conditions as described previously (Miner et al., 2016). The following primers and probe targeting ZIKV were used: 5'-CCACATGTCTCTGCA GACATATG-3'; reverse: 5'-TTCGACAGCGTGTACACAG-3'; and probes: 5'-56-FAM/AGCCTACCTTGACAA GCAGTC/3IABkFQ-3' (Integrated DNA Technologies).

Histology and immunohistochemistry staining

Placentas and fetuses were dissected by cesarean section on E14.5. Harvested samples were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) at room temperature and embedded in paraffin. At least five placentas from different dams with the indicated genotypes or treatments were sectioned and stained with hematoxylin and eosin to assess morphology. Histologic images were captured by use of a Nikon Eclipse microscope equipped with an Olympus DP71 color camera under 2 \times , 20 \times , and 40 \times objectives. Measurement of size and thickness of different placental layers were performed using ImageJ (National Institutes of Health).

For immunohistochemical staining of mouse placentas, deparaffinized tissues were quenched with 3% H₂O₂, blocked

for 2 h, and incubated with primary antibodies anti-p62 (1:500; ab56416; Abcam) overnight. The M.O.M. kit (BMK-2202; Vector Laboratories) was applied for blocking and secondary antibody incubation according to the manufacturer's instructions. The sections were then incubated with the ABC reagent from the Vectastain universal kit (PK-7200; Vector Laboratories) and developed using the DAB substrate kit (SK-4100; Vector Laboratories). Tissues were counterstained with hematoxylin. A no-primary antibody staining was included as a negative control.

RNA ISH

RNA ISH was performed with an RNAscope 2.5 (Advanced Cell Diagnostics) according to the manufacturer's instructions. In brief, formalin-fixed paraffin-embedded tissue sections were deparaffinized and incubated at 60°C for 1 h. H₂O₂ was applied to quench endogenous peroxidases for 10 min at room temperature. Slides were then boiled in RNAscope Target Retrieval Reagents for 15 min and incubated for 30 min in RNAscope Protease Plus. The ZIKV probe (catalog #467871), positive probe (the plr2a probe; catalog #312471) and negative probe (targeting bacterial gene *dapB*; catalog #310043) were designed and synthesized by Advanced Cell Diagnostics. Tissues were counterstained with hematoxylin and mount. Images were captured with standard bright-field microscopy under 2 \times and 20 \times objectives.

Western blotting

Total protein was extracted from frozen mouse placenta samples and homogenized in RIPA buffer (Cell Signaling Technology) with protease inhibitor cocktails (Sigma-Aldrich). For cell cultures, cells were lysed directly in RIPA buffer. Equivalent amounts of total protein, determined by BCA assays (Thermo Fisher Scientific), were separated on 4%–20% Mini-PROTEAN precast gels (Bio-Rad Laboratories) and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% milk in PBS with Tween 20 for 2 h at room temperature and incubated with indicated antibodies in 5% BSA at 4°C overnight. The following primary antibodies and dilutions were used: anti-LC3B (1:1,000; NB600-1384; Novus Biologicals), anti-p62 (1:1,000; ab56416; Abcam), and anti-GAPDH (1:2,000; 3700s; Cell Signaling Technology). ImageJ was used for densitometry of Western blots.

Statistical analysis

GraphPad Prism 5.0 was used for all analyses. The analyses of virologic or histopathological data were conducted using a Mann-Whitney test or ANOVA with a multiple comparison test. P-values < 0.05 were considered to indicate statistical significance.

Online supplemental material

Supplemental material associated with this study provides supporting data for additional time points of ZIKV infection of human trophoblasts, increased autophagic activity in

mouse placentas, and that HCQ treatment of *Atg161^{HM}* mice does not alter ZIKV infection. Fig. S1 shows ZIKV infection in human trophoblasts. Fig S2 shows that ZIKV infection induces autophagic activity in mouse placentas. Fig. S3 shows that HCQ treatment reduces placental and fetal ZIKV infection specifically via autophagy.

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Author contributions: B. Cao and L.A. Parnell conducted experiments. M.S. Diamond provided key reagents, animal facilities, and resources for evaluating infection. B. Cao and I.U. Mysorekar designed the research, analyzed data, and wrote the first draft of the manuscript. All authors reviewed, edited, and approved the paper.

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SUPPLEMENTAL MATERIAL

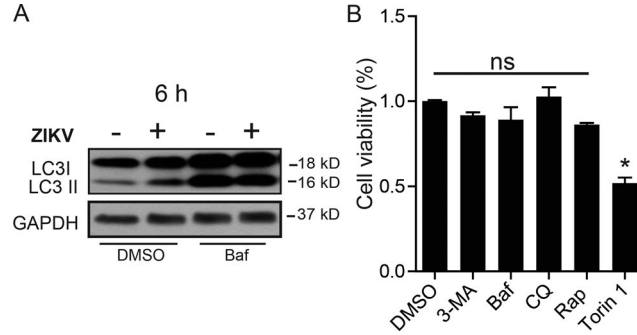
Cao et al., <https://doi.org/10.1084/jem.20170957>

Figure S1. **ZIKV infection in human trophoblasts.** (A) Western blots for LC3-I/II show enhanced autophagy flux upon ZIKV infection at 6 hpi. ZIKV-infected JEG-3 (multiplicity of infection 0.1, 2 h) were cultured for 6 h and harvested. Baf A1 was applied for 30 min before harvesting to monitor autophagic flux. Images represent data from four independent experiments. (B) Cell viability assays of uninfected JEG-3 cells cultured in medium supplemented with indicated autophagy modulators (at same dosage as described in Fig. 1 F) for 48 h; $n = 4$ for each group. Data depict mean \pm SEM. *, $P < 0.05$; ns, not significant (ANOVA with a Dunn's multiple-comparison test). Rap, rapamycin.

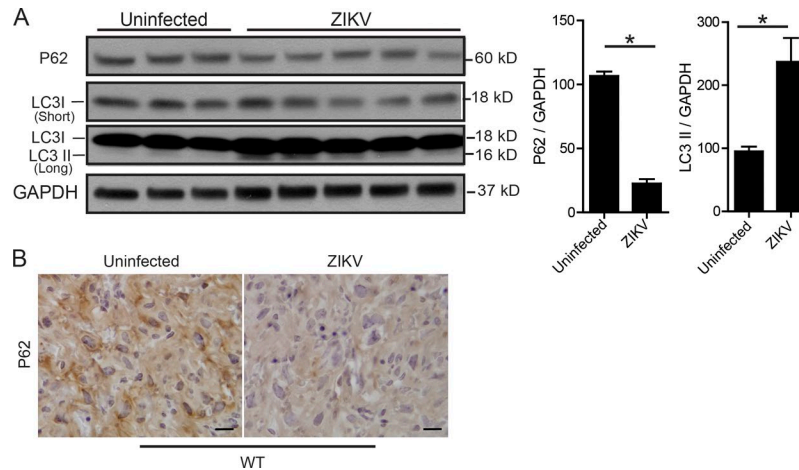


Figure S2. **ZIKV infection induces autophagic activity in mouse placentas.** (A) Immunoblot and quantification of LC3 and p62 show increased autophagic activity in mouse placentas (at E14.5) infected with ZIKV compared with uninfected controls. GAPDH, a loading control; $n = 3-5$. Data depict mean \pm SEM. *, $P < 0.05$, Mann-Whitney test. (B) Representative immunohistochemical staining of p62 in WT placentas with or without ZIKV infection at E14.5. Images represent data from five independent dams. Bars, 100 μ m.

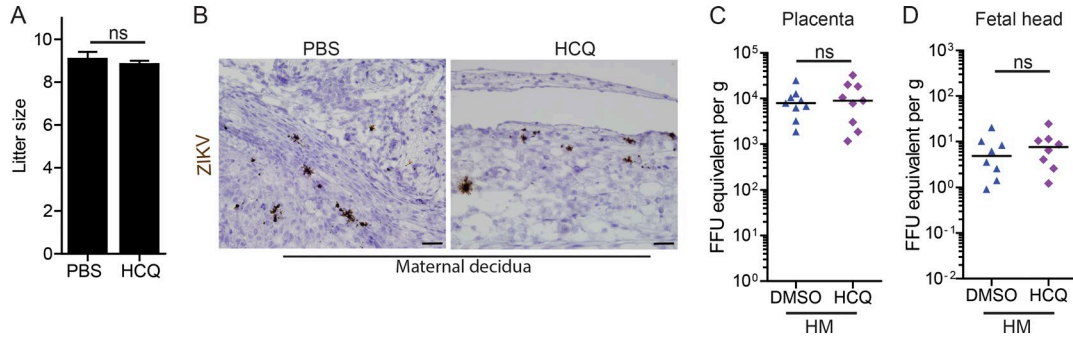


Figure S3. **HCQ treatment reduces placental and fetal ZIKV infection specifically via autophagy.** (A) Litter size at E14.5 with or without HCQ treatment; $n = 6$ per group. Results represent mean \pm SEM, three independent experiments. ns, not significant, Mann-Whitney test. (B) Representative ISH images of ZIKV RNA in indicated maternal uterine decidua. Bars, 100 μ m. (C and D) Pregnant HM female mice were treated with HCQ (40 mg/kg/day via intraperitoneal route) or DMSO as a mock control from day +1 post-ZIKV infection (E10.5) to E14.5. Viral burden of ZIKV in placentas (C) and fetal head (D) were measured by quantitative RT-PCR; $n = 8$ from three independent dams. ns, not significant, Mann-Whitney test.