Trex1 regulates lysosomal biogenesis and interferon-independent activation of antiviral genes

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The sensing of viral nucleic acids by the innate immune system triggers the production of type I interferons, which activates interferon-stimulated viral genes (ISGs) and directs a multifaceted antiviral response. ISGs can also be activated through interferon-independent pathways, although the precise mechanisms remain elusive. Here we found that the cytosolic exonuclease Trex1 regulates the activation of a subset of ISGs independently of interferon. Both Trex1−/− mouse cells and Trex1-mutant human cells had high expression of genes encoding antiviral molecules (‘antiviral genes’) and were refractory to viral infection. The interferon-independent activation of antiviral genes in Trex1−/− cells required the adaptor STING, the kinase TBK1 and the transcription factors IRF3 and IRF7. We also found that Trex1-deficient cells had an expanded lysosomal compartment, altered subcellular localization of the transcription factor TFEB and diminished activity of the regulator mTORC1. Together our data identify Trex1 as a regulator of lysosomal biogenesis and interferon-independent activation of antiviral genes and show that dysregulation of lysosomes can elicit innate immune responses.

Vertebrates are constantly facing challenges from pathogenic microbes that introduce a variety of microbial proteins and nucleic acids into the host cell. To counter this, eukaryotic cells express many different pattern-recognition receptors that detect microbial pathogen-associated molecular patterns, which then activate antiviral interferon and proinflammatory responses. Mammalian pattern-recognition receptors include the Toll-like receptors (TLRs), RIG-I-like receptors, Nod-like receptors, C-type lectin receptors and an emerging category of cytosolic receptors for DNA. During viral infection, viral nucleic acids are the main pathogen-associated molecular patterns detected by the receptors of the host innate immune system, which include RIG-I-like receptors and receptors of DNA in the cytosol and a subfamily of TLRs that localize to the endosomal membrane. The central hub for the cytosolic sensing of DNA is the endoplasmic reticulum membrane adaptor STING (also known as MITA, MPYS or ERIS). Many proteins have been proposed to detect double-stranded DNA in the cytosol and signal through STING, including DAI, IFI16 and DDX41 (refs. 4–6). STING can also directly recognize the cyclic dinucleotide c-di-GMP, which is usually associated with bacterial infection and activates interferon expression. Although interferon has a major role in controlling viral infections, interferon-independent pathways also exist and are vital for antiviral defense. For example, STING can activate the transcription factor STAT6 independently of the interferon pathway, and activated STAT6 induces chemokine expression that primes adaptive immune responses. Infection with enveloped viruses also triggers an interferon-independent pathway that involves the direct activation of a subset of interferon-stimulated genes (ISGs) by the transcription factor IRF3 (ref. 9). A study of innate immune responses to RNA viruses mediated by the signaling adaptor MAVS has demonstrated that interferon-independent induction of genes encoding antiviral molecules (‘antiviral genes’) occurs rapidly after infection and is functionally important for the control of viral replication before the onset of more robust and sustained activation by interferon.

The sensing pathways of the innate immune system are carefully designed to distinguish self ligands from non-self ligands, either by spatial separation (for example, TLR7 and TLR9 reside in endosomes, which are devoid of host nucleic acids) or by stringent ligand specificity (for example, TLR9 recognizes CpG-containing DNA from bacteria, and the RNA heliase RIG-I recognizes RNA containing 5′ triphosphate from viruses). However, how cytosolic DNA-sensing pathways distinguish host DNA and viral DNA remains unclear. Trex1, an exonuclease that resides on the endoplasmic reticulum, is a negative regulator of the sensing of cytosolic human immunodeficiency virus (HIV) DNA by the innate immune system. In Trex1-deficient (Trex1−/−) mouse embryonic fibroblasts (MEFs) and in human CD4+ cells...
T cells and macrophages depleted of Trex1 by RNA-mediated interference, cytosolic HIV DNA accumulates and triggers interferon through the STING–kinase TBK1–IRF3 pathway\(^\text{11}\). Those findings and other studies\(^\text{12}\) suggest that cells rely on negative regulatory molecules such as Trex1 to keep cytosolic DNA-sensing pathways in check.

Trex1 deficiency has been linked to the pathogenesis of autoimmunity. TREX1 mutations in humans are associated with a spectrum of autoimmune and inflammatory phenotypes, including Aicardi-Goutières syndrome (AGS; an inflammatory brain disease that mimics the symptoms of congenital viral infection\(^\text{13,14}\)), systemic lupus erythematosus (SLE), familial chilblain lupus, and retinal vasculopathy with cerebral leukodystrophy\(^\text{15–17}\). Disease in MOI, 2 and 10; infection with two different doses of VSV (multiplicity of infection) has been found in up to 2% of patients with SLE, with an extremely high odds ratio (of 25)\(^\text{18}\); thus, such mutations represent one of the highest disease risks recorded for a single susceptibility gene in complex polygenic SLE\(^\text{14}\). Studies of Trex1\(^{-/-}\) mice have shown that Trex1\(^{-/-}\) cells accumulate cytosolic single-stranded DNA that might be derived from DNA repair in the nucleus or from endogenous retroelements\(^\text{19,20}\).

Genetic evidence has demonstrated that the STING-mediated DNA-sensing pathway is essential for the pathogenesis of autoimmune disease in Trex1\(^{-/-}\) mice\(^\text{13}\). The initiation of interferon expression is detected in only a subset of nonhematopoietic cells in Trex1\(^{-/-}\) mice, which raises the question of what happens to the majority of other cells that also lack Trex1 function. We also wondered whether Trex1 inhibits interferon responses to other viruses beyond HIV and/or if the mere loss of Trex1 function in a cell would elicit innate immune responses and establish an antiviral state.

In this study, we found that Trex1-deficient and Trex1-mutant cells had broad antiviral activity against many RNA viruses. The antiviral activity was provided by higher expression of ISGs in cells that lacked Trex1 function and was mediated through an interferon-independent signaling pathway that involved STING, TBK1, IRF3 and IRF7. We also found that Trex1 regulated lysosomal biogenesis through the transcription factor TFEB and the regulator mTORC1 and provide evidence that dysregulation of lysosomes elicits innate immune responses.

### RESULTS

#### Impaired VSV replication in Trex1-deficient cells

To investigate whether Trex1 is involved in the interferon response to RNA viruses, we infected wild-type and Trex1\(^{-/-}\) MEFs with the Indian strain of vesicular stomatitis virus (VSV; a negative-stranded RNA virus), with VSV glycoprotein (G protein)-pseudotyped HIV\(^\text{11}\) or treated them with mock infection, then measured mRNA encoding interferon-β (IFN-β; Ifnb mRNA) 24 h after infection. As reported before\(^\text{11}\), mock-infected wild-type and Trex1\(^{-/-}\) cells did not have detectable expression of Ifnb mRNA, and infection with HIV stimulated the expression of Ifnb mRNA only in Trex1\(^{-/-}\) cells, not in wild-type cells (Fig. 1a). In contrast, infection with VSV stimulated similarly high Ifnb mRNA expression in wild-type and Trex1\(^{-/-}\) cells (Fig. 1a), which suggested that Trex1 did not regulate the type I interferon response to VSV. However, VSV replication was considerably impaired in Trex1\(^{-/-}\) cells relative to its replication in wild-type cells, even though the induction of Ifnb was indistinguishable in the two cell types (Fig. 1b–d). Specifically, the abundance of the two main forms of VSV RNA, encoding the glycoprotein G and matrix protein M, in Trex1\(^{-/-}\) cells was 12% and 7%, respectively, that in wild-type cells (Fig. 1b). We also detected much lower abundance of VSV proteins in Trex1\(^{-/-}\) cells than in wild-type cells after infection with two different doses of VSV (multiplicity of infection (MOI), 2 and 10; Fig. 1c). VSV titers were also lower in infected Trex1\(^{-/-}\) cells than in wild-type cells (Fig. 1d). To better quantify and visualize VSV replication, we infected wild-type and Trex1\(^{-/-}\) cells with VSV-P-eGFP, a form of VSV in which enhanced green fluorescent protein (eGFP) is fused to the VSV phosphoprotein P, which is usually associated with foci of viral RNA replication in the cell\(^\text{11}\). We observed less replication of VSV-P-eGFP in Trex1\(^{-/-}\) cells (3.2% GFP) than in wild-type cells (22.2% GFP) by flow cytometry (Fig. 1e). Consistent with these data, analysis of infected wild-type cells by fluorescence microscopy showed bright replication foci marked by P-eGFP, whereas we detected very little green fluorescent signal in VSV-P-eGFP–infected Trex1\(^{-/-}\) cells (Fig. 1f). We also infected bone marrow–derived macrophages (BMDMs) generated from wild-type, Trex1\(^{-/-}\) and Trex1\(^{-/-}\) mice and found that only Trex1\(^{-/-}\) cells were resistant to infection with VSV (Fig. 1g,h).

We next assessed whether the entry of VSV was inhibited in Trex1\(^{-/-}\) cells. This seemed unlikely, as the entry of VSV–pseudotyped HIV into Trex1\(^{-/-}\) cells was not impaired\(^\text{11}\), and infection with VSV stimulated indistinguishable expression of Ifnb mRNA in wild-type and Trex1\(^{-/-}\) cells (Fig. 1a). Nonetheless, to rule out the possibility of an entry defect, we labeled wild-type VSV virions with the fluorescent dye Dil and monitored the infection of wild-type and Trex1\(^{-/-}\) cells with Dil-labeled VSV by live cell fluorescence microscopy (Supplementary Fig. 1a). We observed no difference between wild-type and Trex1\(^{-/-}\) cells in intracellular Dil-labeled VSV at 1 h after infection. We also observed similar amounts of RNA encoding VSV G and M at 1 h after infection in both cell types (Supplementary Fig. 1b). These data suggested that VSV replication was blocked at an early stage after entry, such as uncoating or RNA replication, in Trex1\(^{-/-}\) cells. We also found that in contrast to infected wild-type cells, VSV-infected Trex1\(^{-/-}\) cells did not show detectable cytopathic effects (Supplementary Fig. 2), consistent with the idea that Trex1\(^{-/-}\) cells were protected against viral infection.

To investigate whether Trex1 was also required for VSV replication in human cells, we studied wild-type skin fibroblasts and Trex1-mutant skin fibroblasts (homozygous for the TREX1 mutation that produces the R114H substitution of Trex1; called TREX1\(^{R114H/R114H}\) here) from a patient with AGS. Arg114 is a critical residue at the interface of the Trex1 dimer, and the R114H substitution substantially disrupts Trex1 function in vitro\(^\text{22}\). R114H represents the most common Trex1 substitution in patients with AGS and has also been associated with SLE\(^\text{14}\). We infected the cells with VSV or VSV-P-eGFP and measured viral RNA. Infection with VSV or VSV-P-eGFP was lower in Trex1\(^{R114H/R114H}\) cells than in wild-type cells, as reflected by the lower abundance of viral RNA and viral proteins and fewer viral replication foci (Fig. 1i–k). Given these collective results, we concluded that VSV replication was impaired at an early after entry step in both mouse cells and human cells lacking Trex1 function.

#### Broad antiviral resistance of Trex1-deficient cells

To determine whether the replication block in Trex1\(^{-/-}\) and Trex1\(^{R114H/R114H}\) cells was unique for VSV, we infected wild-type and Trex1\(^{-/-}\) MEFs and wild-type and Trex1\(^{R114H/R114H}\) human fibroblasts with the following three additional RNA viruses with negative-stranded genomes: influenza virus (A/WSN/1933 strain), Sendai virus and West Nile virus (WNV-TX02 strain). We then measured viral RNA and proteins, as well as viral titers in supernatants. None of the three viruses replicated efficiently in Trex1\(^{-/-}\) or Trex1\(^{R114H/R114H}\) cells, in contrast to their replication in wild-type cells (Fig. 2). These results demonstrated that cells lacking Trex1 function were resistant to infection with several different types of RNA viruses.
Figure 1 Impaired VSV replication in Trex1-deficient cells. 
(a) Quantitative RT-PCR analysis of *Ifnb* mRNA in wild-type (WT) and *Trex1*–/– MEFs left uninfected (–) or infected for 24 h with VSV–pseudotyped HIV1 or with VSV (MOI, 2). AU, arbitrary units. (b–d) Quantitative RT-PCR analysis of RNA encoding the VSV G and M proteins (b), immunoblot analysis of VSV proteins (c) and viral titers in supernatants (d) of wild-type and *Trex1*–/– MEFs mock infected or infected for 18 h with VSV. ND, not detectable; HMGB1, loading control (throughout). (e) Flow cytometry (e) and fluorescence microscopy (f) of wild-type and *Trex1*–/– MEFs infected for 18 h with VSV-P-eGFP. Numbers above outlined areas indicate percent infected (GFP+) cells. SSC, side scatter; DAPI, DNA-intercalating dye. Original magnification, ×20. (g,h) Fluorescence microscopy (g) and quantitative RT-PCR analysis of RNA encoding VSV G and M proteins (h) in wild-type, *Trex1*–/– and *Trex1*–/– BMDMs infected for 18 h with VSV-P-eGFP (g) or VSV (h). Original magnification, ×20. (i) Quantitative RT-PCR analysis of RNA encoding VSV G and M proteins (i) and immunoblot analysis of VSV proteins (j) in wild-type and *Trex1R144H/R114H* (*TREX1*–mut) primary human skin fibroblasts (isolated from a healthy donor and a patient with AGS, respectively), at 0–18 h after infection with VSV. Arrowhead, nonspecific band; Tub, tubulin (loading control throughout). (k) Fluorescence microscopy of wild-type and *Trex1R144H/R114H* human fibroblasts infected for 18 h with VSV-P-eGFP. Original magnification, ×20. Data are representative of at least three independent experiments (error bars (a,b,h,i), s.d.).

**Trex1 regulates interferon-independent activation of ISGs**

Next we investigated the mechanism of antiviral resistance in *Trex1*–/– cells. We first examined gene-expression profiles by infecting wild-type and *Trex1*–/– MEFs with VSV, influenza virus, Sendai virus or West Nile virus, or mock infecting the cells, followed by isolation of total RNA and analysis by high-throughput sequencing technologies (RNA-Seq) that offer quantitative measurement of both host and viral RNA simultaneously (Fig. 3a). The changes in gene expression confirmed by quantitative PCR were similar to those obtained by RNA-Seq (Fig. 3b, Supplementary Figs. 3 and 4 and data not shown), which emphasized the quantitative power of our RNA-Seq analysis. We first analyzed the gene-expression profiles of uninfected wild-type and *Trex1*–/– samples with Ingenuity pathway-analysis software and found that the gene network most represented in *Trex1*–/– cells relative to its representation in wild-type cells was the network of 'antimicrobial response, inflammatory response, infectious diseases', which consists mostly of ISGs (Supplementary Fig. 5a,b). The networks of 'interferon signaling' and 'cytosolic pattern-recognition receptors' were also

Figure 2 Trex1-deficient cells have broad antiviral resistance. 
(a–c) Quantitative RT-PCR analysis of RNA encoding the influenza virus nonstructural protein 1 (a), immunoblot analysis of influenza virus proteins (b) and viral titers in supernatants (c) of wild-type and *Trex1*–/– MEFs or wild-type and *TREX1R144H/R114H* human fibroblasts left uninfected or infected with influenza virus (MOI, 1). (d–f) Quantitative RT-PCR analysis of RNA encoding the Sendai virus phosphoprotein P (d), immunoblot analysis of Sendai virus proteins (e) and viral titers in the supernatants (f) of wild-type and *Trex1*–/– MEFs or wild-type and *TREX1R144H/R114H* human fibroblasts left uninfected or infected with Sendai virus (SeV; MOI, 10). (g–i) Quantitative RT-PCR analysis of RNA encoding the West Nile virus envelope protein Env (g), immunoblot analysis of West Nile virus proteins (h) and viral titers in the supernatants (i) of wild-type and *Trex1*–/– MEFs left uninfected or infected with West Nile virus (WNV; MOI, 10 (h) or 100 (g–i)). Data are representative of at least two independent experiments (error bars (a,d,g), s.d.).
among the top-ranked canonical pathways with high ‘hit ratio’ (determined by the frequency of genes in a pathway that are represented in a data set; Supplementary Fig. 3c). We then constructed a heat map of genes involved in the ‘antimicrobial response’ network and representative genes from each virus based on the expression values (and standard deviation (s.d.)) of each gene across all samples (Fig. 3a). All four RNA viruses replicated less efficiently in Trex1−/− MEFs left uninfected or infected for 18 h with VSV, influenza virus (Flu), Sendai virus or West Nile virus. (Fig. 3a). We then constructed a heat map of genes encoding members of the IFIT family in uninfected wild-type and Trex1−/− MEFs; results are presented relative to those of the control gene Gapdh. (c) Enzyme-linked immunosorbent assay of IFN-β in supernatants of wild-type and Trex1−/− MEFs left uninfected (Med) or infected with VSV. (d,e) Quantitative RT-PCR analysis of Ifnb1 mRNA (d) and Ifit1 mRNA (e) in wild-type and Trex1−/− MEFs mock infected or infected with VSV, influenza virus, Sendai virus or West Nile virus. (f) Quantitative RT-PCR analysis of selected ISGs and interferon-encoding genes in wild-type MEFs 72 h after transfection with control siRNA or Trex1-specific siRNA (si-Trex1, 1–3); results are presented relative to those obtained with control siRNA, set as 1. (g) Quantitative RT-PCR analysis of the knockdown of Trex1. *P < 0.05 (Student’s t-test). (h) Quantitative RT-PCR analysis of Ift1 mRNA in Ifnar1−/− MEFs 72 h after transfection with siRNA as in f (presented as in f). *P < 0.05 (Student’s t-test). (i) VSV-P-eGFP infection in wild-type and Trex1−/− MEFs transfected for 48 h with control siRNA or siRNA specific for (si-) IFIT1, IFITM3, STAT1 or STAT2 (horizontal axis) and then mock infected (UI) or infected for 18 h with VSV-P-eGFP, analyzed by flow cytometry; results are presented as the frequency of GFP+ cells, *P < 0.05 (Student’s t-test). (j) Quantitative RT-PCR analysis of Ifit1, Ifit7 and Ifnb1 mRNA in spleen, heart and BMDMs isolated from wild-type, Trex1−/− and Trex1−/− mice (presented as in b). *P < 0.05 (Student’s t-test). Data are representative of one experiment (a), at least three independent experiments (b–g) or two independent experiments (h–i, error bars (b–i), s.d.).
of the IFITM family had similar expression in wild-type and Trex1−/− cells (Supplementary Fig. 3). Together our data suggested substantial activation of a subset of ISGs in Trex1−/− cells independently of the interferon response.

To further confirm that the activation of ISGs was specific to the loss of Trex1 function, we knocked down Trex1 expression in wild-type MEFs through the use of three different small interfering RNA (siRNA) constructs and noted significantly higher expression of Ifit1, Ifit3 and Irf7 (also an ISG), but not of Ifitm3, Ifna4 or Ifnb1 (Fig. 3f). We also knocked down Trex1 expression in MEFs deficient in the receptor for IFN-α and IFN-β (Ifnar1−/−/− MEFS) and observed similarly higher expression of Ifit1 and Ifnβ (Fig. 3g), which further suggested that the activation of ISGs regulated by Trex1 was interferon independent. To determine whether the activation of ISGs or the interferon pathway contributed to the control of viral infection in Trex1−/− cells, we transplanted wild-type and Trex1−/− cells with a control siRNA or siRNA specific for the products of two ISGs (Ifit1 and Ifitm3) or two key components of the interferon signaling pathway (STAT1 and STAT2). We then infected cells with VSV-P-eGFP and measured infectivity by flow cytometry (Fig. 3h). Knockdown of Ifit1 or IFITM3 in Trex1−/− cells partially alleviated the blockade of VSV replication, consistent with the known antiviral functions of these two molecules24–26. In contrast, knockdown of STAT1 or STAT2 had no effect on VSV replication, further demonstrating that the interferon response was not required for the control of viral infection in Trex1−/− cells. To determine whether this ISG-induction signature was present in primary cells of the immune response and tissues from Trex1−/− mice, we isolated total RNA from whole spleen, heart and BMDMs from wild-type, Trex1−/− and Trex1−/− mice and measured Ifit1, Irf7 and Ifnb mRNA. We observed an induction of ISGs of up to 30-fold in whole tissues and up to 60-fold in primary cells of the immune system only in Trex1−/− mice, relative to their expression in wild-type mice (Fig. 3i). We also observed very low Ifnb expression in all samples from Trex1−/− mice (Fig. 3i), consistent with published reports showing restriction of interferon activation to a subset of heart-muscle cells12,19.

We also used RNA-Seq to analyze total RNA from uninfected wild-type fibroblasts or fibroblasts from patients with AGS with the TREX1R114H/R114H mutation or other mutations linked to AGS, including the RNASEH2C D39Y/D115fs and SAMHD1 R290H/Q548X mutations27. We again found substantial upregulation of a subset of ISGs, but not interferon-encoding genes, in TREX1R114H/R114H cells (Fig. 4a). Notably, the ISG-activation signature was weak in RNASEH2C D39Y/D115fs cells and was not present in SAMHD1 R290H/Q548X cells (Fig. 4a). To determine whether the same group of ISGs was activated in Trex1−/− and TREX1R114H/R114H cells, we selected 35 ISGs that are expressed in both mouse and human cells and compared their induction in Trex1−/− and TREX1R114H/R114H cells. ISGs that were induced in Trex1−/− MEFs were also induced in TREX1R114H/R114H fibroblasts, with a correlation r2 value of 0.49 (Fig. 4b). We observed weak correlation between gene induction in Trex1−/− cells and in RNASEH2C D39Y/D115fs cells (r2 = 0.14) and no correlation for that in Trex1−/− cells and SAMHD1 R290H/Q548X cells (r2 = 0.04). Our data demonstrated that Trex1 also regulated the activation of ISGs in human fibroblasts.

**Factors for interferon-independent ISG activation**

We next sought to identify factors of the innate immune system required for the interferon-independent activation of ISGs in Trex1-deficient cells. We chose to measure Ifit1 mRNA as an example of a Trex1-regulated ISGs because Ifit1 was the gene upregulated the most in Trex1 deficiency. We first examined the effect of IRF3, which activates ISGs directly28–32. We measured Ifit1 mRNA in wild-type, Trex1−/− and Trex1−/−/Ifit3−/− MEFs and found induction of Ifit1 in Trex1−/− single-deficiency cells and that the induction was inhibited by Trex1−/−/Ifit3−/− double deficiency (Fig. 5a), which suggested that IFI3 was required for activation of Ifit1. To determine whether IFI3 was also required for antiviral activity in the setting of Trex1 deficiency, we infected wild-type, Trex1−/− and Trex1−/−/Ifit3−/− MEFs with VSV or Sendai virus and measured viral proteins by immunoblot analysis or flow cytometry (Fig. 5b–d). Infection with VSV or Sendai virus was inhibited in Trex1−/− cells, and infection with each was restored to close to wild-type amounts in Trex1−/−/Ifit3−/− cells. Therefore, IFI3 was a key component of the antiviral resistance in Trex1−/− cells.

We next explored how innate immune pathway upstream of IFI3 was involved in the activation of ISGs in Trex1−/− cells. IFI3 is activated mainly by cytosolic DNA- or RNA-sensing pathways mediated by STING-TBK1 or RIG-I–MAVS, respectively. Therefore, we knocked down key components of each pathway in Trex1−/− cells through the use of siRNA and measured Ifit1 expression. Knockdown
of IRF3, IRF7, TBK1, STING or IFI204 resulted in significantly lower Ifit1 expression, whereas knockdown of RIG-I or MAVS had no effect (Fig. 5e). We also did not observe any effect on Ifit1 expression in Trex1−/− cells after knocking down TLR7 or TLR9 (data not shown). Knockdown of IRF3, IRF7 or TBK1 or STING diminished the VSV-replication block in Trex1−/− cells (Fig. 5f). These results suggested that the cytosolic DNA-sensing machinery was required for the interferon-independent activation of ISGs in Trex1-deficient cells, but the cytotoxic RNA-sensing machinery was not. Knockdown of STING did not seem to enhance VSV replication in Trex1−/− cells, probably because the VSV-replication assay measures the entire life cycle of VSV, and many host factors may contribute to this, or because STING also regulates many other genes encoding molecules that could be required for VSV replication. The same cytotoxic DNA-sensing pathway was also involved in the activation of interferon-encoding genes during viral infection2,11, whose products can then activate ISGs. Therefore, we did double knockdown of Trex1 plus components of the cytotoxic DNA-sensing pathway in Ifnar1−/− MEFs. Ifit1 expression was higher after knockdown of Trex1, and that was diminished by further knockdown of TBK1, IRF7 or STING (Fig. 5g). Knockdown of Trex1 in Ifnar1−/− MEFs also inhibited VSV replication, and further knockdown of TBK1 or IRF7 alleviated that inhibition (Fig. 5h). Together our data suggested that the core cytotoxic DNA-sensing machinery (STING-TBK1-IRF3-IRF7) was involved in activating ISGs directly in cells with diminished or no Trex1 activity.

**Trex1 regulates lysosomal biogenesis via TFEb and mTORC1**

We next sought to identify the underlying basis for the activation of ISGs in Trex1−/− and TREX1R114H/R114H cells. We first considered the possibility that Trex1 directly inhibits the cytotoxic DNA-sensing machinery. To test this, we used 293T human embryonic kidney cells, in which the overexpression of STING induced sixfold more Ifit1 mRNA (Supplementary Fig. 8). We then coexpressed STING and increasing amounts of Trex1 to determine whether overexpression of Trex1 would inhibit the STING-mediated activation of Ifit1.

We did not observe any effect on Ifit1 induction after overexpression of Trex1 (Supplementary Fig. 8). The same degree of Trex1 overexpression inhibits the HIV-mediated activation of interferon-encoding genes11. These results suggested that Trex1 did not directly inhibit the cytotoxic DNA-sensing machinery.

We then hypothesized that perhaps the accumulation of self-ligands or a cellular abnormality in Trex1−/− or TREX1R114H/R114H cells might be detected by the STING-TBK1-IRF3-IRF7 pathway. We first assessed the abundance and morphology of cellular organelles in wild-type and Trex1−/− cells by immunofluorescence staining of well-defined organelle markers (Fig. 6a). We did not observe substantial differences between those cells in their mitochondria, Golgi apparatus, endoplasmic reticulum or early endosomes. However, late endosomes (identified by staining with antibody to LAMP-1) and lysosomes (identified by staining with the probe LysoTracker Red) seemed to be more abundant in Trex1−/− cells than in wild-type cells (Fig. 6a). We also observed a similarly greater abundance of the late endosome-lysosome compartment in Trex1−/− BMDMs than in wild-type or Trex1+/− BMDMs (Fig. 6b). To determine if this difference was also present in human cells, we stained wild-type and TREX1R114H/R114H human fibroblasts, as well as control HEla human cervical cancer cells and HEla cells in which Trex1 was knocked down (by siRNA), with LysoTracker Red. In both cases, we observed much more LysoTracker Red staining in Trex1-deficient cells than in wild-type cells (Fig. 6a). We also observed a similarly greater abundance of the late endosome-lysosome compartment in Trex1−/− BMDMs than in wild-type or Trex1+/− BMDMs (Fig. 6b). To determine if this difference was also present in human cells, we stained wild-type and TREX1R114H/R114H human fibroblasts, as well as control HEla human cervical cancer cells and HEla cells in which Trex1 was knocked down (by siRNA), with LysoTracker Red. In both cases, we observed much more LysoTracker Red staining in Trex1-deficient cells than in wild-type cells (Fig. 6c and Supplementary Fig. 9), which indicated expansion of the late endosome-lysosome compartment in cells that lacked Trex1 function. We quantified the lysosomal expansion by flow cytometry of live cells with LysoTracker Red and found that Trex1−/− cells and TREX1R114H/R114H cells had three- to fivefold more lysosomes than did wild-type cells (Fig. 6d). We also detected more of the lysosomal membrane proteins LAMP-1 and NPC1 in TREX1R114H/R114H cells and Trex1−/− cells than in wild-type cells by immunoblot analysis (Fig. 6e), which suggested enhanced lysosomal biogenesis in Trex1-deficient cells. To further confirm the expansion of the lysosome compartment, we analyzed wild-type and...
Trex1 negatively regulates lysosomal biogenesis. (a, b) Fluorescence microscopy of wild-type and Trex1−/− MEFs (a) and BMDMs (b) stained for various organelle markers (left margin; antibody or dye in parentheses): mito, mitochondria; ER, endoplasmic reticulum; endo, endosome; lyso, lysosome. Original magnification, ×20. (c) Electron microscopy of wild-type and TREX1R114H mice stained with LysoTracker Red. Original magnification, ×20. (d) Flow cytometry of live wild-type and Trex1−/− MEFs or wild-type and TREX1R114H human fibroblasts stained with LysoTracker Red. (e) Immunoblot analysis of LAMP-1 and NPC1 in wild-type and TREX1R114H human fibroblasts stained with LysoTracker Red and FB and wild-type and Trex1−/− MEFs and BMDMs. (f) Electron microscopy of wild-type and Trex1−/− MEFs: arrows indicate undigested (electron-dense) cellular materials in lysosome vacuoles; arrowheads in inset (enlargement of area outlined in main image) indicate single membrane surrounding a lysosome vacuole. N, nucleus; M, mitochondrion; L, lysosome. Scale bar, 1 µm. (g) Quantification of lysosomal vacuoles in thin sections of MEFs (per cell; n = 20). *P < 0.05 (Student’s t-test). Data are representative of at least three independent experiments (a–e) or one experiment (f, g, s.d.).

Trex1−/− cells by electron microscopy. Trex1−/− cells had significantly more lysosomal vacuolar structures (Fig. 6f,g). Those structures were surrounded by single-layer membranes, some of which contained electron-dense cellular materials commonly found in lysosomes (Fig. 6f, inset). Lysosomes are important organelles for the breakdown and turnover of other cellular organelles (such as mitochondria), proteins and nucleic acids. Of note, we did not observe excessive accumulation of undigested cellular materials in these lysosomes; such accumulation is often found in cells associated with lysosomal storage diseases. We also did not detect more autolysosomes in Trex1−/− cells than in wild-type cells, as measured by electron microscopy, the formation of dots of GFP-tagged autophagy marker LC3, and immunoblot analysis of p62, a ubiquitin-binding scaffold protein, and LC3 (Supplementary Fig. 10 and data not shown).

To determine whether the lysosome-expansion phenotype in Trex1−/− cells was caused by the induction of genes encoding proteins of the lysosome, we measured the expression of Ctsa, Sgsh, Lamp1, Mcoln1 and Tpp1, which encode enzymes or structural proteins of the lysosome. All five genes were upregulated three- to fivefold in Trex1−/− cells relative to their expression in wild-type cells, whereas other nonlysosomal genes were not (Fig. 7a). Many other genes encoding molecules involved in lysosomal biogenesis were also upregulated in Trex1−/− cells relative to their expression in wild-type cells (Supplementary Fig. 11). Lysosomal genes are regulated by TFEB through the recognition of conserved binding sites in their promoters. TFEB is a master regulator of the ‘CLEAR’ gene network (‘coordinated lysosomal expression and regulation’) and overexpression of TFEB results in higher lysosomal gene expression and promotes lysosome expansion. TFEB resides mostly in the cytoplasm and translocates to the nucleus after complex post-translational modifications. We did not observe any difference between wild-type and Trex1−/− cells in their abundance of Tfeb mRNA or TFEB protein (Fig. 7a and data not shown). To examine the subcellular localization of TFEB, we stained wild-type and Trex1−/− cells with an antibody to TFEB and found that endogenous TFEB became mostly nuclear in Trex1−/− cells (Fig. 7b). This result suggested that the higher lysosomal gene expression and expansion of the lysosomal compartment were connected to altered localization of TFEB in Trex1−/− cells. We did not detect any interaction between Trex1 and TFEB by immunoprecipitation of proteins from wild-type MEFs (data not shown), which suggested that TFEB did not regulate the translocation of TFEB through direct binding and retention in the cytosol.

To determine whether TFEB function was required for the activation of ISGs and antiviral activity of Trex1−/− cells, we knocked down TFEB expression in Trex1−/− cells and measured Ifit1 and Ifi32 mRNA in uninfected cells, as well as VSV replication in those same cells. Knockdown of TFEB in Trex1−/− cells resulted in lower expression of both Ifit1 and Ifi32 and more VSV replication (Fig. 7c–e). Knockdown of TFEB in wild-type MEFs did not affect Ifi32 or other genes encoding molecules of the innate immune system predicted to be targets...
Figure 7 Trex1 regulates lysosomal biogenesis via TFEB and mTORC1. (a) Quantitative RT-PCR analysis of lysosomal and nonlysosomal genes (horizontal axis) in wild-type and Trex1−/− MEFs (presented as in Fig. 3b). (b) Fluorescence microscopy of wild-type and Trex1−/− MEFs, assessing the endogenous localization of TFEB (left), and frequency of nuclear TFEB (right; n = 13 cells). Original magnification (left), ×20. (c,d) Immunoblot analysis of the knockdown of TFEB (c) and quantitative RT-PCR analysis of Ift1 and Mcoln1 mRNA (d; results presented as in Fig. 3f) in Trex1−/− MEFs transfected with control siRNA or TFEB-specific siRNA (si-TFEB). (e) VSV-P-eGFP infection in wild-type and Trex1−/− MEFs transfected for 72 h with siRNA as in d and mock infected or infected for 18 h with VSV-P-eGFP, assessed by flow cytometry. (f) Quantitative RT-PCR analysis of Ift1 mRNA in wild-type MEFs transfected for 24 h with various concentrations of empty vector plasmid (pcDNA) or plasmid encoding Myc-tagged TFEB; results are presented relative to those of mock-transfected cells (0 ng), set as 1. (g) Quantitative RT-PCR analysis of the expression of Ift1 and Mcoln1 in wild-type MEFs mock treated (Mock) or treated for 16 h with chloroquine (wedges: 10, 50 or 100 µM); results are presented relative to those of mock-treated cells, set as 1. (h,i) Immunoblot analysis of phosphorylated (p-) and total mTORC1, S6K, S6P and 4E-BP1 in wild-type and Trex1−/− MEFs left uninfected or infected for 16 h with VSV (h; phosphorylated amino acids in parentheses), followed by densitometry analysis, presented relative to results obtained for wild-type cells, set as 1 (i). (j) Quantitative RT-PCR analysis of Ift1 mRNA in wild-type MEFs transfected for 72 h with control siRNA or siRNA specific for Trex1 or mTOR (si-mTOR, 1 and 2); results are presented relative to those of MEFs transfected with control siRNA, set as 1. (k) Immunoblot analysis of phosphorylated and total S6P and 4E-BP1 in wild-type and Trex1−/− MEFs transfected with for 24 h with empty vector or vector encoding Flag-tagged Trex1 (F-Trex1). *P < 0.05 (Student’s t-test). Data are representative of at least three independent experiments (error bars a,b,d,e,i,j), s.d.).

of TFEB31 (Supplementary Fig. 12), which suggested that TFEB did not regulate ISGs directly. Moreover, we found that Trex1−/−Irf3−/− cells had higher expression of lysosomal genes and LAMP-1 protein, similar to that in Trex1−/− cells, whereas ISG expression was much lower in Trex1−/−Irf3−/− cells than in Trex1−/− cells (Supplementary Fig. 11), which suggested that lysosomal biogenesis (regulated by TFEB) acted upstream of ISG expression (regulated by IRF3 and IRF7).

Overexpression of TFEB promotes lysosomal biogenesis32. To determine whether manipulating the expression or nuclear translocation of TFEB in wild-type cells also induced the expression of ISGs, we overexpressed TFEB in wild-type MEFs and found that Ift1 expression was increased in a dose-dependent manner (Fig. 7f). We also treated wild-type MEFs with chloroquine, which induces translocation of TFEB to the nucleus33, and observed a dose-dependent increase in the expression of Mcoln1 (a lysosomal gene) and Ift1 (Fig. 7g). These data further supported the proposal of a link between TFEB function in lysosomal biogenesis and induction of ISGs. Of note, the treatment of Trex1−/− cells with chloroquine did not restore VSV replication (Supplementary Fig. 13), probably because of the known antiviral effect of chloroquine.34–38

One of the upstream regulators of the translocation of TFEB to the nucleus is mTORC1, and inhibition of mTORC1 activity under many conditions promotes the transport of TFEB into the nucleus35,39. We thus examined mTORC1 activity in infected and uninfected wild-type and Trex1−/− cells. We found that inhibition with VSV induced mTORC1 activity in wild-type MEFs (Fig. 7h), consistent with the function of mTORC1 as a proviral factor.40 The activity of mTORC1 was much lower in uninfected and infected Trex1−/− cells than in uninfected and infected wild-type cells (as assessed by phosphorylation of the kinase S6K, the ribosomal protein S6P and the translation initiation inhibitor 4E-BP1; Fig. 7h,i). We also found that knockdown of mTOR (via two independent siRNA molecules) in wild-type MEFs resulted in higher Ift1 expression (Fig. 7). Moreover, expression of Flag-tagged Trex1 enhanced mTORC1 activity in wild-type cells and restored mTORC1 activity in Trex1−/− cells relative to the activity achieved with the vector plasmid control (Fig. 7k). Our data suggested that Trex1 was important for maintaining mTORC1 activity and that a lower abundance of mTOR led to the induction of ISGs. Consistent with our data, lower mTORC1 activity has been associated with antiviral effects.40 Collectively, our data suggested that Trex1 regulated lysosomal biogenesis through TFEB and mTORC1 and that lysosomal biogenesis had a critical role in innate immunity and antiviral defense (Supplementary Fig. 14).

**DISCUSSION**

It is well established that interferon has an important role in antiviral immunity. Cells are equipped with an extensive network of sensing...
mechanisms in the innate immune system for the detection of invading pathogens through recognition by pattern-recognition receptors. When such receptors are engaged, they trigger signaling pathways that often lead to the activation of interferon expression. Infection with enveloped viruses also triggers an interferon-independent pathway that involves the direct activation by IFR3 of a subset of ISGs\(^9,41\). In fact, IFR3 can bind to the promoters of many ISGs in addition to those of interferon-encoding genes\(^41\). The promoters of interferon-encoding genes (such as *Ifnb1*) are complex, containing both positive and negative regulatory elements for transcription factors such as members of the IRF family, NF-κB and AP-1, and a concerted effect by multiple transcription factors is often required for their stimulation. In contrast, the promoters of many ISGs (such as *Ifit1*) are simpler and can be easily turned on by IRF proteins independently of interferon\(^9,41\). Direct activation of antiviral genes is important for 'nonprofessional' interferon-producing cells such as fibroblasts to effectively defend themselves against viral infection or for cells to defend themselves against viruses that have evolved mechanisms to disrupt the interferon response. It is also advantageous for cells to rapidly induce some ISGs after viral infection before a stronger and more sustained response can be established by interferon signaling pathways. A study of a cytosolic RNA-sensing pathway has provided evidence that interferon-independent activation of ISGs mediated by peroxisomal MAVS is functionally important for defense against infection with RNA viruses\(^10\).

Very little is known about whether interferon-independent activation of ISGs occurs in the absence of infection and how it is regulated. Here we have identified Trex1, a cytosolic protein associated with the endoplasmic reticulum, as a key negative regulator of interferon-independent activation of *Ifit1* and other ISGs in uninfected cells. When the function of Trex1 was disrupted, either by genetic deficiency in mice or by homozygous mutation in humans, or by siRNA-mediated knockdown in a variety of cell types, a subset of ISGs were activated independently of interferon, which led to an antiviral state. Notably, the induction of ISGs in Trex1-deficient cells was sustained at a very high degree and achieved an antiviral state similar to that caused by the interferon-dependent pathway. That was in contrast to the interferon-independent response induced by viral infection in wild-type cells that seems to be temporary and less robust\(^11\). We also challenged wild-type and *Trex1*\(^−/−\) mouse cells and *TREX*\(^R_{114H}/R_{114H}\) human cells with a variety of RNA viruses, including VSV, influenza virus, Sendai virus and West Nile virus, and all these viruses failed to replicate in cells that lacked Trex1 function.

We have also identified a pathway in the innate immune system, involving STING, TBK1, IRF3 and IRF7, that was important for the interferon-independent activation of ISGs in Trex1-deficient cells. STING is a critical factor for the sensing of pathogen-associated DNA or cyclic di-GMP in the cytosol and subsequent induction of interferon expression\(^2,7\). Our data have expanded the function of the STING-TBK1-IRF3-IRF7 pathway to include both interferon-dependent and interferon-independent branches as downstream pathways. A published study has also shown that STING activates the phosphorylation of STAT6 after viral infection, which then induces the expression of chemokines such as CCL2, CCL20 and CCL26 and homing of cells of the immune system\(^8\). We did not observe induction of the expression of those chemokines in *Trex1*\(^−/−\) or *TREX*\(^R_{114H}/R_{114H}\) cells relative to their expression in wild-type cells (data not shown). Collectively, our study has expanded the understanding of STING and associated factors of the innate immune system as a versatile machinery that can activate multiple distinct downstream pathways.

Our data have also shed some light on the potential endogenous trigger of interferon-independent activation of ISGs. We found that Trex1-deficient or Trex1-mutant cells had excessive amounts of lysosomal vacuoles and expanded lysosomal compartments, as determined by immunofluorescence and immunoblot analysis of lysosomal markers, quantitative RT-PCR analysis of lysosomal genes, and electron microscopy. Consistent with such enhanced lysosome biogenesis, the master regulator of lysosome genes TFEB translocated to become predominantly nuclear in *Trex1*\(^−/−\) cells. We also found lower mTORC1 activity in *Trex1*\(^−/−\) cells and restoration of mTORC1 activity after expression of Flag-tagged Trex1 in *Trex1*\(^−/−\) cells; this suggested an important role for Trex1 in maintaining the activity of mTORC1, which regulates the translocation of TFEB to the nucleus\(^35,39\). We also provided the following lines of evidence that demonstrated a functional linkage of TFEB-regulated lysosomal biogenesis to the activation of ISGs: knockdown of TFEB in Trex1-deficient cells tempered the activation of ISGs and antiviral immunity; overexpression of TFEB in wild-type cells, which promotes lysosomal biogenesis\(^32\), resulted in higher *Ifit1* expression; treatment of wild-type cells with chloroquine, which induces translocation of TFEB to the nucleus\(^35\) and has antiviral activity\(^36–38\), resulted in higher *Ifit1* expression, up to 15-fold; and knockdown of mTOR by siRNA in wild-type cells also resulted in higher *Ifit1* expression. Furthermore, given our observation of higher expression of lysosomal genes and a greater abundance of lysosomal proteins and lack of excess accumulation of undigested contents, *Trex1*\(^−/−\) cells probably have enhanced lysosomal function. The release of abnormally large amounts of processed peptides or nucleic acids into the cytosol or into the extracellular space (via exocytosis\(^42\)) might break cellular homeostasis or immunotolerance or exceed the threshold for cytosolic sensing of DNA. The exact identity of such cytosolic DNA remains unclear; published studies have indicated that it might be DNA-replication debris\(^20\) or endogenous retroelements\(^39\). Aberrant functions of lysomes have been identified in lupus nephritis, in which lysosomal contents mimic viral particles and activate innate immunity\(^43\). It is also possible that a greater abundance of lysosomal vacuoles could result in a membrane perturbation that would elicit an interferon-dependent or interferon-independent antiviral response\(^8,44\). Further studies are needed to distinguish among these possibilities. Collectively, our work has demonstrated a link between lysosomal biogenesis and activation of ISGs by the innate immune system, as well as a previously unknown role for Trex1 in regulating lysosomal biogenesis through TFEB and mTORC1.

Trex1 inhibits HIV-mediated activation of interferons\(^11\). Here we confirmed that finding and further identified a previously unknown function of Trex1 in the regulation of interferon-independent activation of the innate immune response through lysosomal biogenesis in uninfected cells, which resulted in a broad-spectrum antiviral state in which the replication of several different RNA viruses was inhibited. Both functions of Trex1 shared a similar signaling pathway of the innate immune system that involved STING-TBK1-IRF3, which was able to activate multiple downstream pathways. The upstream stimulus for HIV-mediated interferon activation is HIV DNA from nonproductive reverse transcription\(^11\), whereas the upstream stimulus for the interferon-independent pathway probably involves lysosome function.

Our work has also provided further insight into pathogenetic mechanisms underlying systemic autoimmunity associated with *TREX1* mutation, such as SLE, a prototypical autoimmune disease. Central to SLE pathogenesis is that ineffective waste disposal due to impaired apoptosis or defective clearance of cellular debris leads to...
excessive release of autoantigens, which activate sensors of the innate immune system and trigger immunological responses that lead to the formation of autoantibodies. Our findings have identified a previously unknown cell-intrinsic mechanism for the initiation of autoimmunity due to enhanced lysosome function. Moreover, the constitutive type I interferon–independent ‘ISG-signature’ detectable in a variety of cell types and tissues may potentially represent a valuable biomarker that could be applied as a measure of clinical outcome.

In summary, our study has identified a signaling cascade that involved the biogenesis of a cellular organelle (the lysosome) and cytosolic detection by the innate immune system. Both segments of the cascade functioned together to establish an antiviral state in Trex1-deficient cells independently of interferon activation or viral infection. We have identified many components of this cascade, some of which (TFEB and mTORC1) have not been directly linked to intrinsic antiviral immunity before, to our knowledge. We have also identified previously unknown functions for known regulators of the innate immune system, such as Trex1 and STING. Further understanding of the mechanism by which this signaling cascade is regulated will have implications for the understanding of not only antiviral defense but also the pathogenic mechanisms that underlie autoimmune diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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Author Contributions

M.H. and N.Y. designed and did most of the experiments; J.K. helped with the experiments; M.A.K.P ., J.B. and B.L. contributed reagents and advice; E.K.W . and I.D. M.H. and N.Y . designed and did most of the experiments; J.K. helped with the experiments. Supported by grants AI093795 and AI098569 to N.Y.; CA129387 to J.B.; and AI057156 to B.L., and any associated references are available in the online version of the paper.

Competing Financial Interests

The authors declare no competing financial interests.

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RNA isolation, quantitative RT-PCR analysis and cytokine-detection assay. Total RNA from various mouse tissues was extracted with the RNeasy Mini kit (74104; Qiagen), and total RNA from cells was isolated with TRIzol. The quality of RNA was determined to have an RNA integrity number of 8 or greater by Bio-Analyzer. Total RNA (1 µg) was used for production of the cDNA library according to standard protocols that include cDNA synthesis and fragmentation, the adaptation of adaptors, size selection, amplification and quality control (Illumina). A HiSeq 2000 system (Illumina) was used for SE-50 sequencing (single-ended 50–base pair reads), with over 18 \times 10^6 ‘reads’ per sample. Basic data analysis was done with CLC-Biosystems Genomic Workbench analysis programs to generate quantitative data for all genes, including reads per kilobase of exon per million mapped reads; unique and total gene reads; annotated transcripts and detected transcripts; median coverage; chromosomal location; and putative exons. Data sets for uninfected wild-type and Trex1^{−/−} cells were analyzed by Ingenuity pathway-analysis software (Ingenuity Systems). Heat maps were produced by normalization of the reads per kilobase of exon per million mapped reads for each gene (average and s.d.) across all treatment conditions, followed by the generation of hierarchy-clustered heat maps with Spotfire software.

Transfection and immunoblot analysis. Cells were grown on coverslips were fixed in 4% (wt/vol) paraformaldehyde and were permeabilized and stained by standard protocols. Samples mounted in Vectashield mounting medium containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories) were imaged with a Zeiss Imager.M2 fluorescence microscope equipped with AxioVision software. The following antibodies were used for immunostaining: anti-HSP60 (13966; Santa Cruz), anti-GM130 (610822; BD), anti-calreticulin (Ab4-100; Abcam), anti-EEA1 (ab2900; Abcam), anti-LAMP-1 (ab24170; Abcam) and anti-TFEB (generated in house) with Alexa Fluor 488- and Alexa Fluor 546–tagged secondary antibodies (A21202, A21206, A10036 and A10040; Invitrogen). For live-cell fluorescence microscopy cells were grown in in 35-mm glass-bottomed dish and were imaged with a Zeiss LSM510 confocal microscope. For visualization of VSV infection in live cells, VSV was incubated for 10 min with 2 mM Vybrant Dil Cell labeling solution (Invitrogen) in PBS, followed by purification with a Quick Spin Sephadex G-50 column (Roche) for the removal of residual dye. Dil-labeled VSV virions were subsequently added to target cells, followed by incubation for 1 h before imaging. In some experiments, LysoTracker Green was used for visualization of lysosomes in cells with red-labeled virus. For flow cytometry of lysosomes or VSV-P-eGFP-infected cells, cells were incubated for 1 h with LysoTracker Red (40 nmol/ml) or for the appropriate time with VSV-P-eGFP. Cells were then washed twice with PBS and fixed with 4% paraformaldehyde. Cells were acquired with a FACSCalibur (BD Biosciences). For all samples, 2 \times 10^5 events were computed and analyzed by FlowJo software.

Immunostaining, fluorescence microscopy and flow cytometry. Cells grown on coverslips were fixed in 4% (wt/vol) paraformaldehyde and were permeabilized and stained by standard protocols. Samples mounted in Vectashield mounting medium containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories) were imaged with a Zeiss Imager.M2 fluorescence microscope equipped with AxioVision software. The following antibodies were used for immunostaining: anti-HSP60 (13966; Santa Cruz), anti-GM130 (610822; BD), anti-calreticulin (Ab4-100; Abcam), anti-EEA1 (ab2900; Abcam), anti-LAMP-1 (ab24170; Abcam) and anti-TFEB (generated in house) with Alexa Fluor 488- and Alexa Fluor 546–tagged secondary antibodies (A21202, A21206, A10036 and A10040; Invitrogen). For live-cell fluorescence microscopy cells were grown in in 35-mm glass-bottomed dish and were imaged with a Zeiss LSM510 confocal microscope. For visualization of VSV infection in live cells, VSV was incubated for 10 min with 2 mM Vybrant Dil Cell labeling solution (Invitrogen) in PBS, followed by purification with a Quick Spin Sephadex G-50 column (Roche) for the removal of residual dye. Dil-labeled VSV virions were subsequently added to target cells, followed by incubation for 1 h before imaging. In some experiments, LysoTracker Green was used for visualization of lysosomes in cells with red-labeled virus. For flow cytometry of lysosomes or VSV-P-eGFP-infected cells, cells were incubated for 1 h with LysoTracker Red (40 nmol/ml) or for the appropriate time with VSV-P-eGFP. Cells were then washed twice with PBS and fixed with 4% paraformaldehyde. Cells were acquired with a FACSCalibur (BD Biosciences). For all samples, 2 \times 10^5 events were computed and analyzed by FlowJo software.
Specimens were then embedded in embedding molds and polymerized overnight in an oven at 60 °C. Thick sections (1.0–1.5 µm in thickness) were cut on Leica Ultramicrotome with a glass knife, mounted on glass slides, and stained with toluidine blue stain. Thin sections (60–90 nm) were cut with a Leica Ultramicrotome with a diamond knife, mounted on copper grids, and stained with uranyl acetate and lead citrate. A Hitachi H-7500 transmission electron microscope was used for ultrastructural examination.

Statistical methods. Statistical significance was determined by Student’s t-test. P values of less than 0.05 were considered statistically significant.

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