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# Ebola virus requires phosphatidylinositol (3,5) bisphosphate production for efficient viral entry

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ABSTRACT

For entry, Ebola virus (EBOV) requires the interaction of its viral glycoprotein with the cellular protein Niemann-Pick C1 (NPC1) which resides in late endosomes and lysosomes. How EBOV is trafficked and delivered to NPC1 and whether this is positively regulated during entry remain unclear. Here, we show that the PIKfyve-ArPIKfyve-Sac3 cellular complex, which is involved in the metabolism of phosphatidylinositol (3,5) bisphosphate (PtdIns(3,5)$P_2$), is critical for EBOV infection. Although the expression of all subunits of the complex was required for efficient entry, PIKfyve kinase activity was specifically critical for entry by all pathogenic filoviruses. Inhibition of PIKfyve prevented colocalization of EBOV with NPC1 and led to virus accumulation in intracellular vesicles with characteristics of early endosomes. Importantly, genetically-encoded phosphoinositide probes revealed an increase in PtdIns(3,5)$P_2$-positive vesicles in cells during EBOV entry. Taken together, our studies suggest that EBOV requires PtdIns(3,5)$P_2$ production in cells to promote efficient delivery to NPC1.

## 1. Introduction

Ebola virus (EBOV) is a highly pathogenic zoonotic virus belonging to the *Filoviridae* family and the causative agent of severe hemorrhagic fever disease in humans and non-human primates. Currently, there are no FDA-approved therapeutics for the treatment of Ebola virus disease. Virions harbour the characteristic filoviral filamentous morphology and are surrounded by a host-derived lipid envelope, which is acquired during budding from cells. Studded on the viral envelope is the metastable trimeric EBOV glycoprotein (GP), which is the primary determinant of viral entry (Lee and Saphire, 2009). GP is composed of heterodimers of the subunits $GP_1$, which contains the receptor binding domain shielded by a glycan cap (Lee et al., 2008), and $GP_2$, which contains a hydrophobic fusion loop and heptad repeat regions (Malashkevich et al., 1999). Viral fusion requires a number of triggering factors; these include cleavage of the glycan cap on $GP_1$ by low-pH dependent cysteine proteases, and interaction with a host receptor (Chandran et al., 2005; Côté et al., 2011; Miller et al., 2012; Schornberg et al., 2006).

Identification of Niemann-Pick C1 (NPC1), a late endosomal/lysosomal resident protein, as the filovirus receptor indicated that viral particles require not only initial internalization but also trafficking through the endosomal system for delivery to intracellular compartments containing NPC1 (Carette et al., 2011; Côté et al., 2011). In support of this, recent studies have shown that EBOV displays late entry kinetics due to the need to traffic deep into the endocytic pathway (Mingo et al., 2015). Using live cell imaging to visualize viral-host membrane lipid mixing, Spence et al. and Simmons et al. found that EBOV GP-mediated viral membrane fusion occurs primarily in NPC1 + and Rab7 + compartments (Simmons et al., 2016; Spence et al., 2016). However, which cellular proteins are involved in viral trafficking and whether EBOV actively regulates its delivery to NPC1 + compartments remains largely unexplored.

A previous genetic screen for EBOV entry host factors uncovered a

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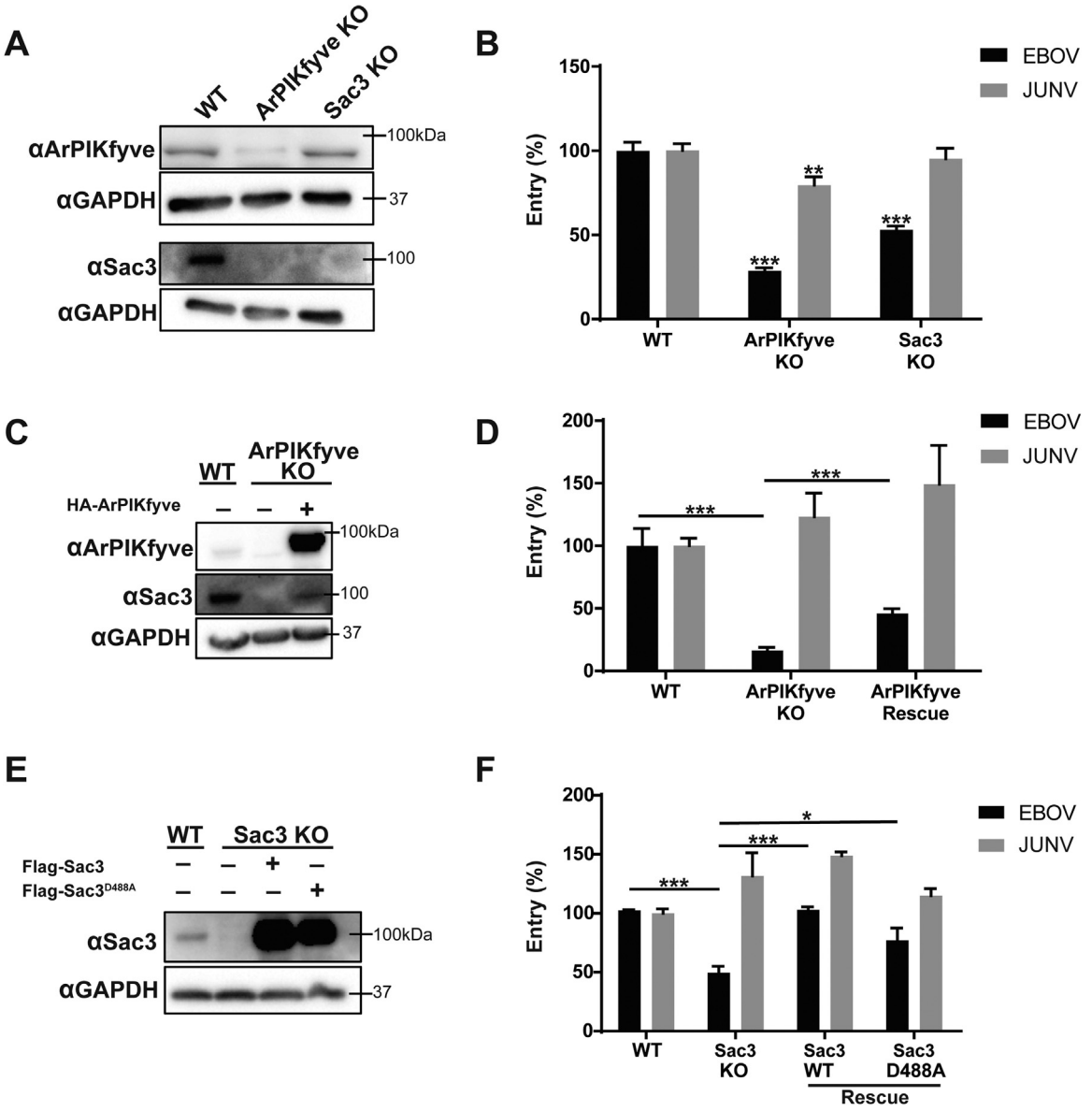


**Fig. 1. EBOV GP-mediated entry requires ArPIKfyve and Sac3 expression, but not Sac3 phosphatase activity.** (A) Expression of ArPIKfyve and Sac3 in HT1080 CRISPR/Cas9 monoclonal KO cells. (B) Entry of βlam-VLPs harbouring EBOV ΔM GP or JUNV GP in Sac3 and ArPIKfyve KO cells was detected by measuring the percentage of cells with cleaved CCF4, normalized to WT cells. Asterisks indicate significant difference in entry compared to WT cells. (C, D) ArPIKfyve KO cells were transfected with plasmids encoding LacZ (mock) or gRNA-insensitive ArPIKfyve$^{WT}$ and infected with βlam-VLPs. Protein expression (C) and viral entry (D) were assessed 48 h post-transfection. (E, F) Sac3 KO cells were transfected with plasmids encoding LacZ (mock) or gRNA-insensitive Sac3$^{WT}$ or phosphatase-deficient Sac3$^{D488A}$ and infected with βlam-VLPs. Protein expression (E) and viral entry (F) were assessed 48 h post-transfection. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

number of genes encoding trafficking factors in addition to genes encoding the triggering factors, cathepsin B and NPC1. Among others, genes of the subunits that form the Homotypic Fusion and Protein Sorting (HOPS) complex, as well as those of PIKfyve and Sac3, were found to be enriched for gene-trap insertions in a cell population resistant to vesicular stomatitis virus expressing EBOV GP compared to unselected control cells (Carette et al., 2011). Interestingly, PIKfyve and Sac3 are found in the same ternary complex, which is scaffolded by the adaptor protein ArPIKfyve (Ikonomov et al., 2009b; Jin et al., 2008; Sbrissa et al., 2008). The PIKfyve-ArPIKfyve-Sac3 (PAS) complex is recruited to phosphatidylinositol (3) phosphate (PtdIns3P)-enriched endosomal membranes via a FYVE domain located at the N-terminal of PIKfyve and plays an essential role in regulating vesicular trafficking (Sbrissa et al., 2002). PIKfyve, a lipid kinase, is the sole known enzyme capable of producing phosphatidylinositol (3,5) bisphosphate (PtdIns(3,5)$P_2$) in mammalian cells, while the phosphatase function of Sac3 hydrolyzes PtdIns(3,5)$P_2$ to regenerate PtdIns3P (Ikonomov et al., 2001). Biochemical characterization of the complex has shown that integrity of the entire complex is critical for maintaining PIKfyve kinase activity, while Sac3 stability depends primarily on association with ArPIKfyve (Botelho et al., 2008; Duex et al., 2006; Ikonomov et al., 2009b, 2010; Morioka et al., 2017).

PtdIns(3,5)$P_2$ is a member of a family of phospholipids collectively called phosphoinositides (PIs), whose precise spatiotemporal localization on membranes throughout the cell allows them to dynamically regulate signalling events by recruiting and activating various effectors (Balla, 2013). PtdIns(3,5)$P_2$, despite composing less than 1% of the total PI pool, is known to be a critical regulator of endosomal membrane homeostasis and progression of cargo through the endosolysosomal trafficking system (Ho et al., 2012; Jin et al., 2016). Cells with reduced PtdIns(3,5)$P_2$ levels display profoundly enlarged endosomes as well as defective autophagy, endosomal maturation and