Proteomics-based approach for target discovery in Zika virus infection: abridged secondary publication

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KEY MESSAGES

1. The proteomics-based approach is effective in rapid identification of functionally relevant targets in virus infections.
2. Deubiquitylases that are specifically activated upon Zika/dengue virus infection are key regulators of immune activation and virus secretion via Src-family kinases (SFK) function.
3. Genetic and pharmacological inhibition of deubiquitylases and SFKs can effectively attenuate production of viral progenies.
4. A biochemical screening strategy combined with in vivo models can provide powerful means of developing drug screening platforms.

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Introduction

Ubiquitylation can dramatically change protein expression and activity.[1,2] Alteration in cellular ubiquitylation is recurrent in most virus infections caused by reorganisation of host intracellular networks that need to be rapidly installed. Among the enzymes of the ubiquitylation machinery, deubiquitylases (DUBs) and E3-ligases regulate critical signalling pathways at the intersection of host immunity and viral pathogenesis. We aim to identify and characterise druggable targets of the ubiquitylation pathway in Zika infection.

Methods

A combination of mass spectrometry, biochemistry, and virology was applied to address how enzymes of the ubiquitylation pathway affect Zika virus infection. A reporter for ubiquitin—HA-Ubvme and E2 charged with HA-Ubvme—was used to isolate deubiquitylases and E3 ligases from mock and infected samples for identification using mass spectrometry. Hepatocytes were treated with perfringolysin O, a pore-forming toxin, to permeabilise the plasma membrane and facilitate delivery.[3,4] Purified reporters were added to permeabilised cells, which were Zika or mock-infected. The function of the highest scoring candidates in Zika infection was validated and characterised. Otub1 and Ataxin-3 were identified to be associated with the RIG-I signalling pathway and the Src-family kinases (SFK), respectively. The SFKs in turn regulated secretion of viral progenies via an unconventional mechanism derived from autophagosomes triggered specifically upon infection. Genetic deletions were generated using the CRISPR/Cas9 strategy and their function characterised in Zika infection. The efficacy of a set of inhibitors as possible therapeutic candidates for targeting the SFKs and the deubiquitylases was evaluated in in vitro and in vivo models.

Results

To identify enzymes of the ubiquitylation machinery that are activated upon Zika virus infection, we applied a ubiquitin charged E2 probe to isolate all activated ubiquitylation enzymes from infected cells (Fig 1). We used a quantitative proteomics screen combined with activity-based E3 ligase profiling to identify E3-ligases that were activated upon infection. The screen revealed that while antigen presentation was suppressed, membrane associated RING-CH (MARCH) family E3-ligases were enriched in Zika and dengue-infected primary human monocytes. Several MARCH E3 ligases emerged as significant hits from the proteomics screen, particularly MARCH9 and 2, which also co-purified with MHC-I and II from dengue-infected cells. The MARCH E3 ligases share sequence homology and domain organisation with the herpesvirus K3 family of E3 ligases that are known to downregulate MHC. We therefore hypothesise that Zika co-opts MARCH E3 ligases to suppress MHC-I and II through similar strategies as the K3 proteins to escape T-cell surveillance.

To isolate DUBs from Zika and IAV-infected cells, mock and virus infected cells were permeabilised using perfringolysin O, and activated

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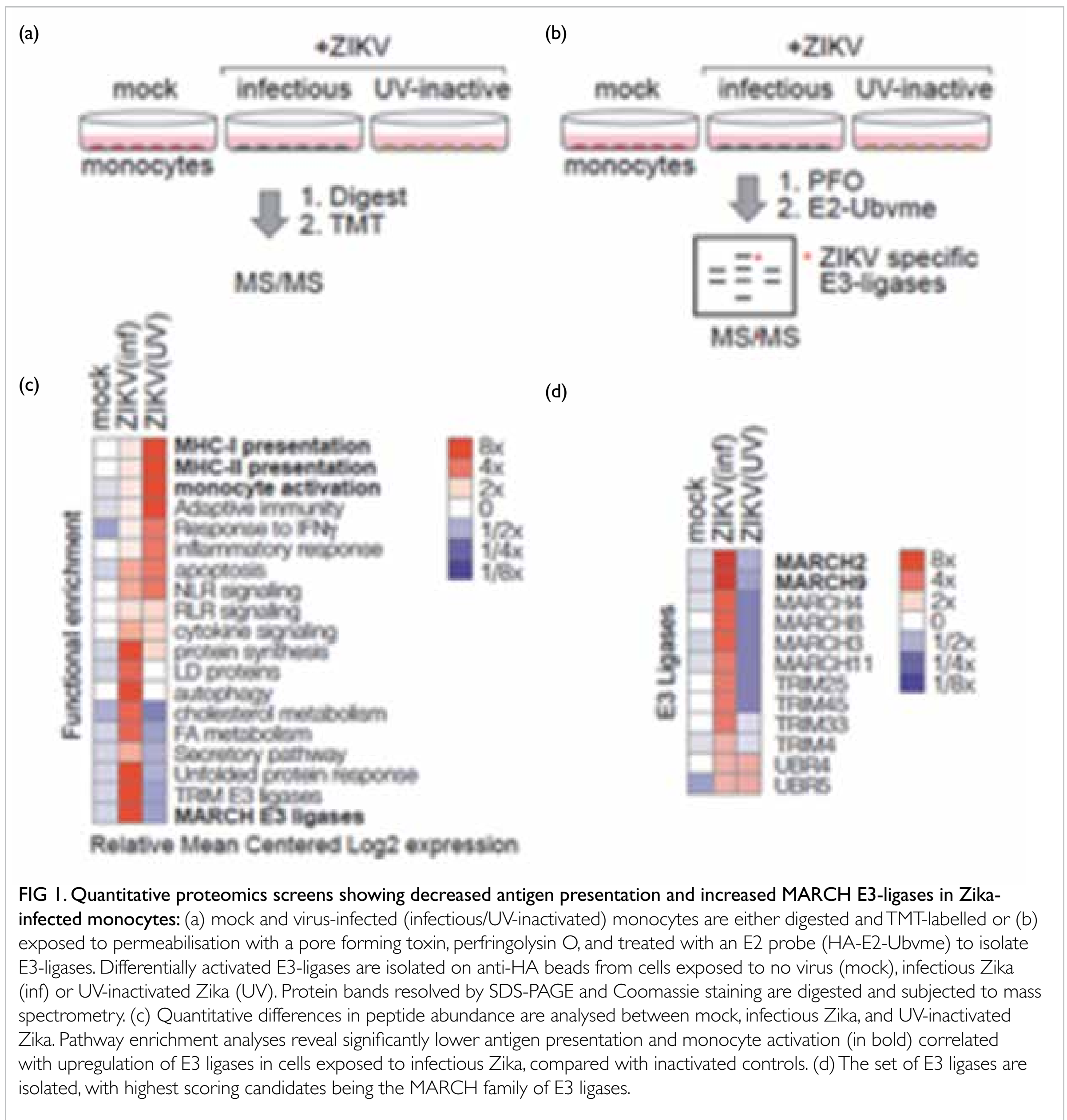

FIG 1. Quantitative proteomics screens showing decreased antigen presentation and increased MARCH E3-ligases in Zika-infected monocytes: (a) mock and virus-infected (infectious/UV-inactivated) monocytes are either digested and TMT-labelled or (b) exposed to permeabilisation with a pore forming toxin, perfringolysin O, and treated with an E2 probe (HA-E2-Ubvme) to isolate E3-ligases. Differentially activated E3-ligases are isolated on anti-HA beads from cells exposed to no virus (mock), infectious Zika (inf) or UV-inactivated Zika (UV). Protein bands resolved by SDS-PAGE and Coomassie staining are digested and subjected to mass spectrometry. (c) Quantitative differences in peptide abundance are analysed between mock, infectious Zika, and UV-inactivated Zika. Pathway enrichment analyses reveal significantly lower antigen presentation and monocyte activation (in bold) correlated with upregulation of E3 ligases in cells exposed to infectious Zika, compared with inactivated controls. (d) The set of E3 ligases are isolated, with highest scoring candidates being the MARCH family of E3 ligases.

DUBs were isolated with Ub-vme carrying a TAMRA or HA-tag (Fig 2). Ub-vme reactive material from control and infected cells were resolved by SDS-PAGE and visualised by fluorescent scanning or enriched on anti-HA beads first and detected by silver staining. Potential candidates were identified by trypsin digestion, mass spectrometry, and spectral counting of peptides on immunoprecipitated material and validated in lysates from infected cells. Candidates identified included Usp25,[3] Usp15,[4] and DUBA, which displayed reduced expression in virus-infected cells and high Ubvme reactivity in IFN-I treated samples, in agreement with a previous study.[5]

Among the DUBs identified in the Ubvme screen, we identified Ataxin-3, which regulates the expression of SFKs in flavivirus infection. We therefore screened for activated SFKs in Zika and dengue-infected cells. There are at least nine members of SFKs expressed in different combinations in all mammalian cells.[6] To identify those that are activated upon Zika infection, we immunoprecipitated phosphorylated SFKs using anti-pSFK antibodies from cell lysates prepared from mock or Zika-infected cells. Phosphorylated SFKs and associated cellular factors were isolated, resolved by SDS-PAGE, and detected by silver staining (Fig 3). The lanes were sliced into 2-mm sections and subjected to trypsin digest for identification by mass spectrometry. We identified three members: Src, Fyn, and Lyn, and several SFK-regulated substrates in Zika-infected samples. Several co-

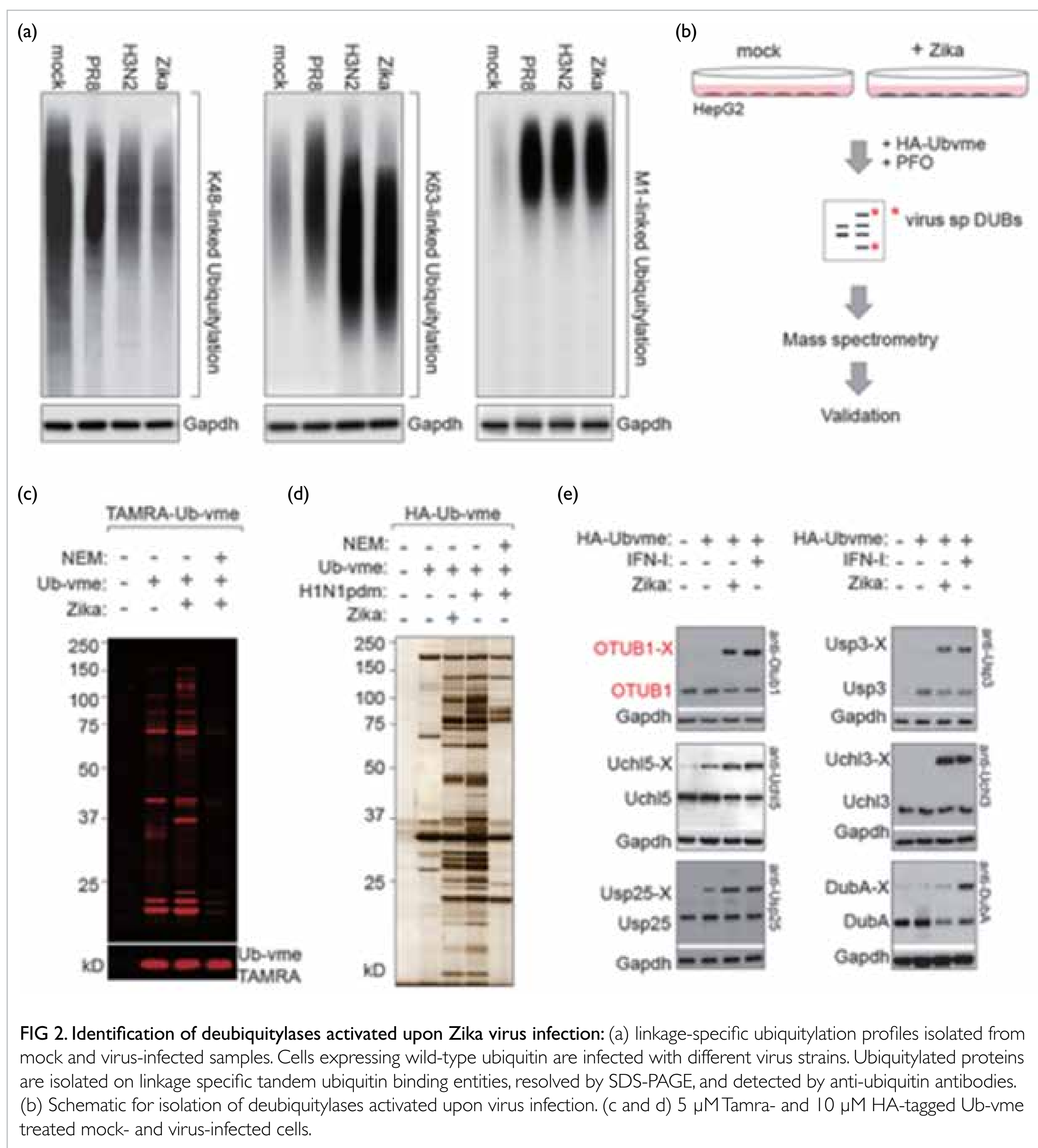

FIG 2. **Identification of deubiquitylases activated upon Zika virus infection:** (a) linkage-specific ubiquitylation profiles isolated from mock and virus-infected samples. Cells expressing wild-type ubiquitin are infected with different virus strains. Ubiquitylated proteins are isolated on linkage specific tandem ubiquitin binding entities, resolved by SDS-PAGE, and detected by anti-ubiquitin antibodies. (b) Schematic for isolation of deubiquitylases activated upon virus infection. (c and d) 5 μM Tamra- and 10 μM HA-tagged Ub-vme treated mock- and virus-infected cells.

immunoprecipitating proteins were components of the secretory pathway, ER/Golgi resident proteins, and vesicular transport machinery including the KDELRs, which have been characterised as important for dengue secretion.[7]

The three SFKs displayed high levels of expression in susceptible cell lines as confirmed by immunoblotting. To confirm their activation, we immunoprecipitated phosphorylated proteins from mock and Zika virus-infected Vero cells using anti-p-tyrosine-antibodies. Eluates from immunoprecipitated material were analysed by Western blotting with specific antibodies against the selected kinases: Src, Fyn, and Lyn. Although expression levels of total SFKs were comparable in mock and infected samples, a significant increase was noted in their phosphorylated form in eluates from Zika virus-infected samples, compared with mock. We used a reciprocal strategy where kinases from mock and Zika-infected samples were first immunoprecipitated on specific antibodies followed by immunoblotting with anti-phospho-tyrosine antibodies to confirm these results. To further quantitate increases in specific SFK activation, we used the Milliplex Map 8-plex SFK activation kit using the Luminex technology to confirm specific activation of Lyn, Src, and Fyn in lysates prepared from dengue and Zika-infected cells.

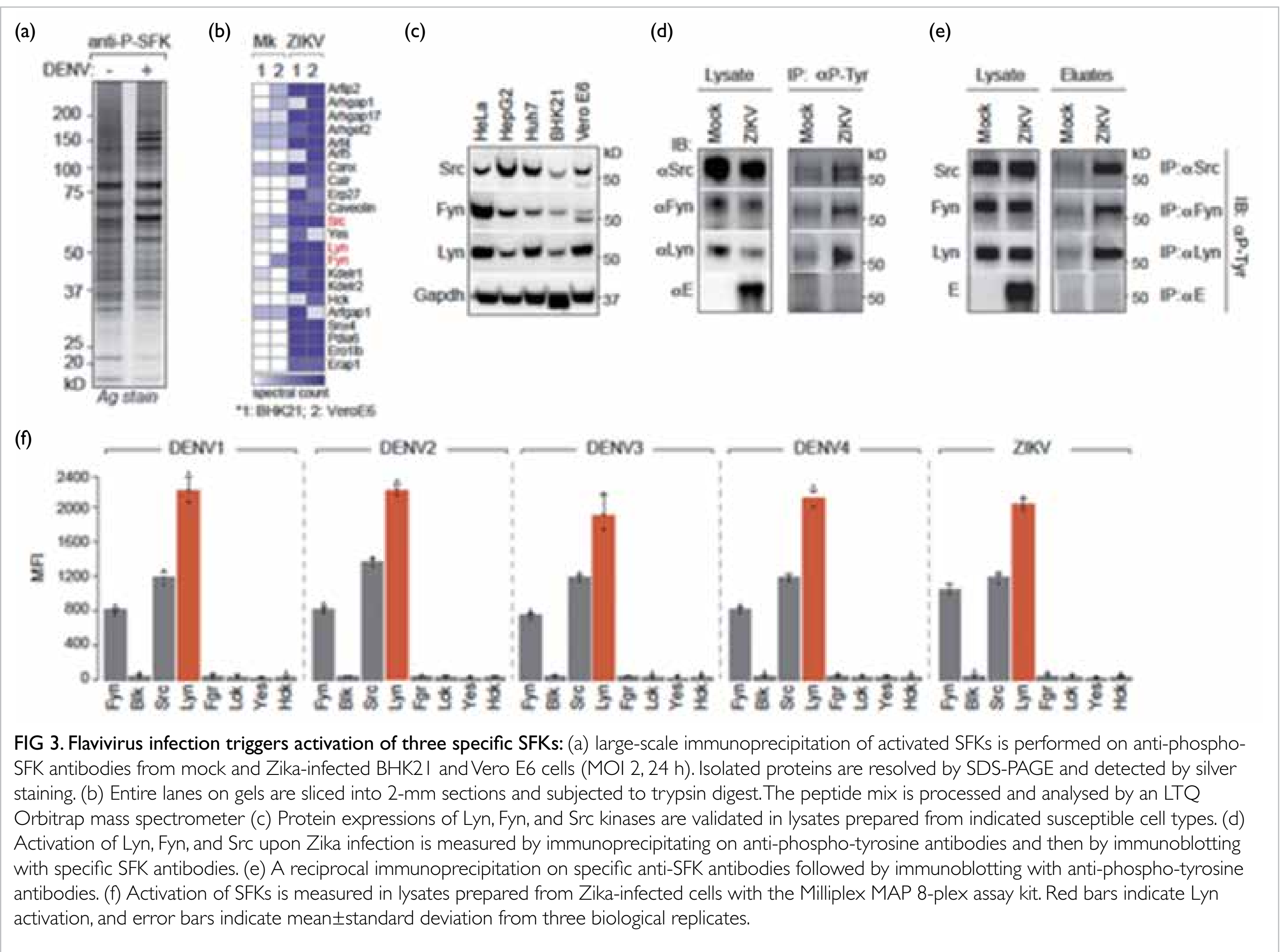

FIG 3. Flavivirus infection triggers activation of three specific SFKs: (a) large-scale immunoprecipitation of activated SFKs is performed on anti-phospho-SFK antibodies from mock and Zika-infected BHK21 and Vero E6 cells (MOI 2, 24 h). Isolated proteins are resolved by SDS-PAGE and detected by silver staining. (b) Entire lanes on gels are sliced into 2-mm sections and subjected to trypsin digest. The peptide mix is processed and analysed by an LTQ Orbitrap mass spectrometer (c) Protein expressions of Lyn, Fyn, and Src kinases are validated in lysates prepared from indicated susceptible cell types. (d) Activation of Lyn, Fyn, and Src upon Zika infection is measured by immunoprecipitating on anti-phospho-tyrosine antibodies and then by immunoblotting with specific SFK antibodies. (e) A reciprocal immunoprecipitation on specific anti-SFK antibodies followed by immunoblotting with anti-phospho-tyrosine antibodies. (f) Activation of SFKs is measured in lysates prepared from Zika-infected cells with the Milliplex MAP 8-plex assay kit. Red bars indicate Lyn activation, and error bars indicate mean±standard deviation from three biological replicates.

Discussion

The ubiquitylation machinery is extensively used during virus infections. Zika infected samples provide proof-of-concept results in underscoring the importance of targeting host factors for development of antivirals that can be administered, especially in virus infections that do not have effective vaccines. ZIKV infection activates different classes of enzymes, particularly DUBs, E3-ligases, and SFKs. Targeting autophagy-dependent secretion specifically triggered during virus infection may prevent host toxicity while selectively blocking secretion of virus progenies. Inhibitors to selective autophagy may provide broad-spectrum protection against infections by positive stranded RNA viruses.

Funding

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