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# New Insights into Chikungunya Virus Infection and Pathogenesis

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PathogenesisVasiliya Kril,<sup>1,\*</sup> Olivier Aïqui-Reboul-Paviet,<sup>2,\*</sup>  
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**Keywords**

chikungunya virus, host cell interactions, nonstructural proteins, pathogenesis, musculoskeletal tissues, immune evasion

**Abstract**

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne alphavirus responsible for major outbreaks of disease since 2004 in the Indian Ocean islands, South east Asia, and the Americas. CHIKV causes debilitating musculoskeletal disorders in humans that are characterized by fever, rash, polyarthralgia, and myalgia. The disease is often self-limiting and nonlethal; however, some patients experience atypical or severe clinical manifestations, as well as a chronic rheumatic syndrome. Unfortunately, no efficient antivirals against CHIKV infection are available so far, highlighting the importance of deepening our knowledge of CHIKV host cell interactions and viral replication strategies. In this review, we discuss recent breakthroughs in the molecular mechanisms that regulate CHIKV infection and lay down the foundations to understand viral pathogenesis. We describe the role of the recently identified host factors co-opted by the virus for infection and pathogenesis, and emphasize the importance of CHIKV nonstructural proteins in both replication complex assembly and host immune response evasion.

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**CHIKV:** chikungunya virus

**IOL:** Indian Ocean Lineage

**nsP:** nonstructural protein

**ORF:** open reading frame

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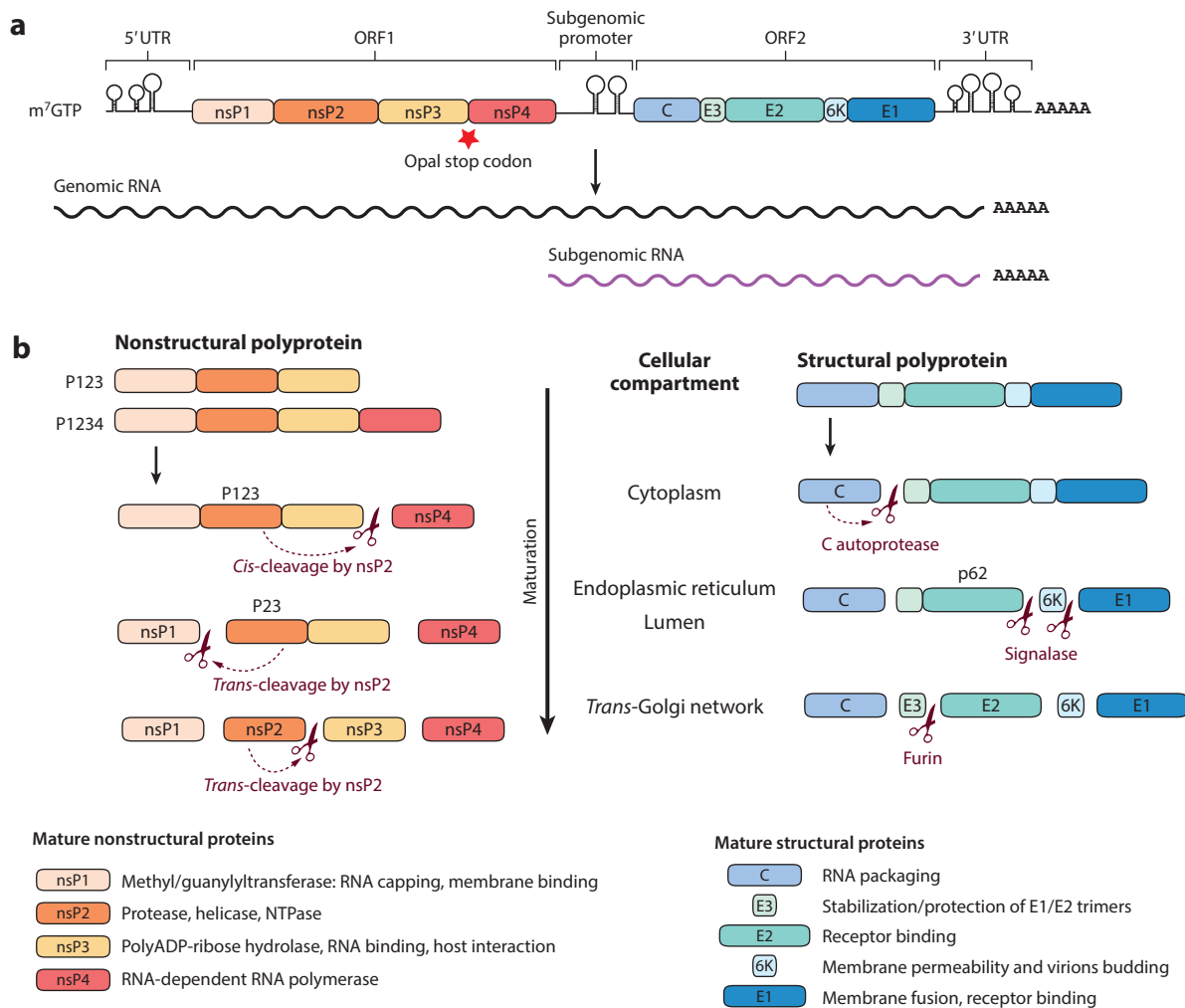
## INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne virus that belongs to the *Alphavirus* genus, a group of enveloped RNA viruses that cause severe diseases in humans and animals. CHIKV is the epidemiologically most prevalent alphavirus that is transmitted to humans by *Aedes* mosquitoes during the blood meal. Phylogenetic analysis identified three distinct lineages of CHIKV corresponding to their respective geographical origins: the West African, the East-Central-South African (ECSA), and the Asian lineages (1, 2). Before 2000, CHIKV circulation was restricted to Sub-Saharan Africa, where sporadic outbreaks have been described (2, 3). CHIKV transmission remained silent until 2004, when an ECSA strain re-emerged in Kenya (4), evolved, and rapidly disseminated to the Indian Ocean islands, causing outbreaks of unprecedented magnitude particularly on Reunion Island (5, 6). This epidemic strain, assigned now to a new lineage termed Indian Ocean Lineage (IOL), spread to Southeast Asia and India, causing more than 1.3 million cases (7), and autochthonous transmission was reported in southern Europe (Italy and France) (8, 9). A second major outbreak occurred when a strain from the Asian lineage emerged in the Caribbean Sea (Saint Martin Island) in December 2013 (10), causing more than one million cases in 50 countries of the South American continent (11). Prior to the 2004 Indian Ocean outbreak, CHIKV was vectored mainly by *Aedes aegypti* mosquitoes. However, the IOL strain contains an adaptive mutation within the sequence coding the E1 glycoprotein, causing an alanine-to-valine substitution at position 226 (E1-A226V), which is responsible for a 40-fold increase in transmission by *Aedes albopictus* without affecting viral fitness in the *A. aegypti* vector (12, 13). This and other *A. albopictus*-adaptive mutations (reviewed in 14) in the IOL CHIKV strain have promoted viral expansion in temperate regions colonized by this mosquito vector.

CHIKV belongs to the arthritogenic Old World alphaviruses (15, 16). CHIKV-infected persons experience a syndrome characterized by fever, rash, arthralgia, and myalgia (reviewed in 17). Importantly, CHIKV-infected patients develop chronic muscle and joint pains that last for months to years after acute infection (17). Currently, there is no CHIKV-specific antiviral or vaccine. Patient management relies only on symptom relief with antalgics (paracetamol) and steroidal and nonsteroidal anti-inflammatory drugs. The identification of new antiviral strategies relies on a better understanding of CHIKV host cell interactions and on the elucidation of the molecular mechanisms and cellular pathways co-opted by the virus to become a successful human pathogen. In recent years, significant progress has been made in the fields of CHIKV molecular and structural virology, immunology, entomology, and epidemiology. However, many aspects of CHIKV biology, including tissue tropism and pathogenesis, remain poorly understood. In this review, we focus on important insights that have emerged into how CHIKV interacts with the host cell and subverts host cellular pathways for productive infection. We discuss the molecular determinants of viral replication and persistence in musculoskeletal tissues and their effect on CHIKV pathogenesis. We also emphasize the recently discovered cellular factors mediating CHIKV infection and discuss the emerging roles of the CHIKV nonstructural proteins (nsPs) in viral replication and immune evasion.

## CHIKUNGUNYA VIRUS: GENOMIC ORGANIZATION AND INFECTIOUS CYCLE

CHIKV is a small (70 nm in diameter) enveloped virus with a single-stranded, message-sense, 5'-capped and 3'-polyadenylated RNA genome (11.8 kb) that is separated into two open reading frames (ORFs) (**Figure 1a**). The 5' ORF encodes a CHIKV nonstructural polyprotein (P1234) that is translated and cleaved in four nsPs forming the RNA replicase complex (18, 19). The 3' ORF is transcribed into a subgenomic positive-stranded RNA and encodes, after subsequent cleavage



**Figure 1**

CHIKV genomic organization and viral proteins. (a) The CHIKV genome consists of a 5'-capped and 3'-polyadenylated positive-stranded RNA molecule divided into two ORFs. Expression of ORF1 and ORF2, encoding for the nonstructural and structural polyproteins, is controlled by the genomic promoter in the 5' UTR and the internal subgenomic promoter, respectively. Genome replication and transcription are *cis*-regulated by RNA stem loops, referred to as conserved sequence elements, located in the 5' UTR, subgenomic promoter, and 3' UTR regions. CHIKV replication results in the accumulation of full-length RNA used as a genome for CHIKV progeny assembly. Subgenomic RNA is transcribed and used as a template for translation of structural polyproteins.

(b) Translation of the CHIKV genome and subgenomic RNA results in the accumulation of nonstructural (P1234) and structural (C-E3-E2-6K-E1) polyproteins. The presence of a leaky opal stop codon at the end of the nsP3 sequence directs the translation of a partial nonstructural polyprotein (P123). P123 and P1234 are sequentially processed in *cis*- and *trans*-reactions by the cysteine protease nsP2 to produce mature nsPs (nsP1–4) forming the replication complex. The structural precursor is first matured by the C protein that possesses *cis*-proteolytic activity and then by cellular proteases (signalases and furin), resulting in the production of E1, E2, and E3 glycoproteins and 6K protein, all contributing to viral particle assembly and budding. Abbreviations: C, nucleocapsid; CHIKV, chikungunya virus; E, envelope; nsP, nonstructural protein; ORF, open reading frame; UTR, untranslated region.

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**C:** nucleocapsid

**E:** envelope protein

**vRC:** viral replication complex

**dsRNA:** double-stranded RNA

**CPV-I:** type I cytopathic vacuole

**CPV-II:** type II cytopathic vacuole

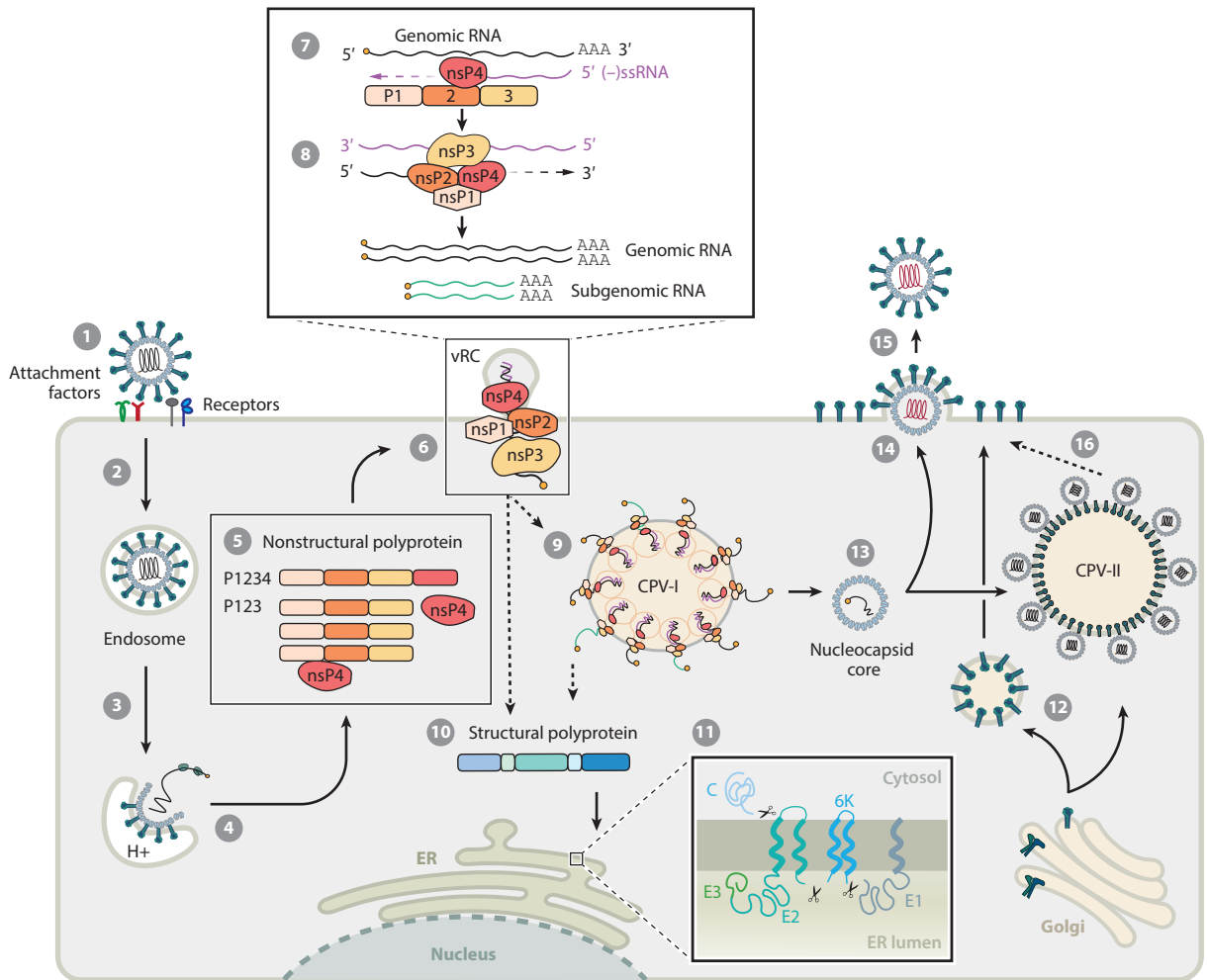
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and maturation steps, six structural proteins: C (nucleocapsid); E1, E2, and E3 envelope viral glycoproteins; 6K and its translational frameshift product, TF (transframe) (**Figure 1b**). A CHIKV virion consists of the viral RNA genome that is surrounded by 240 copies of the C protein and a host lipid bilayer with 80 embedded trimeric glycoprotein spikes. Each spike is formed by three E1-E2 heterodimers that are essential for receptor recognition and binding (E2) and membrane fusion (E1) (20).

CHIKV enters target cells by receptor-mediated endocytosis. Within the endosome, pH acidification triggers major conformational reorganization of E1/E2 heterodimers and unmasks the buried E1 fusion loop, leading to fusion of the viral and endosomal membrane (18, 19). Subsequently, the nucleocapsid disassembles in the cytoplasm, releasing the viral genomic RNA, which is directly translated into the P123 and P1234 precursors. (**Figure 2**). The P1234 polyprotein is rapidly processed at the nsP3/nsP4 junction by the nsP2 protease to release the active nsP4 RNA-dependent RNA polymerase. P123 and nsP4, along with the viral RNA, are recruited to the plasma membrane where, in association with host factors, they form an early short-lived replicase complex. A negative-stranded RNA complementary to the viral genome is synthesized and serves as an intermediate for viral replication (21, 22) (**Figure 2**). P123 accumulation leads to the complete polyprotein processing, and the replicase activity switches to the transcription of new positive-stranded RNA genomes and subgenomic RNA-encoding viral structural proteins. These steps take place in bulb-shaped, viral-induced vesicular structures formed at the plasma membrane, termed replicative spherules or viral replication complexes (vRCs), which isolate the accumulating double-stranded RNA (dsRNA) from innate immune sensing (**Figure 2**). Upon internalization, these compartments can fuse with endosomes, resulting in the formation of large vacuolar structures of 600 to 2,000 nm in diameter, termed type I cytopathic vacuoles (CPV-I), where RNA synthesis continues before diffusion to the cytoplasm through a connecting pore (23). Once in the cytoplasm, the subgenomic RNA is translated into a structural polyprotein. It is processed by the autocatalytic activity of the C protein, which is released in its mature form (22). Direct interaction with the newly synthesized genomes catalyzes C oligomerization and nucleocapsid assembly. The remaining polyprotein (pE3-E2-6K-E1) is translocated and inserted in the endoplasmic reticulum (ER) membrane for processing (**Figure 2**). Release of the accessory 6K protein mediated by host ER signalases allows the association of E1, E2, and E3 into an immature trimeric spike complex, which traffics through the secretory pathway while undergoing conformational changes and post-translational modifications (palmitoylation, N-linked glycosylation). Alternatively, E glycoproteins are arranged and transported from the *trans*-Golgi network to the plasma membrane in type II cytopathic vacuoles (CPV-II), which recruit assembled nucleocapsids on their cytoplasmic face (24). The E3 protein, which stabilizes the E2/E1 trimers by preventing the activation of the E1, is released by a furin-dependent cleavage in the *trans*-Golgi (22). At the plasma membrane, CHIKV particle budding is finally promoted by the interaction of the E2 cytoplasmic domain with the hydrophobic domain of the C protein (**Figure 2**).

## THE KEY ROLE OF THE MUSCULOSKELETAL TISSUE IN CHIKUNGUNYA VIRUS PATHOGENESIS

Viral tropism has a direct effect on pathogenesis, including the nature of symptoms, the duration of infection, and the establishment of a chronic phase (25). In vertebrates, CHIKV displays a large cell and tissue tropism that covers endothelial cells, fibroblasts from almost all organs, and brain cells (26–30). However, viral pathogenesis mainly reflects CHIKV capacity to actively replicate in joints and muscle tissues (29, 31–37). In joints, chondrocytes, osteoblasts, and bone marrow mesenchymal stem cells, which serve as precursors for osteogenic cells, are susceptible to CHIKV



**Figure 2**

CHIKV replication cycle in vertebrate cells. CHIKV infection is initiated by the interaction of E1/E2 glycoprotein heterodimers with cell surface Mxra8 receptor and attachment factors (Step 1). The viral particle is internalized by endocytosis and trafficked to endosomes (Step 2). Acidification of the vacuolar pH results in the unmasking of the E1 fusion peptide and fusion of viral and endosomal membranes (Step 3). The viral C is released in the cytoplasm and rapidly uncoated (Step 4). The CHIKV genome is translated to produce P123 and P1234 polyproteins (Step 5). nsP4 is matured and forms a complex with P123 and an RNA template that traffics to the plasma membrane. This complex reshapes the cell membrane to promote the formation of replication organelles, or spherules (Step 6), in which a negative-stranded full-length RNA [(-)ssRNA] is synthesized (Step 7). Then, P123 is sequentially processed to produce the four mature nsPs, resulting in a shift of replication complex activity toward the synthesis of a positive-stranded RNA [(+)ssRNA] genome and subgenomic RNA (Step 8). To some extent, CHIKV replication compartments are endocytosed and fused with endo-lysosomes to form CPV-I in which internalized spherules remain active (Step 9). Structural proteins are translated from subgenomic RNA in the form of a polyprotein translocated to the ER (Step 10). C is liberated by autocatalytic processing. E glycoproteins and 6K protein are matured by host proteases (Step 11) and trafficked to the plasma membrane through the secretory pathway (Step 12). The C protein and RNA genome form an icosahedral nucleocapsid (Step 13). Viral assembly takes place at the plasma membrane where mature E glycoproteins and nucleocapsids are targeted (Step 14). C/E2 interaction promotes CHIKV particle budding and release (Step 15). Alternative recruitment of assembled nucleocapsids and E glycoproteins to CPV-II additionally contributes to CHIKV virion assembly and budding (Step 16). Abbreviations: C, nucleocapsid; CHIKV, chikungunya virus; CPV-I, type I cytopathic vacuole; CPV-II, type II cytopathic vacuole; E, envelope; ER, endoplasmic reticulum; nsP, nonstructural protein; ORF, open reading frame; ssRNA, single-stranded RNA; UTR, untranslated region; vRC, viral replication complex. Figure adapted from Cell Background Straight by BioRender.com (2020), retrieved from <https://app.biorender.com/biorender-templates>.

**IFN:** interferon

**RANKL:** receptor activator of nuclear factor  $\kappa$ B

infection (38–40). Cellular damages account for CHIKV-induced arthritis and joint pain. As an example, infection of osteogenic cells impairs mineralization and repair capacity, resulting in the dysregulated dynamics of bone homeostasis often reported in patients (40). Muscles are also a privileged site for CHIKV replication as supported by the presence of viral antigens and signs of necrosis, vacuolization, and fibrosis from patients with acute and chronic CHIKV disease (41). In vitro, muscle fibroblasts, satellite cells, and myoblasts (muscle progenitors) are highly susceptible to CHIKV infection (37, 41, 42). Initial reports suggested that terminally differentiated myotubes are poorly infected by CHIKV (41). However, murine skeletal muscle fibers were recently proven to be efficiently infected with effect on the severity of CHIKV infection in mice (43–45). A few studies also suggested that recent epidemic strains could differ in their ability to infect muscle cells and to induce a myopathic syndrome, although it is not formally demonstrated (42, 45). The isolate from the Reunion Island outbreak was shown to induce more severe muscle disease in neonatal mice as compared to an isolate from Senegal circulating in 1983 (45). Whereas both strains equally spread from the inoculation site to distal muscle by infecting connective tissue fibroblasts, the epidemic strain replicates more efficiently in myofibers, resulting in increased muscle disease characterized by severe myonecrosis (45). Whether the increased muscle pathology is due to a more robust immune response is not clear, as no differences were observed in the induction of type I interferon (IFN-I) and proinflammatory cytokines such as IL-1 $\beta$  or IL-6 (45). Recently, Lentscher and colleagues (44) definitively established that viral replication in muscle cells is determinant for CHIKV disease pathogenesis. They engineered a CHIKV strain exhibiting restricted replication in muscles via incorporation of target sequences for skeletal muscle cell-specific miR-206. This microRNA is expressed at detectable levels in skeletal muscle progenitor-satellite cells, strongly induced upon differentiation, and then stably expressed at high levels throughout the life of the muscle fiber. Using this tool, Lentscher and colleagues demonstrated that replication in skeletal muscle cells does not affect the overall viral titers and the global inflammatory status. Instead, it results in attenuated muscle damages reflected by diminished necrosis and local induction of IL-6, IL-1 $\beta$ , TNF $\alpha$ , and IP10, which are biomarkers of disease severity in humans and mice (44, 46).

Besides tissue damage, the attraction of infiltrating monocytes/macrophages is also critical to local inflammation and viral persistence (39, 47–49). More specifically, infection of fibroblast-like synoviocytes results in the secretion of IL-6, IL-8, and CCL2, which attracts phagocytes. It also stimulates secretion of RANKL (receptor activator of nuclear factor  $\kappa$ B), which may contribute to bone loss and to the occurrence of arthritis/arthritis by stimulating the differentiation of monocytes into bone-resorbing osteoclasts (39, 50). In muscles, monocytes/macrophages could be part of the dynamics of CHIKV-induced myositis. The modulation of the monocyte-driven infiltration reduces muscle inflammation while allowing the accumulation of a macrophage subset enhancing muscle repair and recovery (48).

Musculoskeletal tissues are also proposed to participate in CHIKV persistence and chronic condition (34, 38, 51–53). Multiple studies reported that CHIKV RNA persists long after viremia declines (38, 53, 54). Besides synovial macrophages, which are proposed to be a niche for viral persistence (38, 54), muscle fibroblasts and also myofibers that survive acute infection are potential reservoirs for persistent CHIKV RNA in mice (34). Nevertheless, actively replicating CHIKV has not been evidenced so far in tissues exhibiting chronic inflammation, and CHIKV surface antigens failed to be detected in muscle fibroblasts harboring persistent viral RNA (17, 34, 55). Instead, the chronic CHIKV-induced immunopathology seems to be the prolongation of the acute inflammatory process, which persists until clearing of the viral material (17, 56). It is still unknown how CHIKV RNA persists in joints and muscles and to what extent it contributes to chronic CHIKV disease.

## **Mxra8, AN IMPORTANT BUT NOT EXCLUSIVE DETERMINANT OF CHIKUNGUNYA VIRUS ENTRY AND PATHOGENESIS**

CHIKV entry into target cells is a complex multistep process that begins with the interaction of the viral E2 glycoprotein with specific receptor(s) expressed on the host cell surface. The cellular receptor mediating viral entry remained elusive until Zhang and colleagues (57) identified Mxra8 (also called limitrin, DICAM, or ASP3) as a CHIKV entry factor using a CRISPR/Cas9 genome-wide screening strategy. Mxra8 is an adhesion molecule mainly expressed on epithelial and mesenchymal cell types targeted by CHIKV (dermal and synovial fibroblasts, osteoblasts, chondrocytes, and skeletal muscle cells). Mxra8 is the first CHIKV entry molecule identified so far that fulfills the criteria of a virus receptor. Structural studies have revealed that it interacts with the envelope spike in a complex 3:3 binding interaction (58, 59). In this complex, Mxra8 contacts both E2 and E1 proteins to facilitate virus attachment and internalization in the cell. Overexpression of Mxra8 in poorly susceptible cells renders them permissive to CHIKV (57). Conversely, preventing the Mxra8-CHIKV interaction by CRISPR-Cas9-mediated depletion of the *Mxra8* gene or by using neutralizing antibodies or fusion of extracellular Mxra8 domains with an immunoglobulin Fc fragment (Mxra8-Fc) blocked infection in both murine and human cells and reduced CHIKV pathogenesis in experimentally infected mice (57, 60, 61). This indicates that Mxra8 is required for optimal infection, dissemination, and articular pathogenesis (joint swelling and neutrophil infiltration). Interestingly, this function is conserved for arthritogenic alphaviruses, such as Ross River, Mayaro, and o'nyong-nyong viruses (57). The lack of Mxra8 on the surface of some CHIKV permissive cells (57) strongly indicates that other CHIKV receptors exist and remain to be discovered. Consistent with this, some pathogenic IOL strains (e.g., LR-2006) display limited dependency on Mxra8, conversely to Asian CHIKV strains (181/25 and AF15561 strains) (57). Glycosaminoglycans (GAGs), a family of negatively charged polysaccharides, interact with a structurally conserved and positively charged domain in E2. These membrane proteins were proposed to enhance infection by promoting E1/E2 dissociation (62, 63). The functional importance of GAGs was recently reassessed in genome-wide loss of function screens performed in HAP1 cells that identified GAGs' biosynthesis enzymes (B3GAT3, SLC35B2, PAPSS1, NDST1) as critical factors for CHIKV infectivity (64, 65). The need for GAGs binding in order to achieve efficient infection was recently demonstrated for all CHIKV clades (62). Interestingly, the comparison of viruses from the ECSA and Asian lineages revealed that the requirement of GAGs for CHIKV binding and infection was inversely correlated with Mxra8 dependency (62). This suggests that GAG binding may be a compensatory mechanism for the entry of CHIKV strains poorly interacting with Mxra8 (62). However, this model does not seem to be the only scenario allowing for viral entry, as infection was still observed to some extent in the absence of GAGs and Mxra8 (62). The vast array of cell factors reported to facilitate CHIKV entry, including C-type lectins (DC-SIGN and L-SIGN), immunoglobulin and mucin domain-containing proteins 1 and 4 (hTIM1 and hTIM4), and the AXL receptor, which all have also been described to stimulate CHIKV infection (66–68), could be part of the complex mechanism accounting for CHIKV entry and wide tropism.

## **BUILDING MEMBRANE SPHERULES TO ENSURE VIRAL REPLICATION**

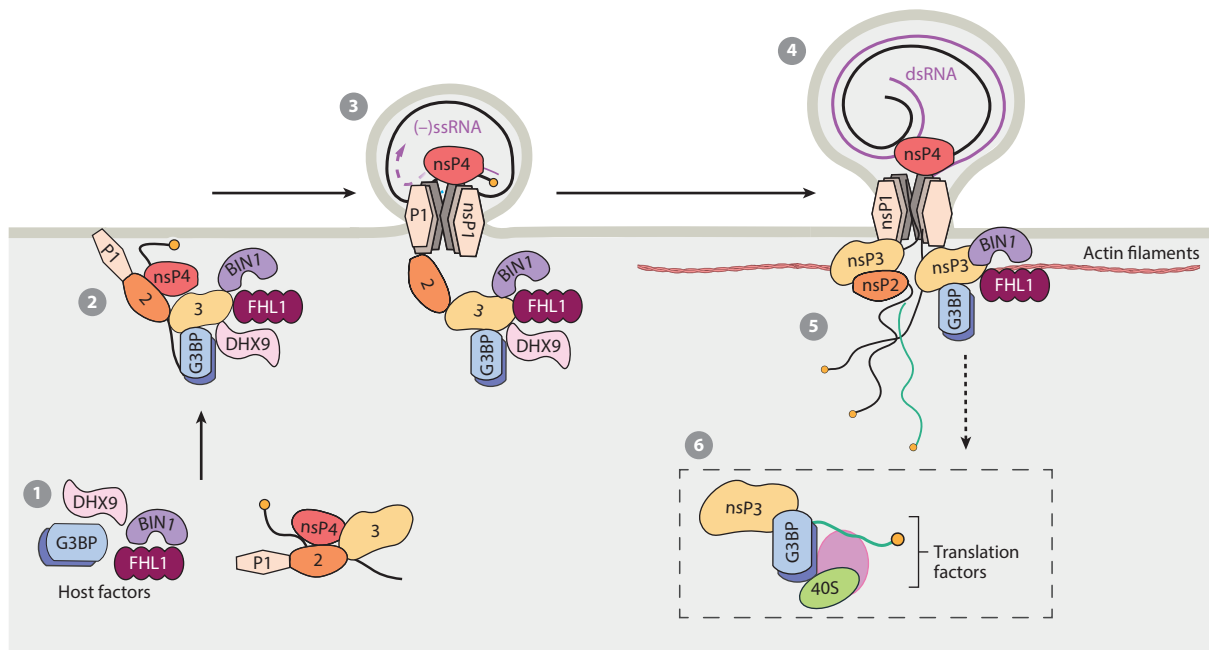
Like other alphaviruses, CHIKV remodels the host plasma membrane into bulb-shaped protrusions of approximately 50 nm in diameter, referred to as spherules. These compartments create an optimal microenvironment for viral replication because they concentrate the viral nsP, genomic RNA, and dsRNA used as genome replication forms (69). Spherules are also supposed to protect



dsRNA and nascent uncapped RNAs from innate sensing and degradation by cellular RNases (69, 70). In this context, the inner spherule is connected to the cytosol by a 7-nm opening that allows the import of metabolites and cofactors and the export of newly synthesized genomic and subgenomic RNAs. Inside the spherule, the CHIKV replication complex (RC) associates with the inner face of membranes through nsP1, the viral capping enzyme that contains unique membrane binding capacity and displays membrane-dependent methyl/guanylyltransferase activities (71–74). Three-dimensional (3D) cryo-electron tomography was successfully applied to resolve the complex spatial organization of replication membranes formed by many positive-stranded RNA viruses (75–81). Conversely, the 3D architecture and biogenesis of alphavirus spherules remain enigmatic. Particularly, the exact localization of the RC within the spherule, stoichiometries of the nsPs in these compartments, and the nature of cellular factors contributing in spherule biogenesis are all questions that remain unanswered.

Jones and colleagues (82) recently solved the structure of the CHIKV nsP1 assembly by single-particle cryo-electron microscopy. They found that, upon membrane binding, recombinant nsP1 assembles in a dodecameric ring, forming a pore-like structure compatible with the trafficking of globular proteins up to 70–90 kDa in size. In this membrane-bound complex, nsP1 is switched from an enzymatically inactive monomer to a methyl/guanylyltransferase dodecameric active form, NsP1, a monotopic membrane protein. Therefore, by interacting with the inner phospholipid leaflet, nsP1 forms a capping pore that may corral the replication vesicle neck and therefore be critical for spherule structure maintenance (**Figure 3**). Further, nsP1 macroassembly might potentially function as a bioreactor simultaneously capping 12 nascent RNA molecules during their export to the cytosol, thereby contributing to the exceptionally high alphavirus replication rate. Striking similarities also exist with the crown-like assembly of nodavirus-encoded replicase at the neck of Flock House virus spherules, which favors the hypothesis of an evolutionarily conserved replication organelle pore structure among alpha-like viruses (83, 84). This information, which provides an incomparable breakthrough to our understanding of alphavirus replication compartment assembly, needs to be refined in CHIKV-infected cells, considering the simultaneous expression of all four nsPs in the context of a sequential matured nonstructural polyprotein. Overall, this model questions the stoichiometry of other nsPs within and near the spherules. It also raises new important questions regarding the role of nsP1 in spherule biogenesis. Indeed, the minimal requirement for alphavirus spherule formation is the expression of a partially cleaved nonstructural polyprotein in the form of nsP4 + P123 (85) (**Figure 3**). This process is modulated to some extent by the viral RNA template length that determines the spherule shape and size (86). nsP1, whose complex interaction with membranes dramatically reshapes synthetic lipid bilayers or cell membranes in the absence of any other viral factor, is certainly pivotal to spherule creation (72, 82, 87–89). Nevertheless, morphological differences in CHIKV spherules and nsP1-induced membrane deformation, seen as filopodia-like protrusions, suggest additional players (**Figure 3**). For instance, the contribution of nsP1-interacting cellular factors in membrane reshaping during spherule biogenesis awaits investigation (90, 91). CHIKV nsP3 was proposed to be involved in spherule assembly by recruiting BIN1/amphiphysin 2, an F-BAR protein involved in membrane curvature, but there is still a lack of clear evidence (92) (**Figure 3**). Furthermore, the contribution of defined membrane lipid species should be considered with a special attention to fatty acid molecular species that determine the fluidity or curvature of the lipid bilayer, depending on the length and saturation of their fatty acyl chains, and to negatively charged phospholipids and cholesterol that regulate nsP1 capping activity and membrane affinity, respectively (74, 93), and could alternatively promote host cofactor coalescence to the replication site to assist spherule assembly.

Finally, the exact replication steps taking place in proximity to CHIKV spherules at the plasma membrane remain poorly defined. In contrast with other alphaviruses, which internalize



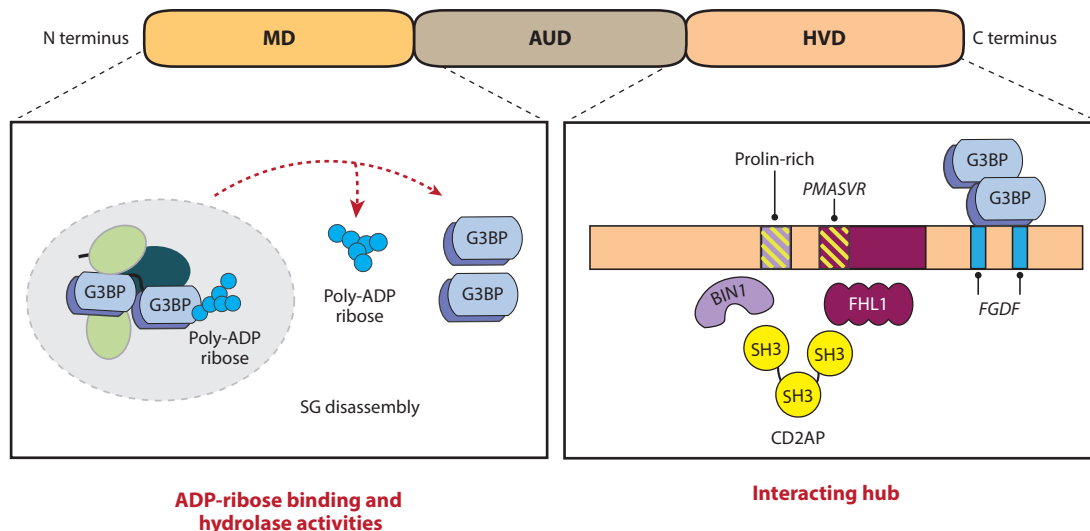
**Figure 3**

Model of CHIKV replication complex assembly. (*Step 1*) G3BP, FHL1, DHX9, and BIN1/Amphiphysin2 (and other SH3 domain replicase and associated host factors) anchor to the plasma membrane through nsP1 membrane-binding properties. (*Step 2*) CHIKV replicase and associated host factors anchor to the plasma membrane through nsP1 membrane-binding properties. (*Step 3*) This complex starts replicating the RNA genome, assisted by DHX9, which is proposed to regulate the early RNA translation-to-replication switch. (*Step 4*) BIN1/Amphiphysin2, CD2AP, SH3KBP1 and FHL1 involved in membrane bending and/or actin cytoskeleton organization may assist the biogenesis of bulb-shaped spherules separating the dsRNA replication intermediate from host innate immune recognition. (*Step 5*) Genomic and subgenomic RNAs synthesized in the replication organelle are then massively exported to the cytoplasm and capped by the nsP1 dodecameric ring connecting the spherule to the cytoplasm. (*Step 6*) G3BP molecules recruited to the cytoplasmic face of these organelles are proposed to recruit the cellular translation machinery in close proximity to membrane vRCs to ensure optimal viral protein synthesis. Abbreviations: CHIKV, chikungunya virus; dsRNA, double-stranded RNA; nsP, nonstructural protein; ssRNA, single-stranded RNA; UTR, untranslated region; vRC, viral replication complex. Figure adapted from images created with BioRender.com.

membrane spherules by activation of the phosphatidylinositol-3-kinase-Akt-mammalian target of rapamycin signaling, CHIKV replication compartments are mostly maintained at the plasma membrane. This feature was assigned to the poor capacity of CHIKV to activate this pathway (94). Currently, the clear benefit of spherule endocytosis is not clearly understood (23). Altogether, viral protein assembly, contributing cell factors, and metabolism pathways involved in spherule biogenesis and architecture represent new promising targets for the development of therapeutics to control CHIKV infection in humans.

### CHIKUNGUNYA VIRUS NONSTRUCTURAL PROTEIN 3: A MULTIFACETED VIRAL PROTEIN ESSENTIAL FOR VIRUS INFECTION AND PATHOGENESIS

The nsP3 molecule is probably the most fascinating and enigmatic CHIKV-encoded protein and has been recognized as essential for both viral replication and adaptation to its host. This viral



**Figure 4**

Organization of CHIKV nsP3 and associated functions. CHIKV nsP3 is structurally separated into three domains: an N-terminal MD that binds and hydrolyzes ADPr and poly-ADPr, a central zinc-finger-containing AUD, and a C-terminal HVD identified as a hub for host factor binding. Mapped binding domains in HVD are indicated. The concerted action of MD-associated ribosylhydrolase activity and HVD results in the removal of poly-ADPr conjugated to G3BP, dissociation of G3BP-positive SGs, and redirection of G3BP to the CHIKV replication complex and nsP3 aggregates. Abbreviations: ADPr, ADP-ribose; AUD, alphavirus unique domain; CHIKV, chikungunya virus; HVD, hypervariable domain; MD, macrodomain; nsP, nonstructural protein; SG, stress granule.

factor is a tripartite phospho-protein composed of a highly conserved N-terminal globular domain termed macrodomain (MD), a central domain forming the alphavirus unique domain (AUD) conserved among alphaviruses, and a C-terminal hypervariable domain (HVD) (Figure 4). During the early phase of the CHIKV life cycle, nsP3 localizes within the vRC and plays a critical role in viral replication (95–98). Several studies showed that nsP3 acts as a platform for the recruitment of multiple host factors through its HVD (91, 92, 95, 99–101). The HVD is intrinsically disordered and consists of multiple small peptides that interact with distinct sets of cellular proteins, which vary depending on both the alphavirus species and the infected cell type (101) (Figure 4). For CHIKV, the major HVD binding molecules identified so far are the G3BP (Ras-GAP SH3 domain-binding proteins, G3BP1 and G3BP2) family proteins, FHL1, DHX9, and several SH3 domain-containing proteins including BIN1/Amphiphysin2, CD2AP, and SH3KBP1, which are involved in membrane bending and cytoskeleton regulation. The roles of G3BP family members during CHIKV replication have been extensively studied. These are essential factors in the assembly of stress granules (SGs), which control viral replication by arresting viral protein translation (reviewed in 102). G3BPs contain RNA-binding domains that self-assemble in macromolecular complexes (103), driving the nucleation of cellular SGs. Disruption of the G3BP-nsP3 HVD interaction or the depletion of both G3BP1 and G3BP2 blocks CHIKV replication (98, 104, 105). According to the current model, in CHIKV-infected cells, G3BPs may interact with the viral P123 precursor and in turn bind the viral genomic RNA to form prereplicative complexes that drive membrane spherule formation and viral RNA synthesis (104). Furthermore, G3BPs interact with the 40S ribosomal subunit, which is thought to recruit the cellular translational machinery in the vicinity of the vRCs for viral protein synthesis (106).

**MD:** macrodomain

**AUD:** alphavirus unique domain

**HVD:** hypervariable domain

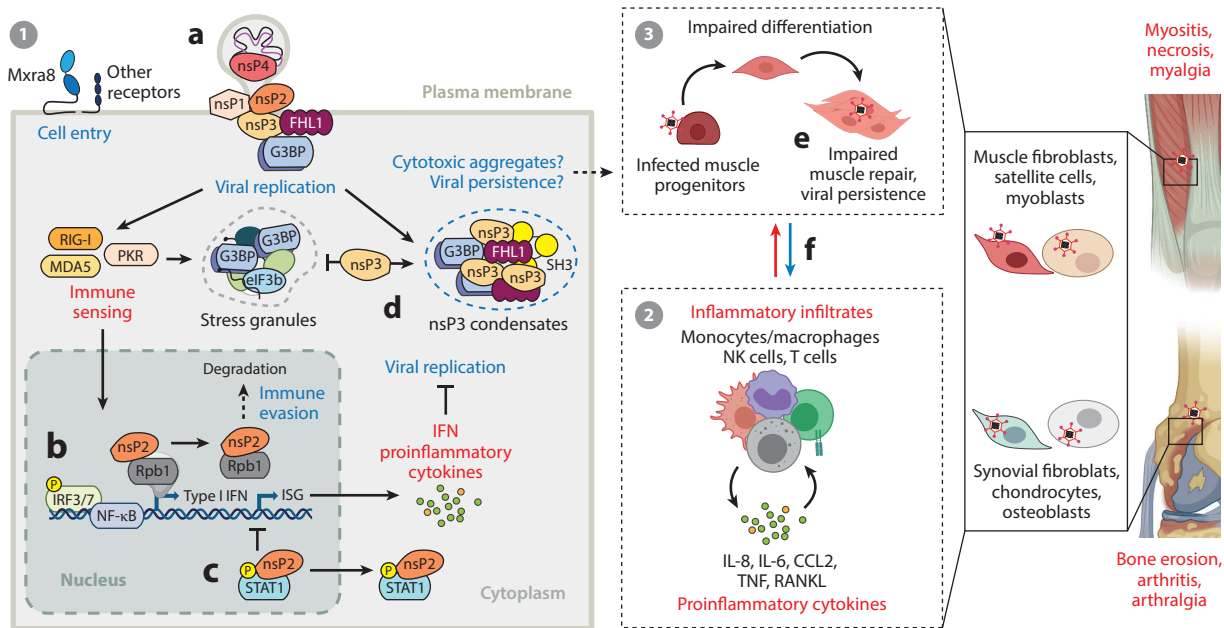
**G3BPs:** Ras-GAP SH3 domain-binding proteins

**SG:** stress granule

Emerging evidence indicates that nsP3 may accomplish a yet-unknown function during the CHIKV life cycle that is distinct from its role in RNA replication and vRC assembly. Several studies showed that a large proportion of nsP3 proteins, expressed either alone or in the context of CHIKV infection, aggregate to form high-density rod-like structures and large spherical granules distinct from the vRC (95, 107, 108). These nsP3 condensates rapidly increase in size and number during virus replication (108). Importantly, CHIKV nsP3 aggregates also contain G3BPs, yet they are different from SGs in morphology and composition (97, 109). Moreover, cells harboring nsP3 aggregates are not able to form bona fide SGs in response to cellular stress, suggesting a role of nsP3 in SG disassembly by trapping G3BPs (97, 98). Importantly, other nsP3 binding proteins such as FHL1, CD2AP, and SH3KBP1 are also found within these condensates (64, 110, 111). By using subdiffractional multicolor microscopy and human cells persistently replicating a CHIKV replicon, Remenyi and colleagues (108, 112) assessed nsP3 spatial and temporal distribution. They demonstrated that nsP3 clusters of different sizes and morphology coexist in cells and can persist for hours to days. The nsP3 clusters contain genomic RNA and are localized either near dsRNA- and nsP1-enriched foci or close to the nuclear envelop and nucleoporins. Moreover, they are detergent resistant and exert liquid-liquid phase separation properties also proposed for SGs (108, 113). Recent studies revealed that, besides the HVD region, the nsP3 MD is important for aggregate formations (**Figures 4 and 5**). The N-terminal MD of nsP3 binds and removes ADP-ribose (ADPr) or poly-ADPr (114, 115), a reversible post-translational modification known to regulate SG formation/disassembly. In CHIKV-infected cells, this activity reverses G3BP-ribosylation, and favors SG disassembly, and recruitment of translation initiation factors within nsP3 condensates (109). Indeed, in the absence of MD ribosylhydrolase activity, nsP3 condensates contain both RNA-binding proteins (G3BPs, TIA-1, TIAR, and others) and translation initiation factors (eIF3, RACK1, and others), thereby corresponding to SGs (109). An important question raised by the abovementioned studies is how these nsP3 aggregates contribute to virus pathogenesis (**Figure 5**). One possibility is that these nsP3 structures might participate in viral persistence by trapping CHIKV RNA or contribute in the attenuation of the antiviral responses by sequestering key players of the innate immunity, thereby facilitating CHIKV replication. Given the importance of nsP3 in CHIKV replication, further studies providing a precise description of the formation, composition, and functions of the nsP3 condensates are required to understand their biological relevance in CHIKV pathogenesis.

## FHL1, A BRIDGE BETWEEN CHIKUNGUNYA VIRUS REPLICATION AND PATHOGENESIS?

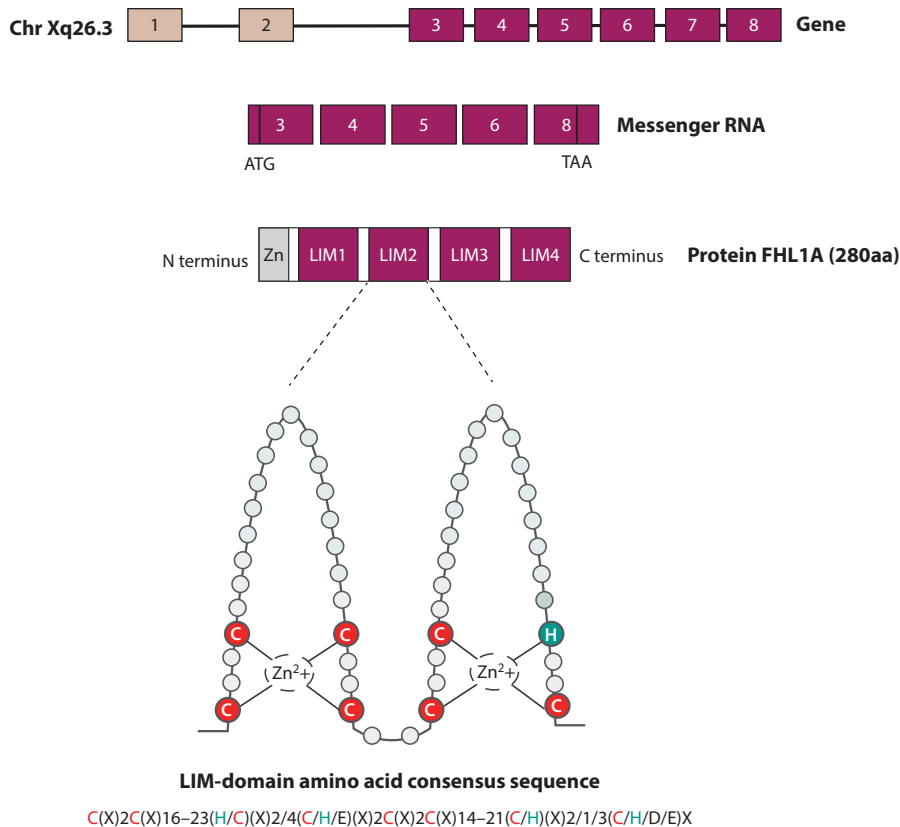
Little is known about the host cellular factors that dictate CHIKV tropism for muscles and joints. A recent genome-wide CRISPR-Cas9 screen identified the FHL1 protein as an important host factor for CHIKV infection and pathogenesis (64). FHL1 is a member of the FHL protein family, which is characterized by the presence of an N-terminal half LIM domain followed by four complete LIM domains (116, 117) (**Figure 6**). Infection studies in FHL1 knock-out cell lines, as well as in primary cells from patients suffering from Emery–Dreifuss muscular dystrophy that lack functional FHL1 proteins, demonstrated that FHL1 is important for CHIKV replication and cell permissiveness (64). Interestingly among the *alphavirus* genus, only CHIKV and its close relative o'nyong-nyong virus use FHL1 for infection. These observations suggest that FHL1 dependency was acquired late during alphavirus evolution. FHL1 interacts with the nsP3 HVD and is important for viral RNA amplification (64). Indeed, ablation of the *fb11* gene severely reduced both CHIKV negative-stranded RNA synthesis and viral spherule formation. Further investigation is required to decipher the exact molecular mechanisms by which FHL1 mediates CHIKV infection



**Figure 5**

Hypothetical model for CHIKV-induced muscular and articular pathogenesis. (*Step 1*) Successful CHIKV replication in muscle and joint cells relies on the concerted action of host factors and viral proteins. CHIKV nsPs allow efficient viral RNA synthesis within the replicative spherules (*a*). CHIKV nsP2, translocated to the nucleus of infected cells, shuts down antiviral genes transcription by (*b*) redirecting the RNA polymerase II Rpb1 subunit to proteasomal degradation and (*c*) inhibiting phospho-STAT1 nuclear accumulation. By sequestering G3BPs, nsP3 counteracts the assembly of cytosolic stress granules and contributes to translational shutoff (*d*). (*Step 2*) The release of cytokines and immune mediators by infected cells attracts monocytes/macrophages to muscles and joints, leading to local inflammation, muscle myositis, and bone resorption. (*Step 3*) CHIKV replication and persistence in muscle progenitor cells impairs muscle repair, contributing to musculoskeletal disease (*e*). Viral material persistence in infected tissues can exacerbate host immune responses, leading to chronic rheumatic symptoms (*f*). Abbreviations: CHIKV, chikungunya virus; IFN, interferon; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; nsP, nonstructural protein; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NK, natural killer; PKR, protein kinase R; RANKL, receptor activator of nuclear factor  $\kappa$ B; STAT1, signal transducer and activator of transcription 1. Figure adapted from images created with BioRender.com.

and to determine whether FHL1 is directly involved in the assembly of spherules or regulates a step in the viral RNA synthesis process. The importance of FHL1 in CHIKV pathogenesis is further supported by *in vivo* studies showing that FHL1 knock-out mice are resistant to CHIKV infection and do not develop disease. Interestingly, FHL1 tissue expression reflects CHIKV tropism. Indeed, FHL1 protein is highly abundant in skeletal muscle and fibroblasts (118, 119). FHL1 is known to participate in muscle development and homeostasis. It is involved in myogenesis, which consists of the activation of satellite cells and their differentiation in myoblasts, which then fuse to create myotubes that finally differentiate in mature myofibers. Muscle satellite cells express high levels of FHL1, which could explain their susceptibility to CHIKV infection. One can speculate that, upon FHL1 hijacking by nsP3, infected muscle satellite cells might be unable to properly regenerate damaged muscle fibers, contributing to CHIKV-induced musculoskeletal disorders (**Figure 5**). An indirect link between FHL1 expression and CHIKV disease severity is supported by the observation that FHL1 seems to be differentially used by CHIKV strains (64, 111). For instance, the pathogenic CHIKV-21 strain isolated from a patient infected during the 2005–2006 CHIKV outbreak on Reunion Island is highly dependent on FHL1 for infection *in vitro* and



**Figure 6**

FHL1 gene and protein organization. FHL1A is encoded by human chromosome Xq26.3 and is expressed with high levels in striated muscle cells and fibroblasts. Exons 1 and 2 (*beige boxes*) are noncoding; exons 3 to 8 (*purple boxes*) encode the full-length FHL1 protein, organized into an N-terminal half LIM domain followed by four complete LIM domains. Each domain consists of two zinc-finger motifs, proposed to be a protein/protein binding interface ensuring the assembly of multimeric protein complexes. The LIM domain amino acid consensus sequence is indicated.

induces severe muscular pathology in mice (64). Conversely, the requirement for FHL1 was less pronounced for the sylvatic CHIKV 37997 strain from the West African genotype (64) and the attenuated CHIKV 181/25 strain (111), which are less pathogenic in mice (45). Understanding the molecular basis for FHL1 usage by CHIKV strains may provide important insights into the muscular pathology associated with CHIKV infection. Furthermore, a study on FHL1 polymorphisms in cohorts of CHIKV-infected individuals could add considerable weight to the *in vivo* relevance of this host factor to CHIKV pathogenesis.

## INHIBITION OF HOST INNATE IMMUNITY BY THE CHIKUNGUNYA VIRUS NONSTRUCTURAL PROTEINS

The ability of CHIKV to successfully establish infection and pathogenesis in its host hinges upon its capacity to counteract the host immune responses. Early after infection, CHIKV elicits the massive secretion of IFNs and numerous proinflammatory chemokines and cytokines that are

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**ISG:**  
interferon-stimulated  
gene

**STAT1:** signal  
transducer and  
activator of  
transcription 1

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critical to the control of viremia and pathogenesis (37, 120) (**Figure 5**). Mice deficient for the IFN  $\alpha/\beta$  receptor rapidly succumb to CHIKV infection, showing that IFN-I signaling is critical in controlling infection (121). This response, initiated by primary sensing of viral RNA via pattern recognition receptors (PRRs) of the RIG-1-like helicase family (RIG-1 and MDA5), controls the expression of hundreds of interferon-stimulated genes (ISGs) (e.g., ISG15, BST2) capable of interrupting CHIKV replication (121–124) (**Figure 5**). As a countermeasure, CHIKV has evolved various strategies to disrupt IFN signaling. In contrast to New World alphaviruses that usually use their C protein to evade innate immunity, the strategies developed by CHIKV to prevent IFN signaling and ISG antiviral effects have been mainly assigned to the viral protease nsP2. In infected cells, CHIKV nsP2 is detected close to the plasma membrane, where it takes part in the RC, and in the nucleus, where it translocates early after infection, thanks to the presence of a noncanonical nuclear localization motif (125, 126). In the nucleus, nsP2 rapidly targets the Rpb1 catalytic subunit of the RNA polymerase II to proteasomal degradation, thereby shutting down cellular gene transcription and avoiding activation of innate immune genes (127) (**Figure 5**). NsP2-mediated Rpb1 degradation is independent of nsP2 enzymatic activities but is abolished by mutation of Proline 718 in the nsP2 C terminus (90, 125–127). Besides this mechanism, proposed as the main strategy to evade the cellular antiviral response, it now appears that nsP2 specifically interrupts IFN signaling independent of general transcriptional shutoff. The nuclear fraction of nsP2 was indeed found to prevent the nuclear accumulation of signal transducer and activator of transcription 1 (STAT1) (128) by promoting its re-export in the cytoplasm through the chromosome region maintenance 1–mediated pathway. This activity involves the nsP2 methyltransferase-like domain (129) (**Figure 5**). Besides nsP2, recent evidence suggests that other nsPs, namely nsP1 and nsP3, also play a role in immune evasion. In this context, nsP3-MD ADP-ribose hydrolase activity was recently reported to reverse nsP2 mono-ADP-ribosylation by the ADP-ribosyltransferase ARTD10, interfering with its auto-proteolytic function (130). In this model, nsP3 ADP-ribose hydrolysis activity would therefore be critical for immune evasion by antagonizing the antiviral activity of the IFN-inducible ARTD10 that efficiently restricts CHIKV protein maturation and efficient replication. nsP1 also appears as one of the countermeasures deployed by CHIKV to avoid the cellular antiviral system, thus contributing to CHIKV-induced musculoskeletal inflammation in mice (131). nsP1 has recently been described as counteracting the IFN-I response by interacting with the cyclic GMP-AMP synthase (cGas), an effector of the cGas-stimulator of IFN genes signaling axis that restricts CHIKV infection (132). Considering these recent reports, CHIKV has seemingly acquired diverse countermeasures to limit host antiviral responses (**Figure 5**). While the most recent investigations suggest redundant mechanisms, the respective importance of nsP1-, nsP2- and nsP3-dependent scenarios in CHIKV global control strategy remains unknown.

## CONCLUDING REMARKS

CHIKV causes a debilitating acute disease that results in persisting arthralgia and myalgia in a large proportion of infected individuals. The mechanisms of CHIKV pathogenesis are complicated and multifactorial, involving both viral and host factors. In the past decades, genomics, proteomics, and structural studies as well as forward genetic screens have generated a plethora of new information about the CHIKV host cell molecular interactions, leading to the identification of several key host molecules important for viral infection. Most of these studies have been performed in immortalized cell lines. In the future, exploring the function of these cellular factors in relevant cellular systems such as primary fibroblasts, musculoskeletal tissues, and animal models of disease will undoubtedly unlock new paradigms of viral pathogenesis. In addition, research will need to uncover the mechanisms of viral RNA persistence in musculoskeletal tissues and joints in

order to uncover at a molecular level why CHIKV frequently evolves to a chronic phase. Studies on CHIKV immunobiology have outlined several elegant mechanisms developed by the virus to counteract host innate immune responses. A detailed understanding of the involved molecular processes and the identification of novel immune evasion strategies would certainly refine our understanding of CHIKV pathogenesis and may be the starting point for the generation of attenuated vaccine candidates and therapeutics to combat CHIKV disease.

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## Errata

An online log of corrections to *Annual Review of Virology* articles may be found at  
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