Host cytoskeletal vimentin serves as a structural organizer and an RNA-binding protein regulator to facilitate Zika viral replication

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27 **ABSTRACT**

28 Emerging microbe infections such as Zika virus (ZIKV) pose an increasing threat to
 29 human health. Current investigations on ZIKV replication have revealed the
 30 construction of replication compartments (RCs) and the utilization of host cellular
 31 endomembranes, without careful examination of the cytoskeletal network. Here, we
 32 investigated the function of vimentin, one of the intermediate filaments (IFs) that play
 33 a central role in basic cellular functions and diseases, in the life cycle of ZIKV
 34 infection. Using advanced imaging techniques, we uncovered that vimentin filaments
 35 have drastic reorganization upon viral protein synthesis, to form a perinuclear
 36 cage-like structure that embraces and concentrates RCs. Genetically removal of
 37 vimentin markedly reduced viral genome replication, viral protein production and
 38 infectious virions release, without interrupting viral binding and entry. Furthermore,
 39 proteomics and transcriptome screens by mass spectrometry and RNA sequencing
 40 identified intense interaction and regulation between vimentin and hundreds of
 41 endoplasmic reticulum (ER)-resident RNA-binding proteins. Among them, the
 42 cytoplasmic-region of ribosome receptor binding protein 1 (RRBP1), an ER
 43 transmembrane protein directly binds viral RNA, can interact with vimentin, resulting
 44 in modulation of ZIKV replication. Together, our work discovered a dual role for
 45 vimentin as being not only a structural element for RCs but also an
 46 RNA-binding-regulating hub in the ZIKV infection model, unveiling another layer of
 47 the complexity between host and virus interaction.

48

49 **KEY WORDS:** intermediate filaments; vimentin; Zika virus; replication
 50 compartments; RNA-binding protein

51

52 INTRODUCTION

53 Zika virus (ZIKV), a mosquito-borne enveloped RNA virus that belongs to the
54 Flaviviridae family, has gained notoriety recently, due to its explosive outbreaks and
55 association with serious clinical diseases such as Guillain-Barré syndrome in adults
56 and microcephaly in newborns (Cao-Lormeau et al. 2016, Pierson and Graham 2016,
57 Rasmussen et al. 2016, Pierson and Diamond 2018). Currently, no ZIKV-specific
58 therapies or prophylactic vaccines are available (Poland et al. 2019). ZIKV genome is
59 a positive-sense, single-stranded RNA (ssRNA(+)) (Musso and Gubler 2016). The
60 viral replication occurs on the surface of the endoplasmic reticulum (ER), where the
61 double strand RNA (dsRNA) is synthesized from viral genomic ssRNA(+) and
62 transcribed into new proteins (Munjal et al. 2017, Lee et al. 2018). The viral genome
63 is translated into a polyprotein which is proteolytically processed into 3 structural
64 proteins (capsid (C), precursor membrane (prM) and envelop (Env)) and 7
65 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), by both
66 host and viral proteases (Shi and Gao 2017, Sirohi and Kuhn 2017).

67

68 Vimentin is the most abundant intermediate filaments (IFs) which generally surrounds
69 the nucleus and extends throughout the cytoplasm, providing help to important
70 biological processes such as organelle positioning, cell migration and cell signaling
71 (Lowery et al. 2015). As a highly dynamic filaments that rapidly respond to
72 physiological stimuli through self-assembly and disassembly (Danielsson et al. 2018),
73 vimentin' role in virus infections has gained increasing attention. For instance, it
74 either acts as a co-receptor to help virus invading target cells, or guides transportation
75 of virus to the replication site, or reorganizes into aggregated structures surrounding
76 replication compartments (RCs), or recruits viral elements to the location of assembly
77 and egress (Dohner and Sodeik 2005, Foo and Chee 2015, Denes et al. 2018, Zhang et
78 al. 2019, Ramos et al. 2020, Zhang et al. 2020). Vimentin network rearrangement has
79 been previously observed in the infection of adenovirus type 2 and type 5 (Defer et al.
80 1990), African swine fever virus (ASFV) (Stefanovic et al. 2005, Netherton and

81 Wileman 2013), coxsackievirus B3 (Matilainen 2016, Turkki et al. 2020), dengue
82 virus type 2 (DENV-2) (Lei et al. 2013, Teo and Chu 2014), foot-and-mouth disease
83 virus (FMDV) (Gladue et al. 2013, Ma et al. 2020), frog virus 3 (Murti et al. 1988),
84 transmissible gastroenteritis virus (Zhang et al. 2015), vaccinia virus (Risco et al.
85 2002), and SARS-CoV-2 (Cortese et al. 2020).

86

87 A common feature for *Flaviviruses* infection is the induction of cellular
88 endomembrane rearrangements to establish viral RCs, which increase the local
89 concentration of viral and cellular factors for efficient viral replication (Neufeldt et al.
90 2018, Cortese et al. 2017, Mohd Ropidi et al. 2020). In the case of ZIKV, infection
91 could induce the rearrangement of F-actin in the cell periphery (Nie et al. 2021), and
92 enwrapping of dsRNA-positive structures by bundled microtubules (MTs) and
93 cytokeratin 8 and nestin IFs (Cortese et al. 2017). Perturbation of F-actin by
94 cytochalasin D or Jasplakinolide enhanced ZIKV infection (Nie et al. 2021), while
95 pharmacological treatment with MTs-stabilizing drug paclitaxel strongly reduced
96 ZIKV titer (Cortese et al. 2017). Despite rearrangement of vimentin has been
97 observed in ZIKV infection (Pagani et al. 2017), the dynamic changes of vimentin IFs
98 during ZIKV lifecycle and its contribution to RCs construction and integrity remain
99 understudied.

100

101 Besides exploiting cytoskeletal networks, ZIKV can hijack ER-resident proteins, such
102 as ER-localized RNA-binding proteins vigilin and ribosome-binding protein 1
103 (RRBP1), to facilitate viral genome replication and viral protein translation (Ooi et al.
104 2019). In addition to provide structural scaffold, there are evidence indicating that
105 cytoskeletal proteins may regulate translational apparatus (Kim and Coulombe 2010).
106 For instance, ribosomes can physically associate with MTs and F-actin in different
107 cells (Hamill et al. 1994, Medalia et al. 2002). Disorganization of F-actin by
108 cytochalasin D impairs local protein synthesis in isolated axoplasmic nerve fibers
109 (Sotelo-Silveira et al. 2008). The interaction between keratin IFs and β subunit of

110 eukaryotic elongation factor-1 (eEF1B β) plays an essential role in protein synthesis
 111 (Kim et al. 2007). MTs can bind to cytoplasmic tail of RRBPI and take part in ER
 112 organization and neuronal polarity (Ogawa-Goto et al. 2007, Farias et al. 2019).
 113 However, information on the spatial and functional relationship between vimentin IFs
 114 and the translational machinery, especially in the context of virus infection, remain
 115 incomplete.

116

117 In this study, we investigated the function of vimentin IFs in ZIKV infection. By
 118 monitoring spatial-temporal responses of cellular vimentin network throughout
 119 various steps of ZIKV life cycle, we demonstrated that ZIKV infection induces
 120 massive rearrangements of cytoplasmic vimentin. When vimentin protein was
 121 genetically depleted from cells, distribution of viral proteins is scattered within
 122 infected cells, and viral RNA replication, protein synthesis, and virion release are
 123 subsequently reduced. Using mass spectrometry and RNA sequencing analysis, we
 124 discovered interactions between vimentin and RNA-binding proteins, and vimentin
 125 binding to RRBPI facilitates ZIKV RNA replication. Thus, our work establishes
 126 important connections among vimentin filaments dynamics, ZIKV RCs, cellular
 127 RNA-binding proteins in highly effective infection.

128 **RESULTS**

129 **ZIKV infection induces drastic cellular vimentin rearrangement and formation** 130 **of cage-like structures**

131 To study whether host cytoskeletal proteins respond to ZIKV infection, we examined
132 vimentin network using an established model with human osteosarcoma cells (U2OS)
133 that express abundant cytoskeletal filaments and are highly susceptible to viruses (Jiu
134 et al. 2015, Rausch et al. 2017, Hackett et al. 2019) (Fig 1A,E). The cells were
135 infected with an ZIKV Asian lineage strain, SZ01, and fixed at different time points
136 post infection (hpi). Viral structural protein envelop (Env) and endogenous vimentin
137 were visualized by immunofluorescence. In mock infected cells, vimentin filaments
138 showed perinuclear localization and radiated towards cell periphery with apparent
139 filamentous structure (Fig 1A). However, the localization of vimentin rearranged
140 markedly and formed compact aggregation together with viral protein near nucleus,
141 without any notable changes on cell size and overall morphology, within 48 hpi (Fig
142 1A,B).

143
144 A time course study showed that viral RNA replication initiated from 8 hpi and
145 reached to a plateau at 36 hpi and onwards, concurrently, viral proteins start to
146 synthesize from 12 hpi and become more pronounced afterwards (Fig 1C,E). It was
147 evident from the quantification that both viral RNA appearance and protein synthesis
148 started before the virus-induced vimentin rearrangement, which was not yet initiated
149 at 16 hpi. The vimentin compartments only appeared to shrink at around 24 hpi,
150 progressively reached to a plateau at 36 hpi (Fig 1B-E). This suggests that vimentin
151 concentration is not required to initiate virus replication. Instead, the emergence of
152 viral proteins may act as a trigger for the vimentin rearrangements to occur.

153
154 At the protein levels, however, cytoskeletal components including actin, tubulin and
155 vimentin were similar between ZIKV infected and mock-infected cells throughout the
156 infection lifecycle (Fig 1E). Different from vimentin, neither actin nor tubulin

157 network show significant redistribution during ZIKV infection (Fig S1C), indicating a
158 vimentin-specific response to infection.

159

160 Considering vimentin reorganization usually accompanies the assembly and
161 disassembly of the filaments regulated by phosphorylation (Inagaki et al. 1987),
162 which modulates vimentin solubility (Snider and Omary 2014), we performed cellular
163 fractionation experiment and western blot analysis. Immunoblotting showed
164 negligible changes of vimentin content in both the cellular (soluble) and cytoskeletal
165 (insoluble) fraction, as well as the phosphorylation levels from 16 hpi onwards (Fig
166 S1A,B), suggesting that the drastic rearrangement of vimentin was not associated with
167 its assembly turnover or posttranslational modification. To further explore whether
168 microtubules (MTs) are the machinery responsible for vimentin transportation
169 towards nucleus, we applied two drugs inhibiting assembly of MTs and the activity of
170 its retro-wards motor dynein, respectively, and found that these drugs did not prevent
171 virus induced vimentin retraction, ruling out the possible involvement of
172 MT-dominated aggresomal transportation system (Fig S1D).

173

174 To describe the detailed dynamics of vimentin filaments rearrangement, we generated
175 a vimentin-mCherry stable expression cell line and performed live-cell imaging
176 analysis. A high MOI of input virus was used to achieve infection of every cell
177 recorded. In mock-infected cells, no apparent changes of vimentin were observed
178 within a 30 h monitoring period (Fig 1D). In contrast, vimentin filaments gradually
179 gathered around nucleus from ~20 hpi and progressively intensified in ZIKV-infected
180 cells (Fig 1D; Video S1,S2).

181

182 ZIKV multiplies in perinuclear replication compartments (RCs) (Welsch et al. 2009;
183 Caldas et al. 2020). In order to gain insight into the spatial relationship between
184 vimentin and viral protein, we took advantage of the three-dimensional-structured
185 illumination microscopy (3D-SIM) for super-resolution visualization. Side view of 3D

186 images discovered that vimentin filaments form a hollow cage-like structure that wrap
187 structural protein Env as well as nonstructural proteins NS1 and NS4B (Fig 1F; Fig
188 S1E). Furthermore, electronic microscopy elaborated events of enrichment of IFs-like
189 filaments next to the concentrated area of viral particles in the perinuclear region (Fig
190 1G; Fig S1G). Together, these imaging observations indicated that ZIKV infection
191 induced vimentin-cage formation around RCs.

192

193 **Exogenous expression of Zika viral protein leads to vimentin enrichment in the** 194 **perinuclear area**

195 To further dissect the role of viral protein in vimentin rearrangement, we transfected
196 vimentin-mCherry-expressing cells with moderate concentrations of EGFP tagged
197 Env protein and monitored vimentin dynamics. The results showed that during 1.5 h
198 real-time monitoring after overnight transfection, vimentin filaments and viral Env
199 protein accumulated next to nucleus synchronously (Fig 2A; Video S3). By tracing
200 the fluorescent intensity of perinuclear region, vimentin was found to be gathered
201 significantly faster (Fig 2B).

202

203 Moreover, the retrograde movement of vimentin filaments gather the scattered viral
204 protein, as visualized by individual fluorescent foci, to the perinucleus region (Fig 2C;
205 Video S4), indicating a potential for vimentin being a pro-viral factor. Together, these
206 results suggest that the cellular machinery recognizing foreign viral protein triggers
207 events leading to varied vimentin arrangement.

208

209 **Host vimentin is required for the integrity of viral replication compartments and** 210 **efficient infection**

211 Vimentin has gained more attention on its roles in various viral infections (Dohner
212 and Sodeik 2005, Foo and Chee 2015, Denes et al. 2018, Zhang et al. 2019, Ramos et
213 al. 2020, Wen et al. 2020, Zhang et al. 2020). To determine whether there is a causal
214 relationship between vimentin rearrangement and ZIKV infection, we used

215 CRISPR/Cas9 method to establish vimentin-knockout (KO) in U2OS cells and Huh7
216 (Fig S2A,D). Results showed that reducing vimentin levels neither affected the cell
217 viability nor cell growth (Fig S2B,C,E). We next used lentiviral system to establish
218 vimentin KO-full length (FL) rescue cells, and verified the levels of vimentin protein
219 express in these cells by western blot (Fig S2A).

220

221 In these cells, we observed not only similar vimentin network shrinkage and
222 formation of a concrete compartment for viral RCs, but also viral protein localization
223 at a subcellular level (Fig 3A). Intriguingly, viral protein staining for Env, NS1 and
224 NS4B displayed a piecemeal distribution in vimentin KO cells (Fig 3A, Fig S2I,J), but
225 rarely seen in wild-type cells. Moreover, depletion of vimentin dramatically increases
226 the proportion of partitioned cellular viral protein compartments up to 85% and
227 reduces the total viral protein area, both of which can be fully rescued when
228 full-length vimentin was reintroduced (Fig 3A-C). Nevertheless, vimentin which
229 forms unit length filaments (ULF) or squiggles was not able to fully rescue the viral
230 dispersion phenotype observed in infected cells (Fig 3A,B).

231

232 How this scattered subcellular distribution of viral protein influences the infectivity?
233 To address this, we subsequently measured viral genome replication by qRT-PCR and
234 viral protein expression by western blot in vimentin KO cells. It is apparent that the
235 absence of vimentin caused significant reduction of viral RNA copies and structural
236 protein Env levels at both 24 and 48 hpi (Fig 3D,E), and reintroducing vimentin
237 partially rescued the viral protein expression (Fig 3E). Virus titer was further
238 measured and as expected, there was at least 20-fold less of viruses released from
239 vimentin depleted cells at 48 hpi (Fig 3F). Similarly, in Huh7 cells, vimentin depletion
240 showed defective infection as that in U2OS cells (Fig S2F-H).

241

242 By immunostaining and flow cytometry, fewer infected cells were detected in
243 vimentin depletion background at 24 and 48 hpi, the fast replication period during

244 viral life cycle (Fig 3G,H), suggesting that vimentin is critical for efficient ZIKV
245 infection. Taken together, depletion of host vimentin leads to compromised viral
246 infection as demonstrated by less genome replication, reduced protein expression, and
247 fewer particle production, most probably as a result of the disruption of the integrity
248 of concentrated perinucleus RCs.

249

250 **Vimentin depletion compromises viral genome and protein synthesis without** 251 **affecting viral binding and entry to the host cell**

252 To further investigate the function of vimentin, a time course of infection experiment
253 was performed. Changes of viral RNA copies in wild-type and vimentin KO cells
254 were confirmed by qRT-PCR. Although the viral RNA numbers were the same at the
255 beginning of infection, their copies in vimentin KO cells were significantly lower than
256 that in wild-type cells during the later period of infection (Fig 4A). Apart from the
257 replication dynamics of viral genome, viral proteins in both backgrounds accumulated
258 gradually, but vimentin depletion led to a slower accumulation and thus much less
259 viral Env protein expression at each time point from 16 hpi onwards (eg. 20, 24, 36,
260 48 hpi), compared to wild-type cells (Fig 4B).

261

262 The observed low levels of viral Env protein expression in vimentin KO cells could
263 be due to a decrease in protein biogenesis and/or an increase in protein degradation.
264 We therefore tested whether vimentin could influence the stability of ZIKV proteins.
265 Plasmid expressing Env-EGFP fusion protein was transiently transfected into cells
266 and analyzed by western blot 48 h later. The results showed that vimentin depletion
267 dramatically reduced the exogenous expression of Env-EGFP (Fig 4D-F). We next
268 treated cells with cycloheximide (CHX, 20 µg/mL for 11 h) to inhibit protein
269 translation, and found that viral Env was relatively stable in both wild-type and
270 vimentin KO cells (Fig 4D). These suggest that vimentin acts on protein biosynthesis
271 but not degradation. The following experiments further confirmed this assertion.

272 There are two classic destinations for cellular protein degradation regulated by
 273 proteasomal and lysosomal pathways. To further analyze whether depletion of
 274 vimentin promotes the degradation of viral protein and via which way, cells
 275 transfected with Env-EGFP were treated with proteasomal specific inhibitor MG132
 276 (10 μ M for 11 h), or lysosomal specific inhibitor ammonium chloride (NH₄Cl) (25
 277 mM for 11 h) (Fig 4C). Immunoblot analysis showed that the Env-EGFP protein level
 278 apparently increased in both wild-type and vimentin depletion cells after MG132
 279 treatment (Fig 4E), but not influenced by NH₄Cl application (Fig 4F). Concurrently,
 280 p21 and LC3, known proteins for proteasome and lysosomal degradation, respectively,
 281 were chosen as positive controls for these experiments. Combing results of the
 282 increased Env upon MG132 treatment and unaltered Env upon NH₄Cl treatment, it is
 283 reasonable to conclude that Env protein was degraded mainly via proteasomal
 284 pathway and vimentin depletion does not interrupt the degradation of Env protein.

285

286 Given that vimentin shrinking was visualized from 24 hpi but not before, we
 287 speculated that the early steps of viral replication were not affected in vimentin
 288 depletion cells. To confirm this, we used two assays to examine binding and entry
 289 steps (Fig 4H), respectively. Cells were infected by ZIKV for 1 h at 4°C, directly or
 290 incubated for 1 more hour at 37°C, washed and then cell lysates collected for
 291 measurement of RNA copies by RT-qPCR (Fig 4G). The results confirmed that both
 292 the binding and entry of ZIKV are similarly efficient in wild-type and vimentin KO
 293 cells (Fig 4H). Collectively, these results suggest that vimentin acts on the production
 294 and accumulation of ZIKV proteins as well as the efficient replication of ZIKV RNA,
 295 without influencing viral internalization.

296

297 **A large number of host RNA-binding proteins interact with and being regulated** 298 **by vimentin during ZIKV infection**

299 Since the perinuclear expression of vimentin is highly associated with ER where viral
 300 RNA replication and protein translation take place, we speculate that aside from being

301 a structural scaffold, vimentin may interact with host factors that regulate viral RNA
 302 transcription and subsequent protein synthesis. To test this, we implemented mass
 303 spectrometry (MS) analysis to identify proteins interacting with vimentin, and
 304 subjected 1050 candidates to gene ontology (GO) analyses by DAVID (The Database
 305 for annotation, visualization and integrated discovery). The recognized vimentin
 306 interactors were classified based on three taxonomic features including molecular
 307 function, cellular component and biological process. From the classification analysis,
 308 we found that a large proportion of candidates interacted with vimentin are RNA
 309 binding proteins and ribosome components (Fig 5A), and they are intimately related
 310 to RNA processing, translation and viral transcription (Fig 5B). Importantly, GO
 311 annotation revealed a significant enrichment of ER components with vimentin (Fig
 312 5C), indicating that vimentin is the principal factor that interacts with ER-associated
 313 RNA-binding proteins (RBPs) in host cells.

314

315 To complement with the interactome assay, RNA sequencing (RNA-Seq) was
 316 subsequently applied to analyze the variations of global gene transcription in both
 317 wild-type and vimentin KO cells infected (or not) with ZIKV. A total of 1408 genes
 318 were significantly affected ($P < 0.05$), including 518 up- and 890 down- regulated
 319 genes in vimentin depletion background upon ZIKV infection (Fig S3C). Among the
 320 downregulated hits, there are many ER-localized genes related to double-strand RNA
 321 binding and transcription regulation (Fig S3A,B), as well as a large proportion of
 322 genes involved in antiviral immune and inflammatory response (Fig 5D). Together
 323 with the MS result, we conclude that aside from contributing to RCs scaffolding
 324 which provides a structural support, vimentin is also involved in processing viral
 325 RNA replication and serving a functional role.

326

327 Given the critical role of ER in ZIKV infection, we more carefully examined
 328 candidates with ER localization in both MS and RNA-Seq results (Fig 5E,F). By
 329 investigating ER annotated candidates, we obtained top 15 vimentin-interacting

proteins by setting the threshold of interacting peptides coverage over 30% from MS results (Fig 5E; Table S1). Concurrently, RNA-Seq data were analyzed and the results demonstrated dramatical changes of the mRNA levels of many ER-associated genes, among them, 80% of significantly differentially expressed genes were downregulated (Fig 5F). By crosschecking MS and RNA-Seq candidates, ER-resident protein ribosome-binding protein 1 (RRBP1, also known as p180) was recognized as the only common hit, which shows high endogenous expression in wild-type and substantially down-regulated expression in vimentin KO cells during ZIKV infection (Fig 5F). Of note, it has been recently reported that RRBP1 play a pronounced role in flaviviruses (eg. DENV, ZIKV) infection by directly binding to viral RNA (Ooi et al. 2019). We thus focused on RRBP1, and asked how the interaction between vimentin and RRBP1 influence ZIKV infection.

342

343 **ER-resident RRBP1 interacts with and regulated by vimentin to catalyze ZIKV** 344 **infection**

RRBP1 contains a short ER luminal domain, a transmembrane domain, and a large cytoplasmic domain containing tandem repeats motif (Reid and Nicchitta 2015) (Fig 6A). RRBP1 peptides interacting with vimentin identified from MS were located mostly in the cytoplasmic coiled-coil region (25 out of 28 recognized peptides) (Fig 6A, Table S2). We subsequently performed the pull-down assay by using purified His-tagged vimentin protein with an RRBP1 antibody as probe. The results confirmed that RRBP1 indeed interacts with vimentin both *in vivo* and *in vitro* (Fig 6B).

352

Next, we explored the correlation between the cellular expression of vimentin and RRBP1 upon ZIKV infection. Immunofluorescence results showed that in wild-type cells, both vimentin and RRBP1 are accumulated around nucleus where ER network resides (Fig 6C). Upon ZIKV infection, both vimentin and RRBP1 aggregated near the nucleus and co-localized with dsRNA-staining positive viral RNA (Fig 6C; Fig S4D), indicating both of them have participated in the viral RCs process.

359 To elucidate the relationship between vimentin and RRBPI, we compared the RNA
 360 level, protein level and the localization of RRBPI in wild-type and vimentin KO cells.
 361 Results showed that depletion of vimentin reduced both the mRNA and protein levels
 362 of RRBPI to around 50%, and enlarged the cellular distribution of RRBPI (Fig 6D,E;
 363 Fig S4A-C,E). In contrast, deprivation of RRBPI neither affect the mRNA nor the
 364 protein level of vimentin (Fig 6G; Fig S4F). Imaging data showed that lacking
 365 vimentin led to an expansion of the subcellular RRBPI in uninfected cells (Fig 6C).
 366 Consistently, upon ZIKV infection, RRBPI turned into scattered segregation in
 367 vimentin KO cells, similar as segregated viral proteins (Fig 3A; Fig 6C), indicating
 368 that disruption of RCs integrity by vimentin influenced not only viral components but
 369 also host viral-binding components. It is subsequently noted that the colocalization
 370 between RRBPI and viral dsRNA were significantly reduced (Fig 6C,F), suggesting
 371 that vimentin depletion reduced the combined abundance of viral-host constituents in
 372 RCs. Therefore, these results suggested that RRBPI is one of the effectors of vimentin
 373 in both normal and infection conditions, through which ZIKV replication was
 374 affected.

375

376 To verify this, we generated RRBPI knockdown (KD) cells in both wild-type and
 377 vimentin KO background by shRNA (Fig 6G; Fig S4E,F) and then infected them with
 378 ZIKV. Consistent with a previous study (Ooi et al. 2019), RRBPI KD reduced viral
 379 RNA copies by about 40% (Fig 6H). In comparison, the effect of vimentin depletion
 380 on the reduction of viral genome replication was much more severe than that of
 381 RRBPI KD; however, the double depletion has no additive effects (Fig 6H),
 382 suggesting that RRBPI and vimentin located in the same regulating cascade during
 383 ZIKV infection. Next, synthesized viral protein was analyzed. The absence of
 384 vimentin resulted in significant reduction of structural protein Env, but the absence of
 385 RRBPI alone showed no obvious effect (Fig 6I), indicating that RRBPI plays a role
 386 in viral RNA replication rather than protein synthesis. Taken together, these results
 387 demonstrate a pivotal role of vimentin as an upstream factor regulating one of the

388 known viral-binding host factor RRBP1 to affect viral RNA replication. Combined
 389 with omics analysis, these data further suggest that aside from contributing to
 390 scaffolding RCs, vimentin also acts as a hub molecule to interact with and regulate
 391 various RBPs. Both functions of vimentin are important for facilitating efficient ZIKV
 392 replication and promoting infection.

393 **DISCUSSION**

394 In this study, we report the spatial-temporal rearrangement of vimentin filaments
395 induced by ZIKV infection. Importantly, we reveal two major functions of host cell
396 vimentin during ZIKV infection: (1) ‘organizer’, as a structural support to increase the
397 local concentration of all necessary factors for high efficiency of viral replication; (2)
398 ‘regulator’, by interacting with and regulating RNA-binding proteins, such as RRBPI,
399 to facilitate viral replication. The latter is the first time that vimentin has been
400 demonstrated to play a non-scaffold function in the context of virus infection.

401

402 **Vimentin cage formation in viral infections**

403 Viruses replicate more efficiently within the viral replication compartments (RCs), but
404 the structural features of such a compartment remain elusive. Our current study uses
405 ZIKV to examine this issue. ZIKV infection induces nestin, one of the intermediate
406 filaments (IFs) in neural stem and progenitor cells, to restructure in the perinuclear
407 region to wrap around viral dsRNA (Cortese et al. 2017). Whether vimentin IFs that
408 expressed in most cell types with greater abundance have a role in ZIKV infection
409 have not been explored. Taking advantage of imaging techniques, we found that ZIKV
410 infection led to vimentin remodeling and formation of cage-like structures that
411 surround the RCs, long after the initiation of viral RNA replication, but following
412 more closely to viral protein production (Fig 1C,E). This kinetic association between
413 host vimentin and viral products has been demonstrated by several lines of evidence:
414 (1) the appearance of vimentin shrinking was approximately 16 h from the
415 commencement of infection, coincides with the period of exponential increase of viral
416 RNA replication (Fig 1C). (2) vimentin filaments gradually accumulated to the site of
417 viral protein synthesis or RCs, to eventually form a whole cage (Fig 1A). (3) the cages
418 could also be formed when cells started to synthesis new viral proteins (Fig 2A,C).
419 Finally, (4) cage formation was not observed in cases where only EGFP vectors were
420 expressed, or medium without live viruses was included (Fig S1F). Despite unable to
421 prove a cause-and -effect mechanism as yet, the above data strongly suggest a link

422 between ZIKV infection and vimentin function.

423

424 In agreement with our data, vimentin rearrangement has been observed in other
425 experimental models of infection. In cells with DENV-2 infection, vimentin has
426 increased the interaction with viral NS4A protein, and vimentin filaments gradually
427 moved towards the nucleus, and finally formed cage structures at around 48 hpi (Teo
428 and Chu 2014). In SARS-CoV-2 infection, the beginning of vimentin retraction falls
429 within the timeframe (around 6.5 hpi) of genome replication (Cortese et al. 2020).
430 Both the time of appearance and the co-localization in the perinuclear area for the
431 replication-assembly organelles, leading us to interpret that vimentin sensed viral
432 replication and formed a scaffold to facilitate viral replication.

433

434 **The underlying mechanisms of vimentin cage formation in viral infections**

435 Several mechanisms have been postulated for vimentin rearrangement during viral
436 infection. One suggests that vimentin rearrangement is due to its phosphorylation on
437 specific sites by viral infection-induced kinase activation. For example, DENV-2
438 infection can induce Rho-associated protein kinase (ROCK) activation and
439 phosphorylation of vimentin at serine 72 (Ser72) (Lei et al. 2013), as well as
440 calmodulin-dependent protein kinase II (CaMKII) activation and phosphorylation of
441 vimentin at Ser39 (Teo and Chu 2014). AFSV replication results in the activation of
442 CaMKII and phosphorylation of vimentin at Ser83 (Stefanovic et al. 2005, Netherton
443 and Wileman 2013). In contrast, we found the phosphorylation levels of vimentin at
444 Ser39, Ser56, Ser83 as well as the soluble/insoluble ratio of vimentin have no
445 significantly fluctuation during ZIKV infection (Fig S1A,B), indicating the
446 phosphorylation mechanism for vimentin reorganization may vary among viruses, and
447 ZIKV induced vimentin remodeling is not through phosphorylation at least on these
448 sites nor the solubility regulation.

449

450 Aside from phosphorylation, crosslinking of neighboring vimentin subunits through

451 cysteine residue at cysteine 328 (Cys328) in response to oxidative stress can result in
 452 reorganization of vimentin network (Perez-Sala et al. 2015). Whether vimentin
 453 Cys328 plays a similar role in respond to massive virus replication remains uncertain
 454 (Ledur et al. 2020).

455

456 Another mechanism is related to the aggresome processing machinery including
 457 dynein, dynactin, and microtubules (MTs). For instance, the capsids of influenza A
 458 virus (IAV), with the help of aggresome processing machinery, can mimic misfolded
 459 protein aggregates and start uncoating in the cytoplasm (Banerjee et al. 2014).
 460 Inhibition of dynein-dependent transport by overexpression of p50 (also known as
 461 dynamitin) block the recruitment of vimentin to the microtubule organizing center
 462 (MTOC) during ASFV infection (Stefanovic et al. 2005). Nevertheless, we found that
 463 neither chemical inhibition of dynein by cilibrevin D nor inhibition of MTs by
 464 nocodazole has influenced the perinuclear aggregation of vimentin and Env protein
 465 during ZIKV infection (Fig S1D).

466

467 Overall, our results exclude the above mechanisms of vimentin reorganization during
 468 ZIKV infection, negating the role of phosphorylation modification or aggresomal
 469 machinery. Therefore, further work is needed to unravel the molecular mechanisms of
 470 vimentin filaments rearrangement in ZIKV infections.

471

472 Finally, direct interaction between viral protein and cellular vimentin may also trigger
 473 the formation of vimentin cage. For instance, human immunodeficiency virus 1
 474 (HIV-1) protease can cleave vimentin bundle and then induce accumulated
 475 perinuclear localization of vimentin filaments (Shoeman et al.1990, Shoeman et al.
 476 2001, Honer et al. 1991). In the case of DENV-2 and FMDV infections, vimentin
 477 interacts with nonstructural protein DENV-2 NS4A and FMDV 2C, respectively,
 478 concurrent with the formation of vimentin cage (Gladue et al. 2013; Teo and chu
 479 2014). Our results are more consistent with the interpretation that ZIKV proteins may

480 directly or indirectly interact with vimentin, which cooperates with other host factors,
481 causing the formation of vimentin cage.

482

483 **The need of vimentin in viral infections**

484 In line with the hypothesis that the cytoskeleton cage observed in ZIKV infection
485 might contribute to spatially concentration of different viral-induced membranous
486 structures (Cortese et al. 2017), we demonstrated that without the cage formation after
487 vimentin depletion, RCs are miss-organized and segregated in the cytoplasm (Fig 3A),
488 leading to less efficient synthesis of viral components, and lower overall infection
489 efficiency (Fig 3A,D-H; Fig 4A,B). In DENV-2 infection, the RCs become diffused
490 throughout the cytoplasm when vimentin was knockdown by siRNA (Teo and Chu
491 2014). Differently, we witnessed that ZIKV RCs are scattered into lumps rather than
492 evenly diffused as in DENV-2 case, indicating a distinct dispersion feature regulated
493 by vimentin upon ZIKV infection. Moreover, disruption of vimentin filaments by a
494 drug Acrylamide significantly reduced the release of both bluetongue virus (BTV) and
495 DENV-2 (Bhattacharya et al. 2007, Kanlaya et al. 2010). Treatment with Withaferin A,
496 a compound that disrupts vimentin network, resulted in a significant reduction in
497 SARS-CoV-2 replication and virion released (Cortese et al. 2020). Thus, it may be a
498 common function in viral infection that vimentin cage organizes the replication
499 structures and provides an optimal niche for the replication/translation to occur.

500

501 The decreased viral protein level within cells may be due to the reduced production or
502 increased degradation. Vimentin has been previously shown to regulate the
503 proteasomal degradation of HCV core protein to affect HCV production
504 (Nitahara-Kasahara et al. 2009). In contrast, by treatment with protein synthesis and
505 degradation inhibitors, we showed that ZIKV Env biogenesis, but not its stability, was
506 reduced in vimentin depleted cells (Fig 4C-F). Thus, why vimentin acts differently in
507 different viral infections remain to be determined.

508

509 **The non-structural role of vimentin in viral infections**

510 The ER is an essential cellular compartment for completion of the virus life cycle.
 511 During ZIKV replication, there is an accumulation of viral components in the ER
 512 (Mohd Ropidi et al. 2020), and viral nonstructural proteins can be incorporated into
 513 ER membrane to create invagination or protrusion vesicles for viral RNA replication
 514 (Neufeldt et al. 2018).

515

516 Since viruses cannot encode all proteins necessary for their life cycle, they usurp
 517 cellular protein biogenesis machineries such as ribosomal proteins (Campos et al.
 518 2017), RNA-binding proteins (RBPs) for viral RNA replication/transcription
 519 (Garcia-Moreno et al. 2018, Dicker et al. 2020, Diosa-Toro et al. 2020), and formation
 520 of ER membrane protein complex (EMC) (Barrows et al. 2019, Lin et al. 2019).
 521 Noncoding subgenomic flavivirus RNA (sfRNA) produced by ZIKV can interact with
 522 over 20 RBPs to regulate multiple cellular post-transcriptional processes and therefore
 523 limit effective response of these cells to viral infection (Michalski et al. 2019, Jansen
 524 et al. 2021). 464 RBPs was identified being associated with DENV or ZIKV gRNAs,
 525 including previously reported candidates (eg. heterogeneous nuclear
 526 ribonucleoproteins (hnRNPs), polyadenylate-binding protein (PABP)) that specifically
 527 associate with DENV RNA (Phillips et al. 2016, Viktorovskaya et al. 2016, Ooi et al.
 528 2019, Scaturro et al. 2019), and recently known RBPs vigilin and RRBPI which were
 529 reported to directly bind to DENV and ZIKV RNA (Ooi et al. 2019).

530

531 In line with these discoveries, using mass spectrometry and RNA sequencing analysis,
 532 we revealed that vimentin interacts with a large number of RBPs and ribosomal
 533 proteins to regulate cellular transcription and translation, enabling efficient ZIKV
 534 replication (Fig 5A-F). Considering the interplay between cytoskeleton and ER
 535 membrane (Terasaki et al. 1986, Risco et al. 2002, Gurel et al. 2014, Zhang 2020), it
 536 is tempting to speculate that virus-induced vimentin cage not only provides physical
 537 space for viral RCs accumulation, but equally important, interacts with molecules

involved in cellular transcription and translation process and thus promotes the efficiency of virus replication from perspectives of both physical support and functional control.

541

542 **The interaction between vimentin and RRBPI**

Among the numerous candidates, we focused on the interaction between vimentin and RRBPI, a positively charged membrane-bound protein found in rough ER (Cui et al. 2012), because: (1) RRBPI was identified as the top candidates in both mass spectrometry and RNA sequencing examinations. (2) RRBPI could directly bind viral RNA and play pronounced role during DENV and ZIKV infection (Ooi et al. 2019).

548

A previous study has identified that RRBPI could mediate the interaction between ER and MTs via the novel MT-binding and -bundling domain MTB-1 of coiled-coil region of RRBPI (Ogawa-Goto et al. 2007). Overexpression of MTB-1 induced acetylated MTs and promoted MT bundling (Ogawa-Goto et al. 2007). Moreover, RRBPI also regulates ER organization and controls axon specification by regulating local MTs remodeling (Farias et al. 2019). Aside from the interplay with MTs, our data demonstrate that RRBPI colocalizes with ZIKV dsRNA, and knockdown of RRBPI in wild-type cells reduces ZIKV RNA replication (Fig 6C,H).

557

Significantly, our results are the first to identify that vimentin can directly bind to RRBPI and influence its cellular localization and expression level in both mock-infected and ZIKV infected cells. This represents an improved understanding of the interplay between cytoskeletal IFs and ER proteins, especially in the context of virus infection. Of note, RRBPI depletion has less effect on viral replication than that of vimentin depletion (Fig 6H,I), and RRBPI expression has no significant influence on the cellular distribution, mRNA and protein expression of vimentin, indicating RRBPI acts through vimentin during ZIKV infection. Further investigation is needed to determine whether other RNA-binding proteins may cooperate with vimentin to

567 modulate ZIKV replication. Identification of these factors not only benefits the
568 characterization of the biogenesis of ZIKV RCs, but also provides potential
569 candidates for developing broad spectrum compounds that restrict viral replication.

570

571 **MATERIA AND METHODS**

572 **Cell culture and virus**

573 Hepatoma Huh7 cells, human osteosarcoma (U2OS) cells and African green monkey
574 kidney epithelial Vero cells were cultured at 37 °C with 5% CO₂ in Dulbecco's
575 modified Eagle's medium (DMEM) (Biological Industries) supplemented with 10%
576 fetal bovine serum (FBS) (Gibco), 1% penicillin and streptomycin. C6/36 cells were
577 cultured in minimum essential medium (MEM) (Gibco) supplemented with 10% FBS
578 and 2% non-essential amino acids (Solarbio, N1250-100) at 28 °C in 5% CO₂. ZIKV
579 strain SZ01 was used in this study (GneBank: KU866423.2). Virus stocks were
580 prepared by virus amplification in C6/36 cells at a multiplicity of infection (MOI) of
581 0.1. Virus-containing supernatant medium were harvested from day 4 post infection
582 and stored at -80 °C. For lentivirus production, the pLKO.1 shRNA plasmid was
583 transfected into HEK293T cells together with psPAX2 packaging plasmid (Addgene
584 #12260) and pMD2.G envelop plasmid (Addgene #12259) by using FuGENE HD
585 (Promega). Supernatants were collected 48 hours post-transfection, filtered through a
586 0.45 µm filter to remove the cells debris and stored at -80 °C. The effectiveness of
587 knockdown of target gene was assessed by qRT-PCR and western blotting.

588

589 **Plasmids and transfection**

590 Constructs expressing mCherry-tagged full-length vimentin and GFP-tagged 'unit
591 length filament' (ULF) vimentin were kind gifts from John Eriksson (University of
592 Turku and Abo Akademi University, Finland). Plasmids encoding ZIKV envelop was
593 amplified by reverse transcription-PCR (RT-PCR) and cloned into the pEGFP-N1
594 vector. All constructs were verified by DNA sequencing. The PCR primers used in
595 this study are summarized in Table S3. Transfection of plasmids at indicated
596 concentrations were performed using jetPRIME transfection reagent (#114-15)
597 following the manufacturer's instructions.

598

599 **Vimentin CRISPR knockout cell line generation**

As previous, vimentin-knockout cells were generated using CRISPR/Cas9 methods (Jiu et al. 2015) based on pSpCas9 (BB)-2A-GFP vector (Addgene #48138) with two targets. Primers for vimentin target 1 were 5'-CACCGTGGACGTAGTCACGTAGCTC-3' and 5'-AAACGAGCTACGTGACTACGTCCAC-3'. Primers for vimentin target 2 were 5'-CACCGCAACGACAAAGCCCGCGTCG-3' and 5'-AAACCGACGCGGGCTTTGTCTGTTGC-3'. Transfected cells were detached at 24 h post-transfection and sorted with FACS Aria II (BD Biosciences) using low intensity GFP-expression pass gating, and then cells were plated onto 96-well plate supplemented DMEM containing 20% FBS and 10 mM HEPES with single cell/well. CRISPR clones were cultivated for two weeks prior selecting clones with no discernible vimentin protein expression by western blotting.

612

613 **Immunofluorescence microscopy**

Cells cultured on glass slides (VWR, #631-0150) were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT), and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then blocked in PBS supplemented with 5% bovine serum albumin (BSA) (ABCONE, #A23088). Both primary and fluorescent-conjugated secondary antibodies were applied onto cells and incubated at RT for 1 h. Cells were mounted in DAPI Fluoromount-G reagent (SountherBiotech, 0100-20) and imaged using either Olympus spinSR10 Ixlore spinning disk confocal microscope or GE DeltaVision OMX SR super-resolved structured illumination microscope. The following primary antibodies were used: vimentin rabbit monoclonal D21H3 antibody (dilution 1:100; #5741, Cell Signaling); vimentin chicken polyclonal antibody (dilution 1:1000; #ab24525, Abcam); tubulin mouse monoclonal antibody (dilution 1:200; #4026, Sigma); ribosome-binding protein 1 (RRBP1) rabbit polyclonal antibody (dilution 1:100; #A303-996A, Bethyl Laboratories); Zika virus envelop mouse monoclonal antibody (dilution 1:1000; #1176-46, BioFront); Zika virus NS4B rabbit polyclonal antibody (dilution 1:1000; #GTX133311, Genetex);

629 Zika virus NS1 mouse monoclonal antibody (dilution 1:1000; #1225-06, BioFront);
 630 dsRNA monoclonal antibody (dilution 1:500; #J2-1909, SCICONS). The following
 631 secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit IgG (H+L) (dilution
 632 1:1000; #A11008, Invitrogen); Alexa Fluor 568 goat anti-rabbit IgG (H+L) (dilution
 633 1:1000; #A11011, Invitrogen); Alexa Fluor 488 goat anti-mouse IgG (H+L) (dilution
 634 1:1000; #A11001, Invitrogen); Alexa Fluor 555 goat anti-mouse IgG (H+L) (dilution
 635 1:1000; #A21422, Invitrogen). F-actin was stained by Alexa Fluor 647 phalloidin
 636 (dilution 1:500; #A22287, Invitrogen).

637

638 **Live cell imaging**

639 Cells were seeded into 35 mm-diameter glass bottom culture dish (MatTek
 640 Corporation) pre-coated by fibronectin (1.5 $\mu\text{g}/\text{cm}^2$) (Sigma-Aldrich, F2006). Cells
 641 were then infected with ZIKV (MOI=5 pfu per cell) for 2 h at 37°C. After removing
 642 the inoculum, 2 mL DMEM containing 2% FBS was added for imaging. Alternatively,
 643 cells were transfected with ZIKV-E-EGFP for 20 h before imaging. Image series were
 644 acquired on an Olympus spinSR10 Ixplorer spinning disk confocal microscope using a
 645 100× U plan apochromat high resolution objective with NA=1.5, with time interval of
 646 10 min for 40 h in infection experiments and 13 s for 1.5 h in transfection experiments,
 647 respectively. For figure 2C, vimentin knockout cells were co-transfected with
 648 ZIKV-E-EGFP and vimentin-mCherry plasmids for 24 h, and image series were
 649 acquired on GE DeltaVision widefield microscope using 60× UPlanXApo objective
 650 with NA=1.42. Image acquisition was performed at time interval of 30 min for 12 h.
 651 All live cell imaging data were further analyzed by Imaris 9.2 (Bitplane, Zurich,
 652 Switzerland) and ImageJ software.

653

654 **Western blotting**

655 Cells were washed two times with PBS and lysed in RIPA lysis buffer (Beyotime,
 656 #P0013B) with protease and phosphatase inhibitors (Beyotime, #P1045). Protein
 657 concentration were measured by BCA (Beyotime, #P0010), adjusted with PBS and

658 6X SDS-sample buffer (Beyotime, #P0015F), and subjected to SDS-PAGE. 5%
659 non-fat milk (BD Difco, #8011939) was used in blocking and PVDF membrane
660 (Millipore, #IPVH00010) was washed by TBST buffer (Tris-buffered saline, 0.1%
661 Tween20). Antibodies were used with the following dilutions in primary antibody
662 dilution buffer (Beyotime, P0023A): vimentin rabbit monoclonal D21H3 antibody
663 (dilution 1:1000; #5741, Cell signaling); phospho-vimentin (Ser39) rabbit antibody
664 (dilution 1:1000; #13614S, CST); phospho-vimentin (Ser56) rabbit antibody (dilution
665 1:1000; #3877S, CST); phospho-vimentin (Ser83) (D5A2D) rabbit antibody (dilution
666 1:1000; #12569S, CST); β -tubulin mouse monoclonal antibody (dilution 1:1000;
667 #T4026, Sigma); β -actin mouse monoclonal antibody (dilution 1:1000; #A5441,
668 Sigma); Zika virus envelop mouse monoclonal antibody (dilution 1:5000; #1176-46,
669 BioFront); ribosome-binding protein 1 (RRBP1) rabbit polyclonal antibody (dilution
670 1:1000; #A303-996A, Bethyl Laboratories); green fluorescent protein (GFP) mouse
671 monoclonal antibody (dilution 1:1000; #G6795, Sigma); p21 Waf1/Cip1 (12D1)
672 rabbit monoclonal antibody (dilution 1:1000; #2947, CST); LC3C (D3O6P) rabbit
673 monoclonal antibody (dilution 1:1000; #14736, CST); GAPDH rabbit monoclonal
674 antibody (dilution 1:5000; #G8795, Sigma). Horseradish peroxidase (HRP)-linked
675 anti-mouse IgG antibody (dilution 1:5000; #7076V, CST) and HRP-linked anti-rabbit
676 IgG antibody (dilution 1:5000; #7074V, CST) were used and chemiluminescence was
677 measured after using western blotting ECL (Tanon, #180-501). The band intensities of
678 blots were measured by ImageJ software. For quantification, the intensities of
679 interested proteins were normalized with the internal control GAPDH, and mock
680 infected cells were set to 1 in each experiment.

681

682 **Real-time RT-PCR**

683 Total cellular RNA was extracted by EZ-press RNA Purification Kit (EZBioscience,
684 #B0004DP) according to the manufacturer's protocols. Total RNA was reverse
685 transcribed by using Color Reverse Transcription Kit (EZBioscience, #A0010CGQ).
686 Real-time RT-PCR was carried out by using 2 $\times$ Color SYBR Green qPCR Master

687 Mix (ROX2 plus) (EZBioscience, #A0012-R2) in QuantStudio 1 system (Thermo).
 688 All readings were normalized to the level of GAPDH. The primers used for real-time
 689 RT-PCR are shown in Table S1.

690

691 Plaque assay

692 Zika virus titers were determined by plaque assay performed on Vero cells. Briefly,
 693 Vero cells were seeded into 24 well plates at a density of 1×10^5 cells/well and washed
 694 with pre-warmed phosphate-buffered saline (PBS). Cells were then infected with
 695 serial 10-fold dilutions of virus supernatants for 2 h at 37 °C with 5% CO₂. Inoculum
 696 was removed and replaced with DMEM containing 1% carboxymethylcellulose
 697 (CMC) (Sigma, #C5678) and 1.5% FBS. After four days post-infection, cells were
 698 washed with PBS and fixed with 4% PFA at RT for 1 h, followed by staining with
 699 crystal violet (Beyotime, C0121) for 10 min. After rinsing with water, the number of
 700 visible plaques was counted, and the virus titers were shown as plaque forming units
 701 (PFU) per milliliter.

702

703 Pull-down assay

704 For preparing the medium, Ni Sepharose 6 Fast Flow (Sigma-Aldrich, GE17-5318-01)
 705 was sedimented by centrifugation at 500×g for 5 min, then washed with distilled
 706 water and binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole,
 707 pH 7.4, filtration through a 0.45 µm filter) for twice, and further resuspended with an
 708 appropriate volume of binding buffer to make a 50% slurry. For binding the sample, 5
 709 µg of His-tagged recombinant vimentin (Sino Biologicals, #10028-H08B) were
 710 incubated with 10 µL of the 50% slurry at 4 °C on a shaker with low speed for 3 h.
 711 Beads were spun down by centrifugation at 500×g for 5 min and washed with cold
 712 binding buffer, then incubated with 1000 µg of filtered whole-cell lysates in 1×
 713 Lysis/Binding/Wash buffer (Cell signaling, #11524S) at 4 °C on a rotator with low
 714 speed overnight. For elution, Beads were spun down and washed with cold binding
 715 buffer for three times, then incubated with cold elution buffer (20 mM sodium

phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4, filtration through a 0.45 µm filter) at 4 °C on a shaker at low speed overnight. The supernatant was boiled in SDS loading buffer (Beyotime, #P0015F) and subjected to SDS-PAGE, followed by western blotting.

Mass spectrometry

Cells were lysed with NP40 buffer (50mM pH8.0 Tris-HCl; 150mM NaCl; 0.5%NP40; 1mM EDTA; protease Inhibitor) on ice for 15 min. Scrape cells and harvest the supernatant by centrifuge at 13,000 rpm for 10 min at 4 °C. Then proceed immunoprecipitation according to the protocol of Dynabeads Protein G (Thermo Fisher, #10004D). Briefly, incubate Dynabeads Protein G with anti-vimentin antibody (Abcam, #ab137321) with rotation for 30 min at RT. Place the tube with Dynabeads-Ab complex on the magnet to remove the supernatant. Then add the cell supernatant and incubate with rotation for 30 min at RT or overnight at 4 °C to allow antigen to bind to the beads-Ab complex. Wash the Dynabeads-Ab-Ag complex 3 times. Then elute target antigen with SDT buffer (4%SDS; 100mM pH8.0 Tris-HCl; 1mM DTT) for further Mass spectrum analyses by Q Exactive (Thermo Fisher).

RNA Sequencing and transcriptome analysis

RNA was extracted from U2OS WT or vimentin KO cells after infection with ZIKV (MOI=1) for 24 h, using TRIzol™ Reagent (Invitrogen, #15596026) according to the manufacturer's instruction. Libraries of RNAs were constructed using Illumina Truseq™ RNA sample prep Kit (Illumina) with the manufacturer's instruction. Illumina HiSeq 2000 was used to sequence the library and FastQC v0.11.4 (Andrews 2010) was utilized to evaluate the quality of the raw reads. Adapters were removed by Cutadapt v1.16 (Martin 2011) and the same software we used to filter the reads with low quality (Q < 20) or short length (< 25 bp). Hisat2 v 2.1.0 (Kim et al. 2019) was used to map the clean reads on human genome (GRCh38). StringTie v 1.1.3b (Pertea et al. 2016) and GRCh38 GTF file (version 92) were used to obtain the counts of

745 genes and edgeR v 3.24 (McCarthy et al. 2012, Robinson et al. 2010) was used to
746 perform the analysis of differential gene expression analysis. FPKM of each gene was
747 calculated and different expression genes which $|\log_2\text{FoldChange}| > 1$ and adjust
748 P-value < 0.05 were selected. Volcano plot and Heat map were generated using
749 TBtools (Chen et al. 2020).

750

751 **GO enrichment analysis**

752 Gene ontology analysis was performed by using the Database for Annotation,
753 Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.8
754 (<https://david.ncifcrf.gov/home.jsp>) (Huang et al. 2009). The molecular function,
755 biological process and cellular component of tested genes were analyzed. Significance
756 was defined as adjust P-value (Padj) less than 0.05. The results were presented with
757 bubble plots drawn by R with ggplot2 package (Wickham 2016).

758

759 **Transmission electron microscopy**

760 Cells were seeded on ACLAR films treated with poly-lysine (100 ug/mL), infected
761 with ZIKV (MOI=5) for 24 h, and fixed in 2.5% glutaraldehyde in PBS with a pH of
762 7.4 at room temperature for 1 hour. Samples were then post-fixed with 1% osmium
763 tetroxide in 0.1 mol/L sodium cacodylate for 1.5 h, and stained with 2% (w/v) uranyl
764 acetate in double distilled water for 50 min to increase the contrast. After washing and
765 dehydration in a graded series of acetone, samples were embedded in Embed 812
766 resin. The embedded samples were sliced into 70 nm sections using a Leica
767 ultramicrotome EM UC6 (Leica, Germany) and sections were collected on the EM
768 grids. Sample grids were imaged under a spirit transmission electron microscope (FEI
769 Company, The Netherlands) operating at 100 Kv.

770

771 **Statistical analysis**

772 Statistical analysis was performed by unpaired Student's *t* test, one-way analysis of
773 variance or two-way ANOVA using the GraphPad Prism v8 software and p values

were indicated by * $p < 0.05$, or ** $p < 0.01$, or *** $p < 0.001$. The histogram data were presented as mean $\pm$ SEM.

ACKNOWLEDGMENTS

We would like to thank John E. Eriksson (University of Turku and Åbo Akademi University, Finland) for providing us the vimentin plasmids, Lan Bao (Chinese Academy of Science, China) and Xueliang Zhu (Chinese Academy of Science, China) for insightful discussion.

Funding

This study was supported by National Natural Science Foundation of China (92054104, 31970660); Shanghai Municipal Science and Technology Major Project (2019SHZDZX02); Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai (KLMVI-OP-202001); “100 talents program” from the Chinese Academy of Sciences; and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13010500).

AUTHOR CONTRIBUTIONS

Y.J. and X.J. designed and supervised the study. Y.Z. carried out experiments and interpretation of the data. Y.X. and F.F. performed the electronic microscopy. J.C. and Y.L. performed the RNA-Seq experiment. S.Z. performed the Mass Spectrometry experiment. Y.J., X.J. and Y.Z. wrote the manuscript with contributions from all other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1 Rearrangement of vimentin filaments in ZIKV-infected cells. (A) Human U2OS cells were infected with ZIKV (MOI=5) for 16, 24, and 48 h. Viral envelop (Env) protein and vimentin were stained with respective antibodies, and nuclear was stained with DAPI. The white dotted line indicates the outline of the cell. Scale bar 15 μ m. (B) Quantification of the proportion of vimentin area versus overall cell area shown in (A). Each point represents a single cell. *** $P<0.001$ (unpaired t -test). (C) Time course of the accumulated intracellular ZIKV RNA (MOI=0.1) levels measured by qRT-PCR (corresponding to the left axis) and relative vimentin area changes (MOI=5) (corresponding to the right axis), upon ZIKV infection. Error bars indicated means $\pm$ SEM from three independent experiments. (D) The dynamic rearrangement of vimentin in vimentin-mCherry-expressing cells infected with ZIKV (MOI=5). Scale bar 30 μ m. (E) Time course of accumulated intracellular ZIKV Env protein level measured by western blot in infected U2OS cells (MOI=0.1). The expression levels of vimentin, tubulin and actin were not affected by ZIKV infection with GAPDH as control. (F) Cells were immunostained for vimentin (red) and viral Env (green) at 48 hpi, and visualized by structured illumination microscopy (3D-SIM). White square in the left panel indicates the magnified area shown in the corresponding color image on the right. Orthogonal sections and 3D reconstruction shows that vimentin encapsulates viral Env to form a cage-like structure. Scale bar 15 μ m in the left panel, 2 μ m in the middle orthogonal view panel, 5 μ m in the XZ panel, 5 μ m in the YZ panel and 5 μ m in the right clipping plane panel. (G) Transmission electronic microscopy images of 70 nm thin sections of resin-embedded cells infected with ZIKV (MOI=5). White square indicates the magnified area shown in the corresponding color image on the right. IFs, intermediate filaments indicated with red lines; MTs, microtubules indicated with green lines; vRCs, viral replication compartments indicated with blue circle. Scale bar 500 nm in the cell images.

Figure 2 Expression of ZIKV envelop protein induces vimentin rearrangement.

1086 (A) The dynamic rearrangement of vimentin in vimentin-mCherry-expressing cells
1087 transfected with ZIKV Env-EGFP plasmid. Scale bar 10 μ m. (B) Plot profile analysis
1088 of Env-EGFP and vimentin-mCherry intensities in the white dotted box in (A) at
1089 corresponding time point. Distance in Axis X represents horizontal distance through
1090 the selection. (C) Vimentin filaments gather scattered viral Env protein towards
1091 peri-nucleus region. N and white box in the left panels indicate cell nucleus and the
1092 magnified area shown in the corresponding right panel. The dotted circles indicated
1093 the positions of one ZIKV-Env foci, and the dotted white lines indicated the shape of
1094 one vimentin filament. Scale bar 2 μ m in the cell images and 1 μ m in the magnified
1095 images.

1096

1097 **Figure 3 Depletion of vimentin results in disruptive replication compartments**
1098 **and subsequent reduced ZIKV infection.** (A) WT, vimentin KO (VIM KO),
1099 vimentin-full-length re-introducing (VIM RES) and vimentin-unit-length-filament
1100 (VIM ULF) re-introducing cells were infected with ZIKV (MOI=5) for 24 h. Cell
1101 were fixed and immunostained to visualize ZIKV Env, vimentin and nucleus. Scale
1102 bar 15 μ m. (B) Quantification of the percentages of cells with segregated ZIKV Env
1103 in WT, VIM KO, VIM RES and VIM ULF conditions. Each point represents an
1104 independent experiment. (C) Quantification of the percentage of total ZIKV Env area
1105 to overall cell area in WT, VIM KO, VIM RES and VIM ULF conditions. Each point
1106 represents an infected cell. (D-F) Intracellular ZIKV RNA (D), ZIKV Env protein
1107 level (E) and titers of ZIKV particles (F) in infected WT and vimentin KO cells
1108 (MOI=0.1) were measured by two-step qRT-PCR, western blotting, and plaque assay,
1109 respectively. Results from three independent experiments are shown. (G) Percentage
1110 of infected WT and VIM KO cells (MOI=1, 48 hpi) measured by flow cytometry. (H)
1111 Percentage of infected WT and VIM KO cells (MOI=5) measured by
1112 immunofluorescence. Scale bar 100 μ m. Error bars indicated means $\pm$ SEM.

1113

1114 **Figure 4 Depletion of vimentin influences the production and stability of viral**

1115 **components after entering cells. (A-B)** Time course of accumulated intracellular
 1116 ZIKV RNA in infected WT and vimentin KO (VIM KO) cells (MOI=0.1) measured
 1117 by qRT-PCR. **(B)** Time course of accumulated intracellular ZIKV Env protein in
 1118 infected WT and VIM KO cells (MOI=0.1) measured by western blots. Numbers in
 1119 the blots indicated the levels of ZIKV Env normalized to GAPDH. **(C)** The schematic
 1120 diagram of drug treatment experiment. Cells were transiently transfected with
 1121 E-EGFP plasmid for 36h, and then treated with MG132 (10 μ M), NH₄Cl (25 mM) and
 1122 CHX (20 μ g/mL) for 11 h. **(D)** Western blotting analysis of viral protein upon CHX
 1123 treatment. **(E)** Western blotting analysis of viral protein upon MG132 treatment,
 1124 where detection of P21 accumulation serves as positive control. **(F)** Western blotting
 1125 analysis of viral protein upon NH₄Cl treatment, where detection of LC3 accumulation
 1126 serves as positive control. **(G)** The schematic diagram of ZIKV binding and entry
 1127 assay. **(H)** ZIKV RNA levels from bound and internalized ZIKV were measured by
 1128 qRT-PCR. The data are from three independent experiments. ***P<0.001 (2way
 1129 ANOVA). Error bars indicated means $\pm$ SEM.

1130

1131 **Figure 5 Interaction between vimentin and ER-localized RNA-binding**
 1132 **components affects ZIKV infection. (A)** The top 10 significant GO terms in
 1133 molecular function are shown in the bubble chart. **(B)** The top 10 significant GO
 1134 terms in biological process are shown in the bubble chart. **(C)** All 17 significant
 1135 cellular components with count number greater than 50 genes are shown in the bubble
 1136 chart. **(D)** Biological process enrichment analysis of significantly down-regulated
 1137 genes in ZIKV infected VIM KO cells compared with infected WT cells (MOI=1, 24
 1138 hpi) by RNA-Seq. The top 10 enriched terms are shown in the bubble chart. In (A-D),
 1139 the color of the bubbles displayed from red to blue indicated the descending order of
 1140 $-\log_{10}(\text{Padj})$. The sizes of the bubbles are displayed from small to large in ascending
 1141 order of gene counts. The x and y axis represent the gene ratio and the GO terms,
 1142 respectively. **(E)** List of ER-located proteins interacting with vimentin by setting the
 1143 coverage peptides threshold over 30% from mass spectrometry assay. **(F)** Heatmap of

1144 significantly differentially expressed genes (ER-annotated) between ZIKV infected
1145 WT and VIM KO cells (MOI=1, 24 hpi) identified by RNA-Seq (n=3 independent
1146 experiments per condition).

1147

1148 **Figure 6 Vimentin interacts with ER-located RRB1 to regulate ZIKV infection.**

1149 (A) The domain structure of RRB1 protein. Red lines represent the peptides
1150 interacting with vimentin identified from mass spectrometry. (B) *in vitro* binding
1151 assay of vimentin with RRB1. Cell lysates were incubated with purified recombinant
1152 his-tagged vimentin protein, and analyzed by anti-RRB1 antibody in western
1153 blotting. (C) Immunofluorescence images of vimentin, RRB1 and ZIKV RNA in
1154 WT and vimentin KO (VIM KO) cells infected with ZIKV (MOI=2) for 24 h, and
1155 stained with anti-dsRNA, anti-RRB1 and anti-vimentin antibodies and DAPI for
1156 nucleus. Scale bar 15 μ m. (D,E) Quantifications of RRB1 intensities (D) and areas
1157 (E) in non-infected (MOCK) WT and VIM KO cells. Each point represents a single
1158 cell. *** P <0.001 (unpaired t -test). (F) Quantification of the colocalization between
1159 RRB1 and dsRNA in ZIKV infected WT and VIM KO cells by Pearson's
1160 coefficients. Each point represents a single cell. *** P <0.001 (unpaired t -test). (G)
1161 Western blots demonstrated that RRB1 was efficiently knockdown by shRNA in
1162 both WT and VIM KO cells. Numbers in the blots indicated the levels of RRB1
1163 normalized to GAPDH. (H) Quantifications of ZIKV RNA copies detected by
1164 qRT-PCR, and normalized to GAPDH, in WT, RRB1 knockdown (RRB1 KD),
1165 VIM KO and RRB1 knockdown; vimentin knockout (RRB1 KD;VIM KO)
1166 conditions. The cells were infected with ZIKV at MOI=0.1 for 24 h, and the data are
1167 from three independent experiments. (I) Protein levels of RRB1, vimentin, ZIKV
1168 Env in WT and vimentin KO cells detected by western blots, which were also probed
1169 with vimentin antibody to confirm the KO efficiency and GAPDH antibody to verify
1170 equal sample loading.

Figure 1

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