

## Reconciling West Nile virus with the autophagic pathway

Miguel A Martín-Acebes,<sup>1,2,\*</sup> Ana-Belén Blázquez,<sup>2</sup> and Juan-Carlos Saiz<sup>2</sup>

<sup>1</sup>Department of Virology and Microbiology; Centro de Biología Molecular “Severo Ochoa” (CSIC-UAM); Madrid, Spain; <sup>2</sup>Department of Biotechnology; Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA); Madrid, Spain

**W**est Nile virus (WNV) is a neurotropic mosquito-borne flavivirus responsible for recurrent outbreaks of meningitis and encephalitis. Several studies analyzing the interactions of this pathogen with the autophagic pathway have reported opposite results with evidence for and against the upregulation of autophagy in infected cells. In this regard, we have recently reported that minimal genetic changes (single amino acid substitutions) in nonstructural proteins of WNV can modify the ability of the virus to induce autophagic features such as LC3 modification and aggregation in infected cells. We think that these results could help explain some of the previously reported discrepancies. These findings could also aid in deciphering the interactions of this pathogen with the autophagic pathway at the molecular level aimed to develop feasible antiviral strategies to combat this pathogen, and other related flaviviruses.

West Nile virus, a neurotropic flavivirus, is maintained in nature in an enzootic infectious cycle between avian hosts and ornithophilic mosquitoes.<sup>1</sup> Since the virus was first reported in the West Nile District of Uganda in 1937, WNV circulation has been associated with recurrent outbreaks of meningitis and encephalitis affecting humans and horses along different areas of Africa, Europe, Asia, and Oceania. In 1999 the virus was detected for the first time on the American continent in New York (NY99 strain),<sup>2</sup> and has spread across the continent during the subsequent years. Nowadays, the virus has become endemic in the USA, and is the leading cause of epidemic encephalitis.<sup>3</sup> Currently, there is no licensed vaccine or

therapy for human use against this pathogen.

Macroautophagy (herein referred as autophagy) is a catabolic mechanism that sequesters cytoplasmic components for degradation.<sup>4</sup> The autophagic pathway can be upregulated to cope with diverse forms of cellular stress, including viral infections.<sup>5</sup> In the case of flaviviruses, the autophagic pathway can play multifaceted roles during the infection of these pathogens that include rearrangements of cellular lipid metabolism, contribution to viral maturation, or involvement in the early steps of the infection.<sup>6–8</sup> However, whereas the upregulation of the autophagic pathway has been well documented for other flaviviruses (for a review see ref. 9), the involvement of autophagy in WNV infection has been controversial. There is evidence supporting an upregulation of autophagy upon WNV infection, mainly based on the increase in the cellular content of the lipidated form of microtubule-associated protein 1 light chain 3 (LC3) and its aggregated forms visualized with GFP-LC3 fusion constructs.<sup>10,11</sup> However, there is also evidence showing that infection by WNV does not induce the lipidation of LC3, thus pointing to a lack of upregulation of the autophagic pathway in infected cells.<sup>12</sup> Furthermore, these reports also showed contradictory results when the effect of the depletion of the autophagy-related (ATG) protein ATG5, a key regulator of autophagy, on the replication of WNV was analyzed. Whereas Beatman et al.<sup>10</sup> and Vandergaast and Fredericksen<sup>12</sup> noted that depletion of ATG5 does not affect replication of WNV, Kobayashi et al.<sup>11</sup> reported that WNV replication is increased in cells depleted of this protein. One could think that these opposing observations could be

**Keywords:** autophagy, flavivirus, infection, LC3, mutants, virus strain, West Nile virus (WNV)

**Abbreviations:** ATG, autophagy-related; GFP, green fluorescent protein; LC3, microtubule-associated protein 1 light chain 3; MEF, mouse embryonic fibroblast; NS, nonstructural; WNV West Nile virus.

\*Correspondence to: Miguel A Martín-Acebes; Email: martin.mangel@inia.es, mamartin@cbm.csic.es

Submitted: 02/03/2015

Revised: 03/24/2015

Accepted: 03/30/2015

<http://dx.doi.org/10.1080/15548627.2015.1037062>

partially the result of different cell lines, viruses, and methodologies used in these studies. In fact, cell-type dependent differences or virus strain-dependent discrepancies in the autophagic effect have been reported for other viral models.<sup>13-15</sup> However, a remarkable feature of the papers addressing the specific case of WNV is that autophagy analyses following infection with viruses from the NY99 genotype lead to different results.<sup>10-12</sup> We have recently revisited the induction of autophagy using a panel of WNV strains that varied geographically and temporally, including viruses belonging to the NY99 genotype. Our results showed that infection with all, except one, of the WNV strains tested induce an increase in LC3 lipidation and aggregation of GFP-LC3 consistent with an upregulation of the autophagic pathway.<sup>16</sup> Remarkably, the virus isolate that failed to induce autophagic features was the NY99 strain<sup>17</sup> included in the assays. Moreover, when a persistent derivative of this parental NY99 strain isolated from a mouse 56 d after vertical infection<sup>18</sup> was tested, this virus (termed mutant B13) also induced LC3 lipidation and aggregation, characteristics of an upregulation of autophagy. The complete genomic sequence of mutant B13 showed only 2 nonsynonymous nucleotide substitutions when compared to its parental NY99 isolate. These nucleotide substitutions are responsible for the introduction of amino acid replacement V67I in nonstructural protein 4A (NS4A) and amino acid replacement I240M in NS4B.<sup>16</sup> Genetically engineered mutant viruses carrying these mutations alone or combined were constructed using an infectious cDNA clone. The introduction of any of these mutations in the recombinant viruses is sufficient to promote an increase in LC3 lipidation and aggregation in comparison to the parental virus recovered from the infectious cDNA clone. These results confirmed that minimal genetic changes in the NS proteins of WNV can modulate the induction of characteristic features of autophagy upregulation in WNV-infected cells.

As noted above, our mutant B13, which induces an increase in LC3 modification/aggregation in comparison to its parental isolate NY99, was isolated from a

persistently infected mouse.<sup>16</sup> The persistence of WNV in infected patients and also in animal models has been well documented (for a review see ref. 19). Unfortunately, the genomic sequence of the viruses detected in most of these studies is rather limited. Nevertheless, it has been noted that WNV recovered from a persistently infected hamster acquires different mutations throughout the genome, including in genes encoding NS proteins.<sup>20</sup> An attractive idea is that the selection of specific mutations could help in the establishment of a persistent infection in vivo. Supporting this idea of viral adaptation to persistent infections, in vitro experiments using WNV replicons have also shown that the selection of mutations in NS proteins (including in NS4B) facilitates persistence.<sup>21</sup> Following this reasoning, we examined whether the mutations found in mutant B13<sup>16</sup> are similar to those observed in WNV recovered from persistently infected hamster.<sup>20</sup> The comparison of the sequences revealed that no common mutations are selected in both persistent viruses. The lack of common mutations could reflect differences between these WNV persistence models and points to the flexibility of adaptation of WNV. Anyway, the role of the mutations selected in these persistent viruses and their connections with the autophagic pathway in vivo still remain to be fully elucidated. However, it has to be also considered that the relationship between viral persistent infections and autophagy has been already documented in other viral models.<sup>22,23</sup> The specific case of hepatitis C virus merits special consideration, since this pathogen is also a member of the *Flaviviridae* family where WNV is classified. This virus induces the selective degradation of mitochondria by autophagy (mitophagy) that has been related to attenuation of viral apoptosis that contributes to persistent infections.<sup>23</sup> Hence, it is possible that the upregulation of the autophagic pathway by mutant B13 could be associated with the establishment of in vivo persistence.

Nowadays, WNV stocks for laboratory usage can be produced in cultured cells from a limited number of infectious clones available, and more commonly, by amplification of diverse tissue culture-

adapted virus isolates. As with any RNA virus, WNV has a high potential for mutation and a relatively high degree of sequence variation among viral isolates occurs. In this way, closely related isolates, even from the same genotype, exhibit differences in the genomic sequence. These differences do not only reflect sequence differences in the source of the infectious virus (infectious clone or isolate), but also the selection of variants that have arisen during the amplification of virus isolates from different origins and passage history. With these considerations in mind, we think that the differences previously observed in the studies analyzing the upregulation or not of autophagy during WNV infection could rely on genetic differences of the viruses utilized in these analyses. Thus, our results could help to reconcile conflicting positions on the relationship between WNV and autophagy. Regarding other flaviviruses, the ability to induce LC3 modification and aggregation has been reported to vary among different strains of Japanese encephalitis virus,<sup>24</sup> which also supports the idea that variations on the ability to upregulate the autophagic pathway of the viral strains could constitute a common feature of the flaviviruses.

Albeit attractive, deciphering the mechanism(s) behind the mutations in NS4A and NS4B and the phenotype of mutant viruses does not seem to be an easy task, since these 2 proteins are multifunctional transmembrane proteins that have been involved in diverse aspects of the flavivirus life cycle. NS4A has been related to WNV-induced intracellular membrane rearrangements and the mechanisms to overcome superinfection exclusion.<sup>25,26</sup> In the case of Dengue virus (another flavivirus) the expression of NS4A has been also related to evasion of the innate immune response and protection against cell death through induction of autophagy.<sup>27,28</sup> On the other hand, NS4B has been associated with flavivirus-induced membrane rearrangements, RNA synthesis, and evasion of innate immunity.<sup>27,29,30</sup> In fact, a WNV mutant carrying a single amino acid substitution in NS4B is attenuated and induces strong innate and adaptive immune responses in vivo.<sup>31</sup> Moreover, the expression of

both NS4A and NS4B in WNV-infected cells has also been associated with the activation of the unfolded protein response, another cellular stress pathway.<sup>32</sup> All these findings suggest that NS4A and NS4B play central roles in the connections between virus replication, membrane rearrangements, autophagy, and immune response in WNV-infected cells. Consistent with this view, these 2 proteins could constitute interesting antiviral targets against WNV and other related flaviviruses.

In our experiments, the viruses inducing different autophagic features share common growth kinetics in cultured cells.<sup>16</sup> This apparent lack of effect of autophagy on in vitro replication of WNV is consistent with the results obtained by other researchers when autophagy-related proteins are depleted in cultured cells.<sup>10,12</sup> However, Kobayashi et al.<sup>11</sup> observed that autophagy-deficient cells (*atg5*<sup>-/-</sup> MEFs) display an increase in virus replication relative to control cells (*Atg5*<sup>+/+</sup> MEFs). This increase in virus replication correlates also with an increase in virus yield when a very low infection dose is used. Based on these observations these authors suggested a protective role of autophagy against WNV infection. This could be consistent with the reported protective role of autophagy against infection with other arboviruses.<sup>33,34</sup> In fact, exogenous activation of autophagy by a proautophagic peptide results in protection against WNV infection in vivo and constitutes a promising antiviral strategy.<sup>35</sup> Along this line, we must remember that the mutant virus differing in autophagy upregulation was isolated from a persistently infected mouse, which might indicate that autophagy plays a role during in vivo infection with WNV, regardless of the results observed in vitro. Considering that the relationship between autophagy and viral persistence has been documented,<sup>22,23</sup> and that persistent infections have to cope with the immune system of the host in a more prolonged way than during acute infections, the immunological role of autophagy during WNV infection in vivo becomes patent. In any case, this does not exclude the possibility that autophagy could play other relevant roles not yet assessed during WNV infection.

Thus, deciphering the interactions between WNV with the autophagic pathway through in vivo experiments, could help with the development of novel antiviral strategies to combat this pathogen. Further in vitro and in vivo characterization of these novel mutants differing in their ability to induce LC3 modification/aggregation, together with the analysis of more genetically divergent viral strains, could provide new clues to understand the molecular mechanisms behind the upregulation of autophagy in WNV-infected cells.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Funding

This work was funded by INIA (RTA2011-0036 and E-RTA2013-00013-C01) and the Comunidad Autónoma de Madrid PLATESA (P2013/ABI-2906). MAMA received a postdoctoral fellowship JAE-Doc from CSIC.

#### References

- Martin-Acebes MA, Saiz JC. West Nile virus: a re-emerging pathogen revisited. *World J Virol* 2012; 1:51-70; PMID:24175211; <http://dx.doi.org/10.5501/wjv.v1.i2.51>
- Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, Crise B, Volpe KE, Crabtree MB, Scherret JH, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* 1999; 286:2333-7; PMID:10600742; <http://dx.doi.org/10.1126/science.286.5448.2333>
- Roehrig JT. West Nile virus in the United States - a historical perspective. *Viruses* 2013; 5:3088-108; PMID:24335779; <http://dx.doi.org/10.3390/v5123088>
- Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* 2008; 451:1069-75; PMID:18305538; <http://dx.doi.org/10.1038/nature06639>
- Jordan TX, Randall G. Manipulation or capitulation: virus interactions with autophagy. *Microbes Infect* 2012; 14:126-39; PMID:22051604; <http://dx.doi.org/10.1016/j.micinf.2011.09.007>
- Heaton NS, Randall G. Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host Microbe* 2010; 8:422-32; PMID:21075353; <http://dx.doi.org/10.1016/j.chom.2010.10.006>
- Mateo R, Nagamine CM, Spagnolo J, Mendez E, Rahe M, Gale M, Jr., Yuan J, Kirkegaard K. Inhibition of cellular autophagy deranges dengue virion maturation. *Journal of virology* 2013; 87:1312-21; PMID:23175363; <http://dx.doi.org/10.1128/JVI.02177-12>
- Panyasrivanit M, Khakpoor A, Wikan N, Smith DR. Linking dengue virus entry and translation/replication through amphisomes. *Autophagy* 2009; 5:434-5; PMID:19202356; <http://dx.doi.org/10.4161/auto.5.3.7925>
- Blazquez AB, Escibano-Romero E, Merino-Ramos T, Saiz JC, Martin-Acebes MA. Stress responses in flavivirus-infected cells: activation of unfolded protein response and autophagy. *Front Microbiol* 2014; 5:266; PMID:24917859; <http://dx.doi.org/10.3389/fmicb.2014.00266>
- Beatman E, Oyer R, Shives KD, Hedman K, Brault AC, Tyler KL, David Beckham J. West Nile virus growth is independent of autophagy activation. *Virology* 2012; 433:262-72; PMID:22939285; <http://dx.doi.org/10.1016/j.virol.2012.08.016>
- Kobayashi S, Orba Y, Yamaguchi H, Takahashi K, Sasaki M, Hasebe R, Kimura T, Sawa H. Autophagy inhibits viral genome replication and gene expression stages in West Nile virus infection. *Virus Res* 2014; 191:83-91; PMID:25091564; <http://dx.doi.org/10.1016/j.virusres.2014.07.016>
- Vandergaast R, Fredericksen BL. West Nile virus (WNV) replication is independent of autophagy in mammalian cells. *PLoS One* 2012; 7:e45800; PMID:23029249; <http://dx.doi.org/10.1371/journal.pone.0045800>
- Cavignac Y, Esclatine A. Herpesviruses and autophagy: catch me if you can! *Viruses* 2010; 2:314-33; PMID:21994613; <http://dx.doi.org/10.3390/v2010314>
- Tang SW, Ducroux A, Jeang KT, Neveu C. Impact of cellular autophagy on viruses: Insights from hepatitis B virus and human retroviruses. *J Biomed Sci* 2012; 19:92; PMID:23110561; <http://dx.doi.org/10.1186/1423-0127-19-92>
- Dumit VI, Dengjel J. Autophagosomal protein dynamics and influenza virus infection. *Front Immunol* 2012; 3:43; PMID:22566925; <http://dx.doi.org/10.3389/fimmu.2012.00043>
- Blazquez AB, Martin-Acebes MA, Saiz JC. Amino acid substitutions in the non-structural proteins 4A or 4B modulate the induction of autophagy in West Nile virus infected cells independently of the activation of the unfolded protein response. *Front Microbiol* 2015; 5:797; PMID:25642225; <http://dx.doi.org/10.3389/fmicb.2014.00797>
- Martin-Acebes MA, Saiz JC. A West Nile virus mutant with increased resistance to acid-induced inactivation. *J Gen Virol* 2011; 92:831-40; PMID:21228127; <http://dx.doi.org/10.1099/vir.0.027185-0>
- Blazquez AB, Saiz JC. West Nile virus (WNV) transmission routes in the murine model: intrauterine, by breastfeeding and after cannibal ingestion. *Virus research* 2010; 151:240-3; PMID:20438776; <http://dx.doi.org/10.1016/j.virusres.2010.04.009>
- Garcia MN, Hasbun R, Murray KO. Persistence of West Nile virus. *Microb Infect* 2015; 17:163-8; PMID:25499188; <http://dx.doi.org/10.1016/j.micinf.2014.12.003>
- Wu X, Lu L, Guzman H, Tesh RB, Xiao SY. Persistent infection and associated nucleotide changes of West Nile virus serially passaged in hamsters. *J Gen Virol* 2008; 89:3073-9; PMID:19008395; <http://dx.doi.org/10.1099/vir.0.2008/003210-0>
- Rossi SL, Fayzuln R, Dewsbury N, Bourne N, Mason PW. Mutations in West Nile virus nonstructural proteins that facilitate replicon persistence in vitro attenuate virus replication in vitro and in vivo. *Virology* 2007; 364:184-95; PMID:17382364; <http://dx.doi.org/10.1016/j.virol.2007.02.009>
- E X, Hwang S, Oh S, Lee JS, Jeong JH, Gwack Y, Kowalik TF, Sun R, Jung JU, Liang C. Viral Bcl-2-mediated evasion of autophagy aids chronic infection of gammaherpesvirus 68. *PLoS Pathog* 2009; 5:e1000609; PMID:19816569; <http://dx.doi.org/10.1371/journal.ppat.1000609>
- Kim SJ, Syed GH, Khan M, Chiu WW, Sohail MA, Gish RG, Siddiqui A. Hepatitis C virus triggers mitochondrial fission and attenuates apoptosis to promote viral persistence. *Proc Natl Acad Sci U S A* 2014; 111:6413-8; PMID:24733894; <http://dx.doi.org/10.1073/pnas.1321114111>

24. Li JK, Liang JJ, Liao CL, Lin YL. Autophagy is involved in the early step of Japanese encephalitis virus infection. *Microb Infect* 2012; 14:159–68; PMID:21946213; <http://dx.doi.org/10.1016/j.micinf.2011.09.001>
25. Roosendaal J, Westaway EG, Khromykh A, Mackenzie JM. Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein. *J Virol* 2006; 80:4623–32; PMID:16611922; <http://dx.doi.org/10.1128/JVI.80.9.4623-4632.2006>
26. Campbell CL, Smith DR, Sanchez-Vargas I, Zhang B, Shi PY, Ebel GD. A positively selected mutation in the WNV 2K peptide confers resistance to superinfection exclusion in vivo. *Virology* 2014; 464–465:228–32; PMID:25104615
27. Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A. Inhibition of interferon signaling by dengue virus. *Proc Natl Acad Sci U S A* 2003; 100:14333–8; PMID:14612562; <http://dx.doi.org/10.1073/pnas.2335168100>
28. McLean JE, Wudzinska A, Datan E, Quaglino D, Zakeri Z. Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication. *J Biol Chem* 2012; 286:22147–59; PMID:21511946; <http://dx.doi.org/10.1074/jbc.M110.192500>
29. Puig-Basagoiti F, Tilgner M, Bennett CJ, Zhou Y, Munoz-Jordan JL, Garcia-Sastre A, Bernard KA, Shi PY. A mouse cell-adapted NS4B mutation attenuates West Nile virus RNA synthesis. *Virology* 2007; 361:229–41; PMID:17178141; <http://dx.doi.org/10.1016/j.virol.2006.11.012>
30. Kaufusi PH, Kelley JF, Yanagihara R, Nerurkar VR. Induction of endoplasmic reticulum-derived replication-competent membrane structures by West Nile virus non-structural protein 4B. *PloS One* 2014; 9:e84040; PMID:24465392; <http://dx.doi.org/10.1371/journal.pone.0084040>
31. Welte T, Xie G, Wicker JA, Whiteman MC, Li L, Rachamalla A, Barrett A, Wang T. Immune responses to an attenuated West Nile virus NS4B-P38G mutant strain. *Vaccine* 2011; 29:4853–61; PMID:21549792; <http://dx.doi.org/10.1016/j.vaccine.2011.04.057>
32. Ambrose RL, Mackenzie JM. West Nile virus differentially modulates the unfolded protein response to facilitate replication and immune evasion. *J Virol* 2011; 85:2723–32; PMID:21191014; <http://dx.doi.org/10.1128/JVI.02050-10>
33. Joubert PE, Werneke SW, de la Calle C, Guivel-Benhassine F, Giodini A, Peduto L, Levine B, Schwartz O, Lenschow DJ, Albert ML. Chikungunya virus-induced autophagy delays caspase-dependent cell death. *J Exp Med* 2012; 209:1029–47; PMID:22508836; <http://dx.doi.org/10.1084/jem.20110996>
34. Orvedahl A, MacPherson S, Sumpter R, Jr., Tallozy Z, Zou Z, Levine B. Autophagy protects against Sindbis virus infection of the central nervous system. *Cell Host Microbe* 2012; 7:115–27; PMID:20159618; <http://dx.doi.org/10.1016/j.chom.2010.01.007>
35. Shoji-Kawata S, Sumpter R, Leveno M, Campbell GR, Zou Z, Kinch L, Wilkins AD, Sun Q, Pallauf K, MacDuff D, et al. Identification of a candidate therapeutic autophagy-inducing peptide. *Nature* 2013; 494:201–6; PMID:23364696; <http://dx.doi.org/10.1038/nature11866>