VP24 Is a Molecular Determinant of Ebola Virus Virulence in Guinea Pigs

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In sharp contrast to human and nonhuman primates, guinea pigs and some other mammals resist Ebola virus (EBOV) replication and do not develop illness upon virus inoculation. However, serial passaging of EBOV in guinea pigs results in a selection of variants with high pathogenicity. In this report, using a reverse genetics approach, we demonstrate that this dramatic increase in EBOV pathogenicity is associated with amino acid substitutions in the structural protein VP24. We show that although replication of recombinant EBOV carrying wild-type VP24 is impaired in primary peritoneal guinea pig macrophages and in the liver of infected animals, the substitutions in VP24 allow EBOV to replicate in guinea pig macrophages and spread in the liver of infected animals. Furthermore, we demonstrate that both VP24/wild type and the guinea pig–adapted VP24/8mc are similar in their ability to block expression of interferon-induced host genes, suggesting that the increase in EBOV virulence for guinea pigs is not associated with VP24 interferon antagonist function. This study sheds light on the mechanism of resistance to EBOV infection and highlights the critical role of VP24 in EBOV pathogenesis.

The Filoviridae family comprises 2 genera, *Ebolavirus* and *Marburgvirus*, which are enveloped negative-sense single-stranded RNA viruses. Among Ebola virus species, *Zaire Ebolavirus* (EBOV) is the most pathogenic for humans and causes sporadic outbreaks of hemorrhagic fever with fatality rates reaching up to 90% [1]. The pathogenesis of EBOV in man is not yet fully understood. Moreover, there are no vaccines or treatments against EBOV infection available for use in humans despite substantial research progress [2–7]. Experimental infection of nonhuman primates (NHPs) has revealed several important aspects of the disease [8–12]. Dendritic cells, macrophages, and monocytes are among the major and primary targets of infection and are responsible for virus spread to areas of massive virus replication later postinfection [11]. At these late stages of infection other cells such as hepatocytes, fibroblasts, and endothelial cells also become involved in virus replication [10–13]. Rapid systemic spread of the virus is associated with the release of virions into the lymph and blood of infected animals. Extremely high viral loads, multiple organ failure, an excessive inflammatory cytokine release, and coagulopathy are among the hallmarks of Ebola hemorrhagic fever.

Despite the extremely high pathogenicity there is evidence that some individuals, although being in direct contact with sick patients and being diagnosed positive for the presence of viral RNA in the peripheral blood mononuclear cells, never develop the symptoms of Ebola hemorrhagic fever [14, 15]. Although these occurrences are rare, the existence of such individuals suggests that asymptomatic Ebola infection can occur in some humans without subsequent massive EBOV replication. The molecular mechanisms involved in the
control of EBOV replication are not yet understood. Unlike humans and NHPs, immunocompetent adult rodents are resistant to infection with wild-type EBOV (EBOV/WT). Importantly, serial passaging of EBOV in mice and guinea pigs has been shown to result in the selection of lethal rodent-adapted variants of this virus [16–19]. Furthermore, it has recently been shown that amino acid substitutions in both NP and VP24 are necessary for the acquisition of EBOV virulence in the mice model. The ability of mutated proteins to overcome the interferon (IFN)—induced antiviral response has been proposed to play a critical role in the pathogenesis of EBOV infection in mice [20]. Serial passaging of EBOV in guinea pigs resulted in the conversion of nonvirulent EBOV/WT into highly pathogenic variants of EBOV [18, 19]. Molecular characterization of the guinea pig–adapted variant EBOV/8mc revealed the presence of 8 nucleotide substitutions leading to 5 amino acid (aa) exchanges: single aa mutations in the NP and L proteins and 3 in VP24 [19]. Elucidation of the molecular bases of resistance to Ebola infection in rodents and the identification of molecular mechanisms involved in the increase of the virulence during serial passaging could aid the development of EBOV treatments.

In this study, using a reverse genetics approach we investigate the effect of individual mutations in guinea pig–adapted EBOV/8mc on EBOV virulence. Recombinant viruses possessing various combinations of wild-type and guinea pig–adapted genes were generated and analyzed both in cell culture and in guinea pigs. We demonstrate that VP24 has a critical role in EBOV pathogenesis and that aa changes in VP24 are essential and sufficient to confer EBOV virulence in guinea pigs. Moreover, the data obtained suggest that increase in EBOV virulence for guinea pigs does not involve VP24’s function as an IFN antagonist.

**MATERIALS AND METHODS**

**Cells**

Vero E6, GPC-16, and HEK 293T cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS). During infections DMEM was supplemented with 2.5% FCS. BSR T7/5, a BHK-21 cell line stably expressing T7 RNA polymerase (a kind gift from K.-K. Conzelmann), was grown in Glasgow medium (Invitrogen) as stably expressing T7 RNA polymerase (a kind gift from K.-K. Conzelmann), was grown in Glasgow medium (Invitrogen) as described elsewhere [21].

**Generation of Recombinant EBOVs**

Plasmids containing overlapping complementary DNA (cDNA) fragments covering the full-length antigenomic sequence of Zaire Ebolavirus strain Mayinga (EBOV) were generated as described elsewhere [21]. Mutations in the coding regions of the NP, VP24, and L were introduced by site-directed mutagenesis of the intermediate plasmids pKSN4, pKSS25, and pKSL23. Plasmids containing full-length cDNAs with mutations in NP, VP24, and L genes were generated, and recombinant Ebola viruses (rEBOVs) were rescued in BSR T7/5 cells and then passaged once and amplified in Vero E6 cells as previously described [21]. The virus stocks were stored at –80°C. Mutations introduced into these viruses were confirmed by sequencing of genomic RNA isolated from the virus stocks. Virus titers were estimated by immunoplaque assay or by 50% tissue culture infectious dose (TCID₅₀) determination using Vero E6 cells [22].

For the immunoplaque assay, cells were infected with 10-fold serial virus dilutions and incubated under overlay containing 0.6% Avicel (FMC BioPolymer), with 2.5% FCS essentially as described elsewhere [23]. Cells were immunostained with an anti-NP monoclonal mouse antibody (1:100 dilution), followed by one-hour incubation with an anti-mouse peroxidase-coupled antibody (1:1,000 dilution; Dako), and the plaques were visualized using True Blue peroxidase substrate (KPL).

**Animal Experiments**

Guinea pigs, strain Hartley (3-week-old females), were infected intraperitoneally with 5000 plaque-forming units or 5000 TCID₅₀ of recombinant virus. Mock-infected controls were inoculated with DMEM. Animals were monitored for clinical manifestations and changes in body weight during 21 days postinfection and were euthanized either when they reached an ethical end point or at the conclusion of the experiment. Samples of liver were collected at necropsy and were analyzed by immunohistochemistry and electron microscopy. For immunohistochemistry, formalin-fixed tissues were embedded in paraffin, sectioned, deparaffinized, rehydrated, rinsed, and placed in phosphate-buffered saline (PBS) containing 3% bovine serum albumin for 20 minutes. EBOV infection in tissues was detected by immunostaining using a mouse monoclonal anti-VP40 antibody used at a dilution of 1:100.

**Electron Microscopy**

Liver samples from EBOV-infected guinea pigs or infected primary peritoneal guinea pig macrophages were fixed with Hank’s balanced salt solution (HBSS) containing 2.5% glutaraldehyde, postfixed with HBSS containing 1% osmium tetroxide, dehydrated, and embedded in an EPON resin. Ultrathin sections were stained using uranyl acetate and lead citrate. Samples were analyzed using a Zeiss 109 transmission electron microscope at 80 kV or a Phillips CM120 electron microscope at 80 kV.

**Harvesting of Primary Peritoneal Guinea Pig Macrophages**

Injection of incomplete Freund’s adjuvant into the peritoneal cavity of guinea pigs was performed to induce the production of macrophages. The animals were euthanized 4 days postinjection, and peritoneal cells were harvested by washing the cavity twice with 20 mL of cold PBS containing 2% guinea pig serum. The cells were clarified by low-speed centrifugation for 10 minutes at 380 × g and washed twice in Roswell Park Memorial Institute medium containing 10% guinea pig serum, 2 μM L-glutamine,
100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μM β-mercaptoethanol. To separate macrophages and nonadherent cells, the cell mixture was placed in 12-well culture plates at a density of 5 × 10^6 cells/mL and incubated for one hour and 30 minutes at 37°C in 5% CO₂. Adherent cells were washed twice with PBS containing 2% guinea pig serum and used for infection with recombinant viruses.

**Immunoblot Analysis**

Samples of cells and culture medium were separated on 10% polyacrylamide gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a polyvinylidene fluoride membrane (Millipore). Western blot analysis was performed using a monoclonal mouse anti-NP antibody (1:2,000) and a rabbit anti-VP24 antibody (1:4,000). Either rabbit anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:20,000; Dako) were used as secondary antibodies. Chemiluminescent reagents (Amersham ECL) were used for detection of specific protein bands.

**Flow Cytometry Analysis**

Peritoneal primary guinea pig macrophages were infected with recombinant EBOV at a multiplicity of infection (MOI) of 0.1. At days 1 and 4 postinfection, infected cells were harvested, washed in PBS containing 2.5% guinea pig serum, and fixed and permeabilized using the Cytofix/Cytoperp kit (BD Pharmingen). Cells were then incubated for 30 minutes at 4°C in 100 μL of diluted KZ32 monoclonal human anti-GP antibody (kindly provided by D. Burton and P. Parren) and monoclonal mouse anti–guinea pig macrophage antibody MCA518S (Serotec). Cells were washed twice in Wash buffer (BD Pharmingen) and incubated for 30 minutes at 4°C with a monoclonal pig antihuman fluorescein isothiocyanate–conjugated antibody (Dako) and a goat anti-mouse Rhodophyta phycoerythrin–conjugated STAR105PE (Serotec) antibody. Flow cytometry analysis was conducted using Beckmann Coulter EpicXL flow cytometer (Beckman) and Explo32 software (Beckman Coulter).

**Reporter Gene Assay**

HEK 293T and GPC-16 cells were grown in 6-well plates for 18 hours and then transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. In total, 1.1 μg of plasmid DNA was used, which included 0.5 μg pISG54-luc with an ISG54 promoter-driven firefly luciferase gene, 0.1 μg of pRL-TK (Promega), a plasmid constitutively expressing renilla luciferase, and either 0.5 μg of the plasmid expressing the corresponding VP24 construct (pCMV-EBOV-VP24) or empty pCMV. Twenty-four hours post-transfection, cells were left untreated or were treated with 1,000 U/mL of IFN (human IFN-β or universal IFN-U; PBL Interferon Source). The cells were harvested 16 hours post-treatment, and firefly and renilla luciferase activities were measured using a dual luciferase assay (Dual-Glo Luciferase, Promega). Firefly luciferase values were normalized by renilla luciferase values for each set of readings.

**RESULTS AND DISCUSSION**

**Adaptive Mutations in VP24 Are Necessary and Sufficient for Increase in EBOV Virulence in Guinea Pigs**

A reverse genetics approach was used [21] to evaluate the impact of individual viral genes on the increase in virulence observed during serial passaging of EBOV in guinea pigs. First, recombinant plasmids encoding the full-length cDNA of EBOV featuring the adaptive mutations found in the guinea pig–adapted variant EBOV/8mc [19], either alone or in combination, were generated (Figure 1A). The recombinant viruses were rescued in BSR T7/5 cells and then amplified in Vero E6 cells in parallel to ensure the same passage history. The multicycle growth kinetics of recombinant viruses were first compared in Vero E6 cells. Growth curves revealed similar virus production for all rescued recombinant viruses (data not shown). To evaluate the virulence of recombinant viruses, groups of outbred Hartley guinea pigs were inoculated intraperitoneally with 5,000 infectious units of each virus, and the animals were monitored daily for changes in weight and the appearance of clinical symptoms during 21 days postinfection. Animals inoculated with the rEBOV/WT did not develop any severe symptoms of infection and survived until the end of experiment (Figure 1B). In contrast, uniform lethality was observed in groups of animals inoculated with viruses encoding VP24 with “8mc” mutations (rEBOV/8mc, rEBOV-NP-VP24/8mc, and rEBOV-VP24/8mc), including that containing only mutations in VP24 (Figure 1A and 1B). In these animals, signs of disease appeared 3–4 days postinfection, eventually leading to death or euthanization on days 5–9. These results suggest that amino acid changes in VP24 (M71I, L147P, T187I) are sufficient for the dramatic increase in EBOV virulence in guinea pigs. Inoculation of animals with recombinant viruses containing 8mc mutations in L (T820A) and/or NP (F648L) genes, rEBOV-NP/L/8mc and rEBOV-NP/8mc, did not show signs of disease except for sporadic appearances of signs of distress and the eventual death of one or 2 guinea pigs in the group inoculated with rEBOV-NP/8mc (Figure 1A). Sequence analysis of virus from the blood of these animals revealed the aa substitution L26F in the VP24. Inoculation of guinea pigs with virus from such animals resulted in their uniform death (data not shown). In order to confirm the importance of VP24 in EBOV virulence for guinea pigs, recombinant EBOV was generated that differed from rEBOV/WT by a single mutation in VP24, rEBOV-VP24/L26F (Figure 1A). Strikingly, all animals infected with rEBOV-VP24/L26F developed signs of EBOV disease approximately 4 days post-infection and either died or were euthanized on days 10–13 (Figure 1B). Importantly, no additional mutations were found...
The viruses recovered from guinea pigs inoculated with either rEBOV-VP24/8mc or rEBOV-VP24/L26F. These results strongly indicate that adaptive mutations in VP24 are necessary and sufficient for an increase in EBOV virulence in guinea pigs and emphasize the role of VP24 in EBOV pathogenesis.

Guinea pig–adapted variants of EBOV other than EBOV/8mc have also been described [17, 19, 24]. In effect, amino acid changes in VP24 have been found in all molecularly characterized guinea pig–adapted variants of EBOV. Notably, the substitutions M71I and L147P are common in EBOV/8mc and EBOV/WT.
GPA-P7 [24], whereas variant EBOV-K5 [19] contains the substitution (H186Y) that is adjacent to T187I in EBOV/8mc. These observations suggest that certain changes in VP24 are required for an increase in EBOV virulence to occur. However, the finding that the substitution L26F is also sufficient suggests that modifications in the VP24 of guinea pig–adapted variants of EBOV are likely to affect the structure of this protein. Interestingly, VP24 mutations are also associated with the adaptation of EBOV to mice [20] but also require an additional mutation of NP. In this study we have demonstrated that the “8mc” mutation in NP per se is not required for the increase in pathogenicity but seems to lead to the accelerated selection of a lethal form of EBOV in which a single mutation in VP24 was able to confer this trait.

Adaptive Mutations in VP24 Restore Efficient Replication of EBOV in Guinea Pigs

Since the pathology of filovirus hemorrhagic fevers is characterized by extensive hepatic involvement [1, 12, 25], liver specimens from guinea pigs infected with rEBOV/WT, rEBOV-VP24/8mc, or rEBOV-VP24/L26F were subjected to immunohistological analysis to evaluate disease severity. Infection of guinea pigs with

![Figure 2. Immunohistochemical analysis of liver from 2 guinea pigs infected with either rEBOV/WT, rEBOV-VP24/8mc, or rEBOV-VP24/L26F. Sections were immunostained with anti-VP40 monoclonal antibodies counterstained by hematoxylin and represent different magnifications.](image-url)
nonadapted wild-type EBOV is known to be characterized by granuloma-like inflammation foci in the liver of infected animals. These foci, composed of monocytes/macrophages, were proposed to play an important role in virus clearance [18]. Immunohistological analysis using anti-VP40 antibodies revealed that in the liver of rEBOV/WT-infected animals small and sparsely located areas containing virus-infected cells were present (Figure 2). These were encircled by noninfected macrophages and thus resembled the granuloma-like inflammation foci described earlier [18]. However, due to the diffused character of the staining it was difficult to conclude if the hepatocytes were indeed infected with virus. In the liver of guinea pigs infected with either rEBOV-VP24/8mc or rEBOV-VP24/L26F, both single infected cells and large foci of infection were seen throughout the liver (Figure 2). Remarkably, numerous infected hepatocytes were also observed in animals inoculated with these 2 viruses. In attempt to identify which cells are involved in viral replication within the infection foci, we performed an electron microscopy analysis (Figure 3). In the liver of rEBOV/WT-infected guinea pigs only a small number of infected macrophages were observed. At 5 days postinfection, these macrophages revealed inclusion bodies containing viral nucleocapsids, a characteristic feature of EBOV infection [26]. Later postinfection, macrophages with at least some signs of infection exhibited aberrant inclusions with atypically condensed or aggregated nucleocapsids (Figure 3). No free virions were found in the extracellular space at early or late stages of infection. Most strikingly, we failed to observe any signs of rEBOV/WT infection in hepatocytes. On the contrary, in the liver of rEBOV-VP24/8mc–infected animals free virus particles were frequently observed, and viral inclusion bodies with viral nucleocapsids were found in both macrophages and hepatocytes (Figure 3). Thus, the lack of virulence exhibited in infections of guinea pigs by EBOV carrying VP24/WT is tied to an inability of the virus to replicate in and/or to be released efficiently from macrophages and even more so hepatocytes. This suggests that varying levels of impairment are associated with different cell types. Failure to massively produce infectious particles by the wild-type EBOV is likely to result in the host defense successfully blocking further virus spread (granuloma-like inflammation foci) and in allowing subsequent virus clearance. Adaptive changes in VP24 restore the ability of EBOV to productively replicate in both macrophages and hepatocytes and thus facilitate systemic virus spread in the infected animals that results in high virulence.

It was also of particular interest to investigate the ability of rEBOV/WT and rEBOV-VP24/8mc to replicate in primary peritoneal guinea pig macrophages. In parallel experiments Vero E6 cells and guinea pig macrophages were infected at a similar MOI with either rEBOV/WT or rEBOV-VP24/8mc. As previously mentioned, both viruses showed similar growth kinetics in Vero E6 cells (Figure 4). Analysis of culture supernatants collected from infected macrophages revealed radical differences
between the 2 viruses. Recombinant EBOV-VP24/8mc replicated in macrophages almost as efficiently as in Vero E6 cells. In sharp contrast, no increase in virus titers was observed during 6 days postinfection with rEBOV/WT (Figure 4). Flow cytometry analysis demonstrated that the number of macrophages expressing EBOV GP did not increase during 4 days postinfection with rEBOV/WT remaining at the level of ~20%, indicating that this virus did not propagate and spread in these macrophages (Figure 4). On the contrary, 4 days postinfection a majority of the macrophages expressed EBOV GP in the case of rEBOV-VP24/8mc, which initially (day 1 postinfection) also showed ~20% of infected cells. These data confirm the ability of rEBOV-VP24/8mc to replicate and spread in primary peritoneal macrophages. An apparent reduction in total number of cells, in this case, is explained by the death of virus-infected cells late postinfection (Figure 4). Western blot analysis, in agreement with the flow cytometry, showed that the levels of NP and VP24 expression at day 1 postinfection are very similar for both viruses, but the viruses showed dramatic differences at day 4 postinfection (Figure 4). An increase in the levels of the viral proteins in cell lysates and culture medium was detected with rEBOV/8mc, whereas no such increase was seen in the lysates from rEBOV/WT-infected macrophages and no viral proteins were detected in the culture supernatants. Taken together, the data obtained indicate that despite a similar capacity to infect peritoneal macrophages, replication of rEBOV/WT in these cells was severely impaired, resulting in the absence of infectious virus release.

In an attempt to understand the nature of the differences between the recombinant viruses, electron microscopy analyses of the primary guinea pig macrophages infected with either virus were performed. As expected, macrophages infected with rEBOV-VP24/8mc showed characteristic inclusion bodies containing viral nucleocapsids (Figure 4). Contrarily, we failed to detect typical viral inclusions in macrophages infected with rEBOV/WT. In this case, massive protein inclusions were present, which did not, however, contain typical viral nucleocapsids (Figure 4). These data correlate with the absence of rEBOV/WT release from the primary macrophages and suggest that wild-type VP24 is somehow incapable of participating in assembly of viral nucleocapsids. The role of VP24 in nucleocapsid assembly has been addressed in several studies, but it has
not as yet been clearly defined [27–29]. Nucleocapsid-like structures in cells transiently expressing viral proteins resemble those formed in virus-infected cells for which the synthesis of the 3 viral proteins NP, VP35, and VP24 is indispensable [28]. In this regard it is reasonable to propose that VP24 participates in the assembly and/or the proper condensation of viral nucleocapsids. It has also been shown that reduction of VP24 expression by specific siRNAs prevents both the formation of viral nucleocapsids and the release of EBOV in virus-infected cells [30]. While an increase in virulence in guinea pigs could be promoted by only mutations in VP24, the presence of mutations in both NP and VP24 of rodent-adapted viruses [19, 20, 24] pointed to a possible interaction between these proteins.
VP24/WT and VP24/8mc Are Similar in Their Ability to Block Expression of IFN-induced Genes

The VP24 protein of EBOV has been demonstrated to interfere with type I and type II IFN signaling pathways by interacting with the cellular karyopherins α-1, α-5, and α-6 and by blocking STAT1 translocation into nuclei [31–33]. It had also been speculated that VP24’s ability to counteract the IFN system plays a role in the increase of EBOV virulence during the virus’s adaptation to mice [20]. Interestingly, the substitution L147P found in the VP24 of guinea pig-adapted EBOV/8mc is located in close proximity to the domain encompassing amino acids 142–146, which recently was shown to be involved in karyopherin binding and required for an efficient control of the IFN response [33]. Thus it was of interest to investigate whether adaptive mutations described in this study affect the IFN antagonist function of VP24. For that purpose, plasmids expressing VP24/WT, VP24/8mc, and VP24/L26F were generated and used in an ISG54 promoter-driven firefly luciferase gene assay. To assess the ability of recombinant VP24 proteins to inhibit IFN signaling 2 different cell lines were used: human HEK 293T and guinea pig GPC-16 (Figure 5). Data obtained demonstrated that the ability of VP24 to inhibit IFN signaling was not significantly affected by the mutations associated with EBOV adaptation to the guinea pig model. In both human and guinea pig cells VP24/WT was even more efficient in inhibiting activation of the IFN-induced promoter than both guinea pig-adapted variants of VP24 (Figure 5). Previously it was proposed that adaptive mutations are required for efficient control of the IFN response by EBOV in the mouse model [20]. In effect, this hypothesis correlates well with data indicating that abrogation of the type I IFN system makes adult mice susceptible to EBOV infection [16, 34]. However, replication of rEBOV/WT and mouse-adapted ZEBOV in mouse macrophages was similarly affected by IFN treatment, which reduced the virus titers by 2 log for both viruses [20]. Moreover, in agreement with our findings, VP24/WT and VP24 of mouse-adapted EBOV were shown to be similar in their ability to block IFN-induced gene expression [32].

In conclusion, in this study we demonstrate that an increase in virulence during serial passaging of EBOV in guinea pigs is associated with changes in the structural protein VP24 but is not linked with the IFN-antagonist function of this protein and can be explained by an inability to control assembly of viral nucleocapsids in infected macrophages. Since the effect of mutations in VP24 on EBOV replication has an apparently species-specific character, involvement of an as yet unidentified cellular protein in the process of nucleocapsids assembly is predicted. We also hypothesize that the same mechanisms are involved in the resistance of guinea pigs to wild-type EBOV replication as in asymptomatic EBOV infections in man [14, 15] and are associated with the ability of primary cell targets to control EBOV replication.

Funding

This work was supported by INSERM, Ministère français de la Recherche (04G5537), Agence Nationale de la Recherche (ANR-07-MIME-006-01), Fondation pour la Recherche Médicale en France (FRM DMI20091117323), and Deutsche Forschungsgemeinschaft (SFB 593 to V. V.). M. M. was supported by Région Rhône-Alpes Cluster 10 "Infectiologie" and by a bourse FRM FDT20090916821.

Acknowledgments

We thank D. Burton and P. Parren for providing the human monoclonal anti-GP antibody KZ52. We are grateful to Hugues Contamin and Philipp Loth (Pasteur Institute, Lyon) for providing technical support in...
conducting experiments with the virus-infected animals and to members of the Centre Technique des Microstructures (UCBL1) and Anipath for help in experiments with electron microscopy and immunohistochemistry. We also thank Christopher F. Basler, Lawrence W. Leung, and St. Patrick Reid for helpful discussions and Robin Buckland for critically reviewing the manuscript.

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