

Two Mutations Were Critical for Bat-to-Human Transmission of Middle East Respiratory Syndrome Coronavirus

Yang Yang,^a Chang Liu,^a Lanying Du,^b Shibo Jiang,^{b,c} Zhengli Shi,^d Ralph S. Baric,^e Fang Li^a

Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota, USA^a; Lindsley F. Kimball Research Institute, New York Blood Center, New York, New York, USA^b; Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai, China^c; Center for Emerging Infectious Diseases, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China^d; Department of Epidemiology, University of North Carolina, Chapel Hill, North Carolina, USA^e

To understand how Middle East respiratory syndrome coronavirus (MERS-CoV) transmitted from bats to humans, we compared the virus surface spikes of MERS-CoV and a related bat coronavirus, HKU4. Although HKU4 spike cannot mediate viral entry into human cells, two mutations enabled it to do so by allowing it to be activated by human proteases. These mutations are present in MERS-CoV spike, explaining why MERS-CoV infects human cells. These mutations therefore played critical roles in the bat-to-human transmission of MERS-CoV, either directly or through intermediate hosts.

Since its emergence in 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) has infected over 1,100 people, with a case fatality rate of ~38% (1, 2). Bats are considered the natural reservoir of MERS-CoV because several coronaviruses, including HKU4, have been isolated from bats and are genetically related to MERS-CoV (3–5). An envelope-anchored spike protein mediates coronavirus entry into host cells. It first binds a host receptor through its S1 subunit and then fuses membranes through its S2 subunit (6–8). For membrane fusion, the spike must be cleaved at the S1/S2 boundary by one or more host proteases (9–13). We recently showed that both HKU4 and MERS-CoV spikes recognize host receptor dipeptidyl peptidase 4 (DPP4) (14). Furthermore, only MERS-CoV spike, and not HKU4 spike, mediates viral entry into human cells because the former, but not the latter, can be activated by human endogenous proteases (14). Here we further identified two residue differences between the two spikes that account for their different capabilities to mediate viral entry into human cells. Our results have revealed a likely evolutionary pathway for the emergence of MERS-CoV as a human pathogen, either from bats directly or through intermediate hosts.

By comparing the sequences of MERS-CoV and HKU4 spikes, we identified two regions in MERS-CoV spike that may serve as human protease target motifs but that differ from the corresponding regions in HKU4 spike (Fig. 1). The first region is a motif for human proprotein convertases (hPPC motif) (15, 16), but the critical Arg748 in MERS-CoV spike corresponds to Ser746 in the HKU4 spike, which deviates from the hPPC motif. The second region is a motif for human endosomal cysteine proteases (hECP motif) (17, 18), but the motif Ala763-Phe764-Asn765 in MERS-CoV spike corresponds to Asn762-Tyr763-Thr764 in HKU4 spike, which likely introduces an N-linked glycosylation site and blocks the access of human endosomal cysteine proteases. These residue differences in the two human protease motifs between MERS-CoV and HKU4 spikes may affect the capabilities of the spikes to mediate viral entry into human cells.

To evaluate the potential genetic changes required for HKU4 to infect human cells, we reengineered HKU4 spike, aiming to build its capacity to mediate viral entry into human cells. To this end, we introduced two single mutations, S746R and N762A, into HKU4 spike. The S746R mutation was expected to restore the

hPPC motif in HKU4 spike, whereas the N762A mutation likely disrupted the potential N-linked glycosylation site in the hECP motif in HKU4 spike. To confirm that the S746R mutation restored the hPPC motif, we produced retroviruses pseudotyped with HKU4 spike (referred to as HKU4 pseudoviruses) in human cells and showed that HKU4 spike containing the S746R mutation was partially cleaved during the molecular maturation process, whereas wild-type HKU4 spike remained intact (Fig. 2A). Confirming that the N762A mutation disrupted the N-linked glycosylation site in the hECP motif was technically challenging because of the large size and heavy glycosylation of HKU4 spike (the trimeric HKU4 spike has 78 predicted N-linked glycosylation sites and a total molecular mass of ~530 kDa). Nevertheless, we managed to identify a slight downward shift of HKU4 spike with the N762A mutation using Western blot analysis (Fig. 2B), consistent with successful removal of the N-linked glycosylation site. We do not currently have direct evidence to show that the spikes are cleaved right at the hPPC and hECP motifs by human proteases. However, both of the hPPC and hECP motifs in the spikes have been well documented to be the cleavage sites for human proteases (16–18). Moreover, mutations in these motifs in coronavirus spikes have demonstrated dramatic effects on viral entry into human cells (see below). Thus, the identified hPPC and hECP motifs are likely to be cleaved by human proteases, although we cannot completely rule out the possibility that alteration of these motifs may affect protease cleavages at a spatially adjacent site on the spikes. In either case, our study revealed that both of these motifs play crit-

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Address correspondence to Fang Li, lifang@umn.edu.

Y.Y. and C.L. contributed equally to this article.

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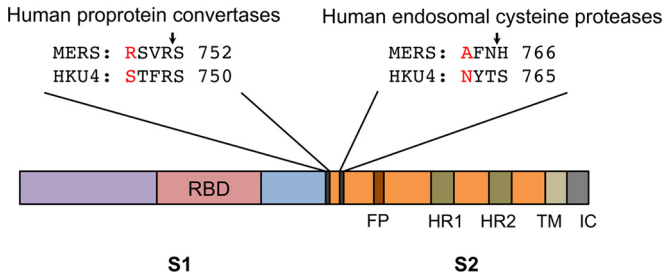


FIG 1 Domain structure of MERS-CoV and HKU4 spike proteins. The spikes contain a receptor-binding S1 subunit, a membrane-fusion S2 subunit, a transmembrane anchor (TM), and an intracellular tail (IC). S1 contains the receptor-binding domain (RBD) that binds DPP4 receptor. S2 contains the fusion peptide (FP), heptad repeat 1 (HR1), and heptad repeat 2 (HR2), all of which are essential structural elements for the membrane fusion process. The S1/S2 boundary in MERS-CoV spike (defined as the region between the RBD and the fusion peptide) contains one established human protease motif that is recognized by proprotein convertases (hPPC) (15, 16); it also contains one established human protease motif that is recognized by endosomal cysteine proteases (hECP) (17, 18). Sequence alignments of these regions in MERS-CoV spike; *AFS88936.1* for MERS-CoV spike; *ABN10839.1* for HKU4 spike) are shown, with the critical residue differences labeled in red. Arrows indicate the predicted sites of cleavage by human proteases in MERS-CoV spike.

ical roles in HKU4- and MERS-CoV-spike-mediated entry into human cells (see below).

We examined the capability of the mutant HKU4 spike to mediate viral entry into three types of human cells (Fig. 3A for HEK293T cells; data not shown for Huh-7 and MRC-5 cells), using a pseudovirus entry assay as previously described (14). In the absence of exogenous protease trypsin, HKU4 pseudoviruses bearing either the reengineered hPPC motif or the reengineered hECP motif were able to enter human cells, whereas HKU4 pseudoviruses bearing both of the reengineered human protease motifs entered human cells as efficiently as when activated by exogenous trypsin (Fig. 3A). In contrast, wild-type HKU4 pseudoviruses failed to enter human cells. Therefore, the reengineered hPPC and hECP motifs enabled HKU4 spike to be activated by human endogenous proteases and thereby allowed HKU4 pseudoviruses to bypass the need for exogenous proteases to enter human cells. These results reveal that HKU4 spike needs only two single mutations at the S1/S2 boundary to gain the full capacity to mediate viral entry into human cells.

To confirm the functions of the reengineered human protease motifs in HKU4 spike, we used protease inhibitors to probe the human proteases that activate HKU4-spike-mediated viral entry into human cells. Human proprotein convertase (PPC) inhibitor and endosomal cysteine protease (ECP) inhibitor both blocked human cell entry of HKU4 pseudoviruses bearing the reengineered hPPC motif and the reengineered hECP motif, respectively (Fig. 3B). This result verified that the gained capability of HKU4 pseudoviruses to enter human cells was due to the reengineered human protease motifs in HKU4 spike.

To investigate the functions of the two human protease motifs in MERS-CoV-spike-mediated entry into human cells, we reengineered MERS-CoV spike in the opposite way, aiming to reduce or eliminate its capability to mediate viral entry into human cells. To this end, we introduced an R748S single mutation and A763N/F764Y/N765T triple mutations into MERS-CoV spike (Fig. 1).

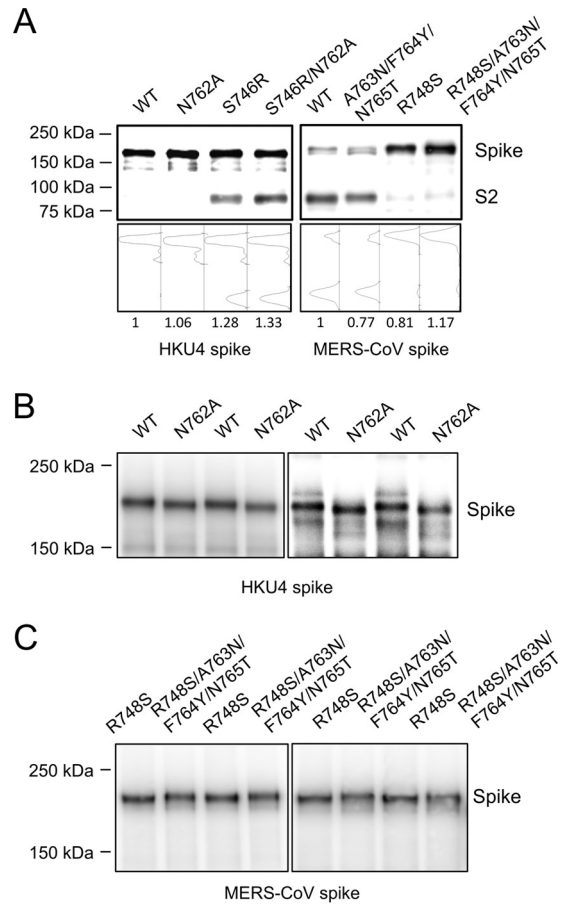


FIG 2 Characterization of two protease motifs in MERS-CoV and HKU4 spike proteins. (A) Western blot analysis of HKU4 and MERS-CoV spikes in pseudovirus particles. Retroviruses pseudotyped with HKU4 spike (i.e., HKU4 pseudoviruses) or MERS-CoV spike (i.e., MERS-CoV pseudoviruses) were prepared in HEK293T (human embryonic kidney) cells as previously described (14). The incorporations of wild-type (WT) and mutant HKU4 and MERS-CoV spikes into pseudovirus particles were measured by Western blotting using antibody against their C-terminal C9 tags. Plots below the Western blot images correspond to quantifications of the band intensities from the cleaved and uncleaved spikes combined. The numbers below the plots indicate the relative amounts of spikes incorporated into pseudovirus particles compared to wild-type HKU4 and MERS-CoV spikes, respectively. All quantifications were done using ImageJ software (National Institutes of Health). (B) Glycosylation state of HKU4 spike at the hECP motif. HEK293T cells exogenously expressing wild-type (WT) and mutant HKU4 spikes were lysed and subjected to Western blot analysis. To improve the separation of high-molecular-mass spikes, 3 to 8% NuPAGE Tris-acetate gels (Life Technologies) were used for gel electrophoresis. To improve the accuracy of the result, each of the mutant and wild-type spikes was run in two lanes that alternated between samples. The experiment was also repeated in a separate gel. Compared with wild-type HKU4 spike, the downward shift in the band of HKU4 spike bearing the mutated hECP motif (i.e., mutation N762A) is consistent with the removal of glycosylation. (C) Glycosylation state of MERS-CoV spike at the hECP motif. Compared with MERS-CoV spike bearing the reengineered hPPC motif (i.e., mutation R748S), the upward shift in the band of MERS-CoV spike bearing both the reengineered hPPC and hECP motifs (i.e., mutations R748S/A763N/F764Y/N765T) indicates the introduction of glycosylation.

The R748S mutation destroyed the hPPC motif in MERS-CoV spike (Fig. 2A), whereas the A763N/F764Y/N765T triple mutations successfully added an N-linked glycosylation site to the hECP motif in MERS-CoV spike (Fig. 2C). MERS-CoV pseudo-

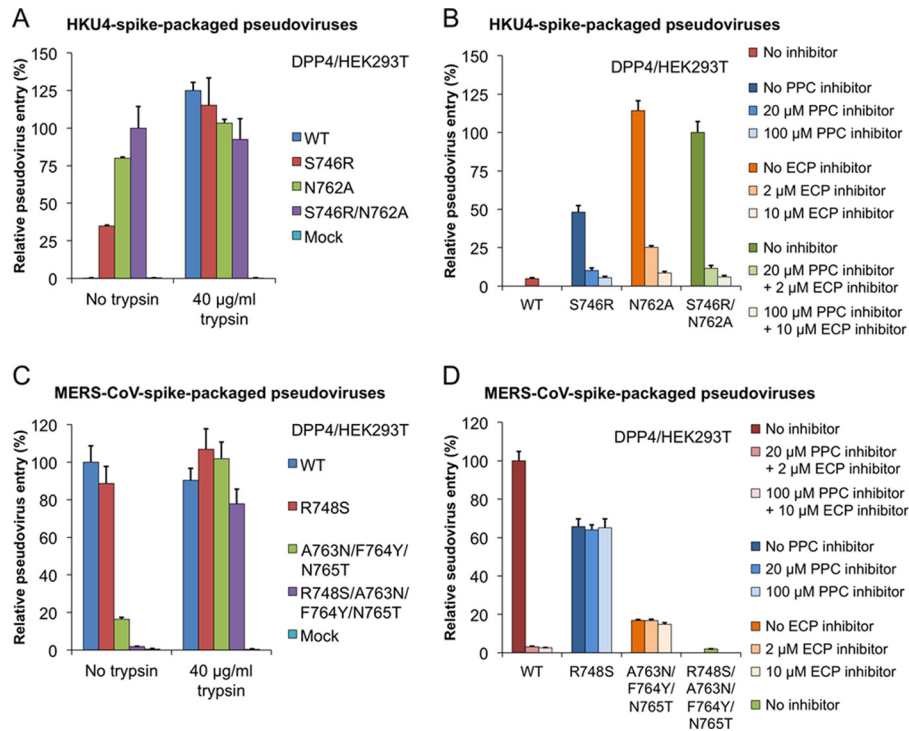


FIG 3 HKU4- and MERS-CoV-spike-mediated pseudovirus entry in human cells. (A) HKU4 pseudoviruses bearing no mutation, the reengineered hPPC motif (S746R), the reengineered hECP motif (N762A), or both of the reengineered motifs (S746R/N762A) were prepared in HEK293T (human embryonic kidney) cells and were then used to infect HEK293T cells exogenously expressing human DPP4 (GenBank accession no. [NP_001926.2](#)). The infections were carried out in the presence or absence of exogenous trypsin. The pseudovirus entry efficiencies were characterized by analysis of the levels of luciferase activity accompanying the entry and were normalized against the relative amounts of spikes incorporated into pseudovirus particles (Fig. 2A). The pseudovirus entry mediated by HKU4 spike bearing both of the reengineered motifs in the absence of exogenous trypsin was taken as 100%. (B) Pseudovirus-producing HEK293T cells were treated with proprotein convertase (PPC) inhibitor (dec-RVKR-CMK) 5 h after transfection of plasmids that encode HKU4 spike containing the reengineered hPPC motif (S746R). Pseudovirus-targeting HEK293T cells were treated with endosomal cysteine protease (ECP) inhibitor (E-64d) before being infected by HKU4 pseudoviruses bearing the reengineered hECP motif (N762A). Both of the inhibitors were used for HKU4 pseudoviruses bearing both of the mutations. (C) MERS-CoV pseudoviruses bearing no mutation, the mutated hPPC motif (R748S), the mutated hECP motif (A763N/F764Y/N765T), or both of the mutated motifs (R748S/A763N/F764Y/N765T) were used to infect HEK293T cells exogenously expressing human DPP4. The pseudovirus entry mediated by the wild-type MERS-CoV spike in the absence of exogenous trypsin was taken as 100%. (D) Pseudovirus-producing HEK293T cells were treated with dec-RVKR-CMK 5 h after transfection of plasmids that encode MERS-CoV spike containing the mutated hPPC motif (R748S). Pseudovirus-targeting HEK293T cells were treated with E-64d before being infected by MERS-CoV pseudoviruses bearing the mutated hECP motif (A763N/F764Y/N765T). Both of the inhibitors were used for MERS-CoV pseudoviruses bearing both of the mutated motifs. Error bars indicate standard errors of the means (SEM) ($n = 4$).

viruses bearing either the mutated hPPC motif or the mutated hECP motif demonstrated a decreased capability to enter three types of human cells (Fig. 3C for HEK293T cells; data not shown for Huh-7 and MRC-5 cells). MERS-CoV pseudoviruses bearing both of the mutated human protease motifs were unable to enter human cells. Exogenous trypsin was able to fully rescue the capability of mutant MERS-CoV pseudoviruses to enter human cells. Moreover, PPC inhibitor and ECP inhibitor both blocked human cell entry of wild-type MERS-CoV pseudoviruses but had no effect on human cell entry of MERS-CoV pseudoviruses bearing the mutant hPPC motif and the mutant hECP motif, respectively (Fig. 3D). Thus, the mutations in the hPPC and hECP motifs together eliminated MERS-CoV-spike-mediated viral entry into human cells. These results demonstrate that the two functional human protease motifs in MERS-CoV spike played a critical role in the bat-to-human transmission of MERS-CoV.

After examining HKU4- and MERS-CoV-spike-mediated viral entry into human cells, we investigated how these spikes mediate viral entry into bat cells. Because of the low transfection efficiency of bat cells, we were unable to package pseudoviruses in bat cells.

Instead, we packaged HKU4 and MERS-CoV pseudoviruses in HEK293T cells and subsequently performed HKU4- and MERS-CoV-spike-mediated pseudovirus entry into two types of bat cells: RSKT (*Rhinolophus sinicus* bat kidney cells) and Tb1-Lu cells (*Tadarida brasiliensis* bat lung cells) (Fig. 4). Wild-type HKU4 pseudoviruses entered bat cells efficiently, whereas HKU4 pseudoviruses bearing the reengineered hECP motif (which removed the N-linked glycosylation site) demonstrated an enhanced capability to enter bat cells (Fig. 4A and B). On the other hand, wild-type MERS-CoV pseudoviruses entered bat cells efficiently, whereas MERS-CoV pseudoviruses bearing the mutant hECP motif (which added the N-linked glycosylation site) demonstrated a reduced, but still significant, capability to enter bat cells (Fig. 4C and D). Moreover, most of HKU4- and MERS-CoV-spike-mediated pseudovirus entry into bat cells could be blocked by the ECP inhibitor, also suggesting that bat endosomal cysteine proteases activate coronavirus spikes bearing a glycosylated hECP motif (Fig. 4). These results indicate that, unlike human endosomal cysteine proteases, bat endosomal cysteine proteases are capable of recognizing and efficiently cleaving the hECP motif containing a glyco-

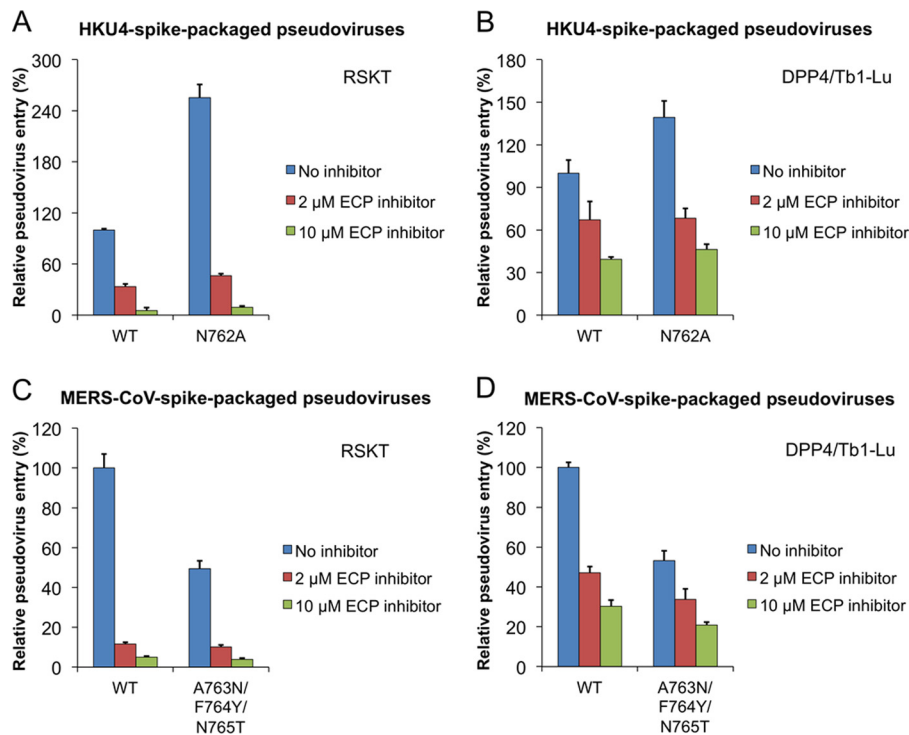


FIG 4 HKU4- and MERS-CoV-spike-mediated pseudovirus entry into bat cells. (A) HKU4 pseudoviruses bearing no mutation or the reengineered hECP motif (N762A) were prepared in HEK293T cells and were then used to infect RSKT cells endogenously expressing bat DPP4 (*Rhinolophus sinicus* bat kidney cells [24]). The cells were pretreated with indicated concentrations of ECP inhibitor (E-64d) before being infected by pseudoviruses. (B) The same HKU4 pseudoviruses were used to infect Tb1-Lu cells (*Tadarida brasiliensis* bat lung cells) exogenously expressing bat DPP4 (GenBank accession no. KC249974). (C) MERS-CoV pseudoviruses bearing no mutation or the mutant hECP motif (A763N/F764Y/N765T) were prepared in HEK293T cells and were then used to infect RSKT cells. (D) The same MERS-CoV pseudoviruses were used to infect Tb1-Lu cells. The pseudovirus entry efficiencies were characterized by analysis of the levels of luciferase activity accompanying the entry and were normalized against the relative amounts of spike proteins incorporated into pseudovirus particles. The pseudovirus entry mediated by the wild-type HKU4 spike (for panels A and B) or wild-type MERS-CoV spike (for panels C and D) in the absence of the inhibitor was taken as 100%. Error bars indicate SEM ($n = 4$).

ylation site. The molecular and structural differences between human and bat hECPs accounting for their functional differences will be investigated in future research.

Understanding the molecular mechanisms for cross-species transmissions of viruses is critical for evaluating their emerging disease potentials and for preventing or controlling their spread in human populations. Here we examined the different cell entry activities of the spike proteins from human-infecting MERS-CoV and a closely related bat coronavirus, HKU4. Although MERS-CoV and HKU4 spikes share high sequence homology and recognize the same host DPP4 receptor, only MERS-CoV spike, and not HKU4 spike, mediates viral entry into human cells. Our study revealed that introduction of two single mutations, S746R and N762A, into HKU4 spike at the S1/S2 boundary fully instilled its capability to mediate viral entry into human cells. MERS-CoV spike already contained both of these mutations, explaining why MERS-CoV is capable of infecting human cells. Thus, these two mutations in the spike are essential for MERS-CoV to transmit from bats to humans by allowing MERS-CoV spike to be activated by human cellular proteases. Viral adaptation to human cellular proteases is critical for viral infection of human cells because human cellular proteases, particularly endosomal proteases, are more reliable sources than some extracellular proteases to activate viral entry. Previous research also identified two mutations in SARS-CoV spike that led SARS-CoV to transmit from palm civets

to humans (19–22). These mutations increased the capability of SARS-CoV spike to bind human receptor angiotensin-converting enzyme 2. Thus, different entry factors appear to have played the most critical roles in the cross-species transmission of MERS-CoV and SARS-CoV: adaption to human cellular proteases by MERS-CoV and adaption to human receptor by SARS-CoV. Although MERS-CoV spike might also need to adapt to human DPP4 receptor upon infecting human cells (14, 23), such adaptations might have only incremental effects on the infectivity of MERS-CoV in human cells. In contrast, the two mutations adaptive to human cellular proteases transformed MERS-CoV spike from completely lacking to fully possessing the capacity to mediate viral entry into human cells, and thus they likely played the most critical role in the bat-to-human transmission of MERS-CoV, either directly or through intermediate hosts.

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