Recurrent emergence of SARS-CoV-2 spike deletion H69/V70 and its role in the variant of concern lineage B.1.1.7


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Wuhan-hu-1
B.1.1.7

Spike
Cleaved Spike
ACE2

S1/S2 cleavage
Spike incorporation
Infectivity
Fusogenicity

WT-PV
ΔH69/V70-PV

69 70
A I H V S G

GCTATACATGTCTCTGGG
GCTAT------------CTCTGGG

"up" RBD
"down" RBD
NTD
4A8 epitope
29-77 loop
Recurrent emergence of SARS-CoV-2 spike deletion H69/V70 and its role in the variant of concern lineage B.1.1.7

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Abstract
We report SARS-CoV-2 spike ΔH69/V70 in multiple independent lineages, often occurring after acquisition of the receptor binding motif replacements such as N439K and Y453F known to increase binding affinity to the ACE2 receptor and confer antibody escape. *In vitro*, we show that whilst ΔH69/V70 itself is not an antibody evasion mechanism, it increases infectivity associated with enhanced incorporation of cleaved spike into virions. ΔH69/V70 is able to partially rescue infectivity of S proteins that have acquired N439K and Y453F escape mutations by increased spike incorporation. In addition, replacement of H69 and V70 residues in B.1.1.7 spike (where ΔH69/V70 naturally occurs) impairs spike incorporation and entry efficiency of B.1.1.7 spike pseudotyped virus. B.1.1.7 spike mediates faster kinetics of cell-cell fusion than wild type Wuhan-1 D614G, dependent on ΔH69/V70. Therefore, as ΔH69/V70 compensates for immune escape mutations that impair infectivity, continued surveillance for deletions with functional effects is warranted.
**Background**

SARS-CoV-2’s spike surface glycoprotein engagement of human angiotensin-converting enzyme (hACE2) is essential for virus entry and infection (Zhou et al., 2020), and the receptor is found in respiratory and gastrointestinal tracts (Sungnak et al., 2020). Despite this critical interaction and the imposed constraints, it appears the receptor binding domain (RBD) is relatively tolerant to mutations (Starr et al., 2020b; Thomson et al., 2020), raising the real possibility of virus escape from past infection or vaccine-induced immunity (Cele et al., 2021; Collier et al., 2021; Gupta, 2021; Madhi et al., 2021) and monoclonal antibody treatments (Starr et al., 2021). Spike mutants exhibiting reduced susceptibility to neutralising antibodies have been identified in *in vitro* screens (Greaney et al., 2021; Greaney et al., 2020; Starr et al., 2020a), and some of these mutations have been found in clinical isolates (Choi et al., 2020).

Studying SARS-CoV-2 chronic infections can give insight into virus evolution that would require many chains of acute transmission to generate. This is because the majority of infections arise as a result of early transmission during pre or asymptomatic phases prior to peak adaptive responses, and virus adaptation not observed as the virus is usually cleared by the immune response (He et al., 2020; Mlcochova et al., 2020b). We recently documented *de novo* emergence of antibody evasion mutations mediated by S gene mutations in an individual treated with convalescent plasma (CP) (Kemp et al., 2021). In addition, a chronically infected immune suppressed individual was recently reported in Russia with emergence of Y453F, along with ΔH69/V70 (Bazykin et al., 2021). Deletions in other parts of the N-Terminal Domain (NTD) have been reported to arise in chronic infections (Choi et al., 2020) and to reduce sensitivity to NTD-specific neutralising antibodies (McCallum et al., 2021; McCarthy et al., 2021).

Here we analyse global SARS-CoV-2 data and find ΔH69/V70 occurs independently often emerging after a significant RBD amino acid replacement such as Y453F and N439K, that are known to facilitate neutralising antibody escape or to alter ACE2 binding whilst incurring an infectivity defect according to some reports (Motozono et al., 2021; Sungnak et al., 2020).
Although structural modelling indicates the H69/V70 is in an exposed loop that contracts post deletion, potentially altering an antigenic site, we report that the ΔH69/V70 does not confer significantly reduced susceptibility to convalescent sera or mAb. Functionally, we find that ΔH69/V70 does increase spike infectivity and compensates for an infectivity defect resulting from RBD replacements N439K and Y453F. The infectivity increase is driven by higher levels of spike incorporation into virions. We demonstrate that the deletion is required for optimal infectivity of the 501Y.V1 (B.1.1.7) spike protein. We show that although B.1.1.7 and wild type (WT) spike pseudotyped virus (PV) have similar infectivity on a range of target cell types, the B.1.1.7 spike protein alone induces more rapid cell-cell fusion and formation of multinucleated cells. Repair of the two amino acids leads to not only reduced B.1.1.7 S incorporation into virions and impaired infectivity, but also reduces cell-cell fusion kinetics back to WT levels.

Results

Multiple occurrences and transmission of spike ΔH69/V70 with and without S mutations

The deletion H69/V70 is present in over 600,000 SARS-CoV-2 genome sequences worldwide, and has seen global expansion, particularly across much of Europe, Africa and Asia (Figure 1). ΔH69/V70 is observed in multiple global lineages (Figure 1A). While variants with deletions in this region of spike are observed in GISAID, the earliest unambiguous sequences that include the ΔH69/V70 were detected on a D614 background in January 2020 (USA and Thailand). The earliest ΔH69/V70 detected on a D614G background was in Sweden in April 2020. Prevalence of ΔH69/V70 has increased since August 2020 (Figure 1B). Further analysis of sequences revealed, firstly, that single deletions of either H69 or V70 were uncommon and, secondly, some lineages of ΔH69/V70 alone were present, as well as ΔH69/V70 in the context of other mutations in spike, specifically those in the RBM (Figure 1).

ΔH69/V70 is not a neutralising antibody escape mechanism

We hypothesised that ΔH69/V70 might confer reduced susceptibility to neutralising antibodies. We first examined the protein structural context of ΔH69/V70 for clues regarding alterations in epitopes (Figure 2A, B). In the absence of experimentally derived structural data for ΔH69/V70, the protein structure of the NTD possessing the double deletion was
modelled *in silico*. The ΔH69/V70 deletion was predicted to alter the conformation of a protruding loop comprising residues 69 to 76, pulling it in towards the NTD (Figure 2B). In the post-deletion structural model, the positions of the alpha carbons of residues either side of the deleted residues, Ile68 and Ser71, were each predicted to occupy positions 2.9Å from the positions of His69 and Val70 in the pre-deletion structure. Concurrently, the positions of Ser71, Gly72, Thr73, Asn74 and Gly75 are predicted to have changed by 6.5Å, 6.7Å, 6.0Å, 6.2Å and 8Å, respectively, with the overall effect of these residues moving inwards, resulting in a less dramatically protruding loop.

This predicted change in the surface of spike could be consistent with antibody evasion. To test this we explored whether ΔH69/V70 conferred reduced susceptibility to neutralising antibodies in sera from fifteen recovered individuals (Figure 2C, D). We performed serial dilutions of sera before mixing with lentiviral particles pseudotyped with Spike proteins with and without ΔH69/V70 (with virus input normalised for infectivity). We plotted infection of target cells as a function of serum dilution (Figure 2D). All but two sera demonstrated clear titratable neutralisation of both wild type and ΔH69/V70 virus. There was no overall change in susceptibility to serum neutralisation for ΔH69/V70 relative to wild type (Figure 2C), but there was a proportion of individuals with slightly increased neutralisation sensitivity of ΔH69/V70 (Figure 2C, D). To further explore the role for ΔH69/V70 in inducing immune escape, we tested the binding of 12 NTD-mAbs to wildtype and ΔH69/V70 NTD by biolayer interferometry (Figure 2E-G). All the NTD-mAbs showed less than 2-fold decrease in binding to ΔH69/V70 compared to WT. These data suggest that ΔH69/V70 does not represent an important antibody escape mechanism.

**ΔH69/V70 spike enhances infectivity associated with increased cleaved S incorporation**

We hypothesised that the deletion might alternatively enhance virus infectivity. In the absence of virus isolates we used a lentiviral PV approach to test the impact of ΔH69/V70 on virus spike protein mediated entry. A D614G bearing Wuhan-1 spike expressing DNA plasmid (WT) was co-transfected in HEK 293T producer cells along with plasmids encoding lentiviral capsid and genome for luciferase. Infectivity was adjusted for input reverse transcriptase activity; we observed a two-fold increase in PV infectivity of ΔH69/V70 as compared to WT in HeLa cervical epithelial cells stably expressing human ACE2 (Figure 3A, B). We observed similar fold increases with ΔH69/V70 in a range of other target cells, both
in context of over-expression of ACE2/TMPRSS2 (HEK 293T cells transiently transfected with ACE2, or ACE2 and TMPRSS2 and A549 lung cells stably expressing ACE2 and TMPRSS2 (Rihn et al., 2021)) or endogenous levels of receptors in Calu-3 lung adenocarcinoma cells (Figure 3A).

Western blotting for S2 spike indicated that a higher amount of cleaved spike in ΔH69/V70 bearing virions and in the 293T producer cell lysates. We also noted a corresponding reduction in uncleaved full length (FL) spike (Figure 3C). Densitometric analysis of spike and p24 from western blots in multiple experiments showed almost a two-fold increase in spike:p24 ratio as well as an increased ratio in S2:FL cleavage for the ΔH69/V70, indicating increased spike incorporation into virions might explain the increase in infectivity (Figure 3D, E). To verify that this increased in S from producer cells was not specific to HEK 293T cells we also transfected the human lung epithelial cell line H1299 (Zhang et al., 2020) with spike and lentiviral packaging plasmids. We again observed that viruses from these cells had a two fold increased infectivity in target cells (Figure 3F). In addition the increased total and cleaved S levels were recapitulated both in the cell lysates and purified virions from these lung cells (Figure 3G, H). Therefore we conclude that the increased S cleavage and its incorporation observed in producer cells and pseudotyped virions is a generalised phenomenon for ΔH69/V70 S. In order to explore whether D614G was required for this enhanced spike cleavage and infectivity, we generated PV bearing D614 spike with and without the ΔH69/V70 followed by infection in HEK293T cells. We observed a similar two fold enhancement of infection and a proportional increase in spike incorporation as we did for D614G spike pseudotyped viruses (Supplementary Figure 1A, B). Finally, to exclude the possibility that increased incorporation of S was specific for pseudotyped lentiviral particles, we generated coronavirus-like particles by co-transfection of WT or ΔH69/V70 S with SARS-CoV-2 (M)embrane, (E)nvelope and (N)ucleocapsid proteins as previously described (Swann et al., 2020; Yurkovetskiy et al., 2020). Compared with levels of N, levels of cleaved S in coronavirus-like particles were again enhanced in the presence of the ΔH69/V70 deletion (Supplementary Figure 1 C,D).

Enhanced infectivity of ΔH69/V70 spike is not correlated with cleavage or entry route

SARS-CoV-2 entry into target cells is thought to take place by two distinct routes following binding to ACE2 (Figure 4A). Firstly an endosomal route where cathepsin is able to cleave
spike with pH dependent fusion in the endosome. The second route involves fusion at the plasma membrane with cleavage via the plasma membrane associated protease TMPRSS2.

In order to determine the mechanism by which increased spike cleavage in the context of ΔH69/V70 might impact entry, we used inhibitors of furin cleavage (CMK) and protease inhibitors specific to endosomal (ED64D) and plasma membrane fusion (camostat) entry routes (Figure 4A). We firstly treated producer cells with CMK and found that indeed CMK inhibits spike S1/S2 cleavage in the producer cells transfected with S ΔH69/V70 plasmid, and that the spikes with altered S1/S2 cleavage are incorporated onto the virions (Figure 4B). We found that CMK treatment, whilst reducing S1/S2 cleavage, did not decrease the PV infection in a variety of target cells (Figure 4C) suggesting the increased infectivity in ΔH69/V70 is not due to more efficient cleavage of spike. To confirm our findings, we generated a spike lacking the polybasic cleavage site with or without ΔH69/V70 and tested PV infectivity on 293T cells overexpressing ACE2 and TMPRSS2. We found that deletion of the PBCS led to increased infectivity of the PV, as observed previously for mutated PBCS. As expected, deletion of the PBCS did not alter the enhancing effect of ΔH69/V70 on PV infectivity (Figure 4D).

The altered level of S1/S2 cleavage in SARS-CoV-2 has been linked to its dependence on viral entry through either membrane fusion or endocytosis in 293T and A549 cells (Peacock et al., 2020; Winstone et al., 2021). We therefore hypothesised that the increased spike cleavage of ΔH69/V70 S could influence the route of entry. To probe this, spike pseudotyped lentiviruses bearing either WT spike, ΔH69/V70 spike or VSV-G were next used to transduce 293T-ACE2 or 293T-ACE2/TMPRSS2 cells in the presence of either E64D or camostat at different drug concentrations (Figure 4E). As expected, the VSV-G pseudotyped particles were not affected by addition of either E64D or camostat. Consistent with previous observations, WT PV utilised endocytosis in the absence of TMPRSS2 (Peacock et al., 2020) but where TMPRSS2 was expressed, plasma membrane fusion became the dominant route (Papa et al., 2021; Winstone et al., 2021). However, there were no differences between WT and ΔH69/V70 in relative utilisation of the endosomal versus plasma membrane entry routes. We conclude that the enhanced spike cleavage, whilst notable in ΔH69/V70, does not appear responsible for the increased infectivity of ΔH69/V70 spike observed in these cell line based experiments.
ΔH69/V70 spike compensates for reduced infectivity of RBD escape mutants

We next examined in greater detail the SARS-CoV-2 lineages where S mutations in the RBD were identified at high frequency and where ΔH69/V70 co-occurs. For example, N439K, an amino acid replacement reported to be defining variants increasing in numbers in Europe and other regions (Thomson et al., 2020) (Figure 1 and 5A) now mostly co-occurs with ΔH69/V70. N439K appears to have reduced susceptibility to some convalescent sera as well as monoclonal antibodies targeting the RBD, whilst increasing affinity for ACE2 in vitro (Thomson et al., 2021). The first lineage possessing N439K (and not ΔH69/V70), B.1.141 is now extinct (Thomson et al., 2020). A second lineage with N439K, B.1.258, later emerged and subsequently acquired ΔH69/V70 leading to the initial rapid increase in the frequency of viruses possessing this deletion, spreading into Europe (Figure 1A) (Brejová et al., 2021).

The second significant cluster with ΔH69/V70 and RBD mutants involves Y453F, another spike RBD mutation that increases binding affinity to ACE2 (Starr et al., 2020b) and has been found to be associated with mink-human infections (Munnik et al., 2020). Y453F has also been described as an escape mutation for mAb REGN10933 and shows reduced susceptibility to convalescent sera (Baum et al., 2020; Hoffmann et al., 2021), and is possibly a T cell escape mutation (Motozono et al., 2021). The ΔH69/V70 was first detected in the Y453F background on August 24th 2020 and thus far appears limited to Danish sequences (Figure 1, Figure 5B), although an independent acquisition was recently reported along with ΔH69/V70 in an immune compromised Russian individual with chronic infection (Bazykin et al., 2021).

We hypothesised that ΔH69/V70 might have arisen after Y453F and N439K in order to compensate for potential loss of infectivity that has been reported for these RBD mutants previously (Motozono et al., 2021; Sungnak et al., 2020). We therefore generated mutant spike plasmids bearing RBD mutations Y453F and N439K (Figure 5C) both with and without ΔH69/V70 and performed infectivity assays in the lentiviral pseudotyping system. RBD mutations reduced infectivity of Spike relative to WT by around 2 fold (Figure 5D) and was partially rescued by ΔH69/V70. Based on observations of the impact of ΔH69/V70 on spike incorporation in WT (Figure 3D), we predicted that the mechanism of increased infectivity for ΔH69/V70 in context of RBD mutations might be similar. The analysis of virions from cell supernatants and cell lysates indeed showed increased ratio of spike:p24 (Figure 5E, F).
As observed for WT, we also observed increased cleaved S2:FL when ΔH69/V70 was present along with the RBD mutants in PV (Figure 5E, G).

**ΔH69/V70 is required for optimal B.1.1.7 spike S2 incorporation and infectivity**

A lineage containing the ΔH69/V70 deletion was first detected in the UK with the RBD mutation N501Y along with multiple other spike and other mutations (Figure 1, Supplementary Figure 2). These UK sequences were subsequently named as B.1.1.7, termed a variant of concern (VOC), as they are associated with higher transmission rate (Volz et al., 2021b). Subsequently B.1.1.7 has spread rapidly to over 100 countries exemplifying a new chapter in the pandemic, with additional VOCs detected in other geographical locations. In addition to RBD N501Y + NTD ΔH69/V70, B.1.1.7 is defined by further S mutations across S2 (T716I, S982A and D1118H), and S1 (ΔY144, A570D, P681H), see figure 6A (Rambaut A., 2020). The available sequence data did not enable determination of whether the B.1.1.7 mutations N501Y + ΔH69/V70 arose as a result of a N501Y virus acquiring ΔH69/V70, or vice versa, though a UK B.1.1.7 sequence was identified with N501Y, A570D, ΔH69/V70 and D1118H (Supplementary Figure 2).

In order to ascertain whether H69V70 represented a target for neutralising antibodies in the context of B.1.1.7, we first tested 12 NTD-specific mAbs isolated from 4 individuals that recovered from WT SARS-CoV-2 infection with an in-vitro PV neutralisation assay using WT SARS-CoV-2 S, the B.1.1.7 S or the B.1.1.7 S with reversion of H69/V70 deletions (B.1.1.7 H69/V70) pseudotyped viruses in VeroE6 target cells expressing TMPRSS2. (Supplementary Table 2). We found that 7 out of 12 NTD-specific mAbs (58%) showed a marked decrease or complete loss of neutralising activity to both B.1.1.7 and B.1.1.7 H69/V70 (>30 fold-change reduction), suggesting that in a sizeable fraction of NTD antibodies the H69/V70 deletion is not responsible for their loss of neutralising activity (Supplementary Figure 3). The remaining 5 mAbs showed a partial reduction (2-to-10 fold) in B.1.1.7 neutralisation that was not rescued by reversion of H69/V70 deletions.

Given our data on introduction of ΔH69/V70 into WT (Figure 3), we hypothesised that ΔH69/V70 was selected in the evolution of B.1.1.7 in order to increase viral entry. We predicted that the replacement of H69 and V70 would impair the infectivity of B.1.1.7 PV and reduce total spike levels. To examine this, we compared the infectivity of B.1.1.7 spike
PV versus B.1.1.7 PV with H69 and V70 restored to B.1.1.7 spike. We observed that infectivity of B.1.1.7 infectivity was slightly lower than WT (Figure 6B). As expected, we observed a significant reduction in infectivity for viruses where the H69 and V70 had been re-inserted across a number of cell types, including H1299 expressing endogenous levels of ACE2 and TMPRSS2 receptors (Figure 6B, C). When we measured spike incorporation into virions we found that the reduced infectivity of the B.1.1.7 with replaced H69 V70 was associated with reduced spike:p24 and S2:FL ratio as expected (Figure 6D-G).

B.1.1.7 spike mediates faster syncytium formation and is ΔH69/V70 dependent

Previous reports have shown that SARS-CoV-2 spike protein localises to the cell host plasma membrane and possesses high fusogenic activity, triggering the formation of large multinucleated cells (named syncytia) in vitro and in vivo potentially providing an additional and a more rapid route for virus disseminating among neighbour cells (Bussani et al., 2020; Cattin-Ortolá et al., 2020; Papa et al., 2021). The role of syncytium formation in viral replication and pathogenesis of severe COVID-19 has been reported and may be druggable process to treat COVID-19 pathology (Braga et al., 2021). We expressed B.1.1.7 spike and a B.1.1.7 with restored H69 and V70 together with the mCherry fluorescent protein in 293T cells and labelled Vero cells with a green fluorescent dye (Figure 7A). All spike constructs showed similar protein expression and achieved similar cell-cell fusion by 16 hours. B.1.1.7 appeared to mediate more cell-cell fusion events over earlier time points with the colour overlap area being 2-3 times greater for B.1.1.7 as compared to wild type at 6 hours post mixing. Interestingly, this enhancement was abrogated by re-insertion of H69 and V70 residues (Figure 7B-D). We conclude that B.1.1.7 spike mediates faster fusion kinetics than wild type bearing D614G Wuhan-1 spike, and that this is dependent on ΔH69/V70.

ΔH69/V70 does not enhance infectivity of bat coronavirus RaTG13 spike

Finally, to investigate the importance of this part of spike beyond SARS-CoV-2 to other coronaviruses with zoonotic potential, we examined the 69/70 region of spike in a set of other known sarbecoviruses (Supplementary Figure 4A-C). We observed substantial variability in the region, resulting in frequent indels, with some viruses including SARS-CoV having 6-7 amino acid deletions (Supplementary Figure 4B). This is indicative of high structural plasticity in this protein region that could allow the sarbecoviruses to alter their Spike conformation. RaTG13 is the evolutionary closest relative to SARS-CoV-2 for this region,
and after RaTG13 is the cluster of 5 CoVs sampled in trafficked pangolins in the Guangxi province (Lam et al., 2020). Inspection of the 69/70 region in these virus sequences raises the interesting observation that one of the five viruses in the cluster, P2V, has amino acids 69H and 70L present, while the other four have a double amino acid deletion (Supplementary Figure 4A-C). Given that SARS-CoV-2 and RaTG13 have the homologous HV insertion at these positions, one explanation is that the proximal common ancestor between SARS-CoV-2 and the Guangxi pangolin cluster had the insertion, which was then lost while circulating in the pangolin population, similar to observations with SARS-CoV-2 in humans. Yet, the fact that P2V was cultured in Vero E6 cells prior to sequencing (contrary to the other 4, sequenced directly from the pangolin sample) raises the possibility of this being an independent insertion, favoured as a monkey cell line specific adaptation. Interestingly, the double amino acid indel in the pangolin viruses is in-frame in contrast to SARS-CoV-2.

Furthermore, the two almost identical bat viruses recently sequenced from Cambodia samples – RShSTT182 and 200 (Hul et al., 2021) possess an H69V70 insertion despite being more distantly related to SARS-CoV-2 for this region of Spike (Supplementary Figure 4A-C). This independent occurrence of the insertion is suggestive of context dependent selective pressures playing a role in recurring gain and loss of these two residues in the Sarbecoviruses. To test whether the fitness effect associated with acquisition of ΔH69/V70 is specific to SARS-CoV-2 and not other Sarbecovirus spike backgrounds, we cloned full length S from RaTG13 and generated pseudotyped lentiviruses expressing RaTG13 Spike protein as well as a RaTG13 SΔH69/V70 counterpart. We observed that cleaved and uncleaved S expression levels in PV did not differ between WT and the ΔH69/V70 RaTG13 spike, and that there was no difference in infectivity on target cells expressing ACE2 or both ACE2 and TMPRSS2 (Supplementary Figure 4D-E). This result suggests that as one would expect, the enhancing effect of ΔH69/V70 on spike levels and infectivity is specific to spike-background.

Discussion
We have presented data demonstrating multiple, independent, and circulating lineages of SARS-CoV-2 variants bearing spike ΔH69/V70. This recurring deletion spanning six nucleotides is due to an out of frame deletion of six nucleotides, and occurs in the terminal loop of a helix loop motif within the predicted RNA structure, as do other NTD deletions observed in new variants such as the UK B.1.1.7, South African B.1.351, Brazilian
P.1(Supplementary Figure 5). Stable helix loop motifs are associated with pausing/dissociation events in reverse transcriptase(Harrison et al., 1998). Since all nucleic acid polymerases have a common ancestor with homologous dNTP binding motifs and similar global structures(Delarue et al., 1990; Ollis et al., 1985; Sousa et al., 1993) it is probable that all RNA polymerases use similar mechanisms for transcript termination(Reeder and Lang, 1994). A recent in-cell biochemical analysis of SARS-CoV2 RNA structure showed nucleotide reactivity consistent with this model within these stem-loops(Huston et al., 2021). These analyses provide a rationale for preferential emergence of ΔH69/V70 and other deletions such as the well described NTD-antibody escape deletion ΔY144(Chi et al., 2020; McCarthy et al., 2020, 2021) (in B.1.1.7 and the recently reported B.1.525) at the terminal loops of helical loop motifs. ΔH69/V70 itself has frequently followed immune escape associated amino acid replacements in the RBD (eg N439K and Y453F), and is specifically found in the B.1.1.7 variant known to have higher transmissibility(Volz et al., 2021a) and possibly pathogenicity(Davies et al., 2021).

We find that ΔH69/V70 does not significantly reduce sensitivity of spike to neutralising antibodies in serum from a group of recovered individuals or binding of multiple mAb directed against the NTD. In addition we have shown that repair of ΔH69/V70 does not appreciably alter the potency of NTD antibodies against the B.1.1.7 spike. Thus the deletion is unlikely to represent an immune escape mechanism. Instead, our experimental results demonstrate that the ΔH69/V70 deletion is able increase infectivity of Wuhan-1 D614G spike pseudotyped virus, as well as pseudotyped virus bearing the additional RBD mutations N439K or Y453F, explaining why the deletion is often observed after these immune escape mutations that carry infectivity cost(Motozono et al., 2021; Sungnak et al., 2020). We show that the mechanism of enhanced infectivity across the RBD mutations tested is associated with greater spike incorporation into virions where the ΔH69/V70 deletion is present. The phenotype is also independent of producer cell used. Importantly, we were able to recapitulate the ΔH69/V70 phenotype in a spike protein that did not have the D614G mutation, indicating that D614G is not involved in the mechanism. These observations are supported by ΔH69/V70 being observed in D614 viruses in Jan 2020 both in the US and Thailand. Although we did not use a replication competent system, a recent pre-print reports ΔH69/V70 mutated Washington strain virus isolate as conferring increased replication in cell lines and higher viral loads in hamsters(Liu et al., 2021). Indeed, ΔH69 has been observed in
cell culture during remdesivir selection experiments with replication competent virus, consistent with a replication advantage (Szemiel et al., 2021).

We have found consistent differences in S, as well as cleaved S, in the producer cell and its incorporation into PV particles when comparing ΔH69/V70 to a Wuhan-1 spike (both with D614G). This could be explained by stability during intracellular trafficking or the route taken to the surface, differences in post-translational modification of the S-protein, or membrane characteristics at the site of virus budding or virus like particles. As the amount of S incorporation into virions reflects the S in the cells, virion formation is likely unaffected by ΔH69/V70. Interestingly, although pharmacological inhibition of furin by CMK in producer cells did prevent S1/S2 cleavage, and altered the balance of S2:FL spike in cells and virions, PV infectivity was not reduced by drug treatment. These data suggested that the increase in entry efficiency conferred by S ΔH69/V70 is independent of spike S1/S2 cleavage. Similar findings on lack of relationship between balance of S2:FL and infectivity were reported in the context of furin knockout cells rather than furin inhibition with CMK (Papa et al., 2021). In addition, a recent report on the S1/S2 cleavage site mutation P681H demonstrated enhanced cleavage of Spike P681H was not associated with increased PV infectivity or cell fusion relative to WT (Lubinski et al., 2021). There may however be differences in vivo.

We explored entry route of ΔH69/V70 spike using cathepsin inhibition to block endosomal entry and camostat to block entry via plasma membrane fusion. ΔH69/V70 spike was equally sensitive to camostat and the cathepsin inhibitor ED64 as WT, arguing that the efficiency of entry route usage is similar despite differences in cleaved spike. Although S1/S2 cleavage allows avoidance of endosome-associated IFITM restriction and appears critical for transmission in animal models (Peacock et al., 2020), cleaved spike may be less infectious if S1 is shed prematurely, therefore possibly conferring a disadvantage under some circumstances. In addition, Peacock et al showed that S1/S2 cleavage in the producer cell, conferred by a polybasic stretch at the cleavage site, is advantageous in cells expressing abundant TMPRSS2 but deleterious in cells lacking TMPRSS2 (Peacock et al., 2020).

The recently emergent lineage (B.1.1.7), bearing seven S mutations is now responsible for a new pandemic phase with a demonstrably more pathogenic (Davies et al., 2021) and transmissible variant (Volz et al., 2021a). The detection of a high number of novel mutations
suggests this lineage has either been introduced from a geographic region with very poor sampling or viral evolution may have occurred in a single individual in the context of a chronic infection (Kemp et al., 2020).

We show that whilst B.1.1.7 spike has similar infectivity as wild type D614G spike, consistent with data on live B.1.1.7 virus in human airway epithelial cells (Brown et al., 2021), but in contrast to another study that showed a difference in live virus with the 8 spike mutations (Liu et al., 2021). Importantly, however, we demonstrate loss of infectivity when the H69/V70 amino acids are replaced in B.1.1.7 S, accompanied as expected by reduced S1/S2 cleavage and reduced S incorporation into virions. These data point to epistatic interactions between observed mutations in spike of B.1.1.7, with a trade-off between mutations that incur virus entry cost with those that contribute to other activities such as immune evasion.

Of greatest potential importance is our observation that B1.1.7 spike mediates faster syncytium formation and that this enhanced cell-cell fusion activity is dependent on ΔH69/V70. Syncytium formation is a key feature of severe and fatal COVID-19 (Bussani et al., 2020), and implicated in elevated viral replication (Braga et al., 2021). We speculate that the increased fusogenicity of B.1.1.7 spike may contribute to higher mortality (Davies et al., 2021) and transmissibility of B.1.1.7 (Volz et al., 2021a).

**Limitations**

While we have combined epidemiological, evolutionary, protein and RNA structure and experimental data in our study, a limitation is the experiments were conducted with pseudotyped viruses (PV) and coronavirus like particles, rather than replication competent viruses. We also carried out experiments in cells overexpressing receptors, although results were recapitulated in lung cell lines expressing endogenous levels of ACE2 and TMPRSS2.

The detection and surveillance of B.1.1.7 has been facilitated in the UK by the phenomenon of SGTF (S gene target failure) due to primers in the Thermofisher SARS-CoV-2 diagnostic qPCR assay used by a significant number of testing facilities. The S gene target (binding in the region of H69/V70) is one of three and therefore a marker for the spread of B.1.1.7 has been tracked by the loss of signal in the S gene target (Volz et al., 2021b). However recent reports from the US and central Europe caution against use of SGTF as a sole marker for
B.1.1.7 detection as a significant ΔH69/V70 lineage without other mutations in spike is circulating in the US, and a B.1.258 lineage with N439K with ΔH69/V70, circulating in Slovakia/Czech republic (Brejová et al., 2021; Larsen and Worobey, 2020). Such examples highlight the need for genome sequencing to accompany novel approaches to diagnostics for variants.

Given the emergence of multiple clusters of variants carrying RBD mutations and the ΔH69/V70 deletion, limitation of transmission takes on a renewed urgency. As a further example, a new variant of concern bearing ΔH69/V70 with E484K was recently identified (B.1.525). Comprehensive vaccination efforts should be accelerated in order to limit transmission and acquisition of further mutations, and future vaccines could include ΔH69/V70 in order to close this route for virus evolution, assuming that effective neutralising antibodies to this region are generated. Fortunately, our experiments with RaTG13 demonstrate that the ΔH69/V70 may not enhance infectivity of other bat Sarbecoviruses with zoonotic potential.

In summary we have found that a two amino acid deletion, ΔH69/V70 promotes SARS-CoV-2 spike incorporation into viral particles and increases infectivity by a mechanism that remains to be fully explained. This deletion has arisen multiple times and often after spike antibody escape mutations that reduce spike mediated entry efficiency. Critically, B1.1.7 spike mediates faster syncitium formation and this enhanced cell-cell fusion activity is dependent on ΔH69/V70. In addition, B.1.1.7 spike requires ΔH69/V70 for optimal infectivity and we conclude therefore that ΔH69/V70 enables SARS-CoV-2 to tolerate multiple immune escape mutations whilst maintaining infectivity and fusogenicity.

Author contributions

Declarations of interest
A.D.M., C.S., K.C., E.C., L.P. and D.C. are employees of Vir Biotechnology and may hold shares in Vir Biotechnology. RKG has received consulting fees from UMOVIS lab, Gilead Sciences and ViiV Healthcare, and a research grant from InvisiSmart Technologies.

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Figure titles and legends

Figure 1. A. Global phylogeny of SARS-CoV-2 whole genome sequences highlighting those with specific mutations in spike: ΔH69/V70, N439K, Y453F and N501Y. The tree is subsampled and tips are coloured by geographic region (see key). Grey bars on the right show the presence or absence of the deletion ΔH69/V70 (del) and amino acid variants N439K, Y453F, and N501Y. PANGO Lineages are shown. Cumulative occurrences of SARS-CoV-2 sequences with the ΔH69/V70 deletion by month for B. ΔH69/V70 with or without N439K/ Y453F and C. ΔH69/V70 with N501Y. Indicated frequencies by month of the ΔH69/V70 deletion are from the GISAID database (accessed 21st May 2021) by reporting country and sampling date.
Figure 2. Spike ΔH69/V70 does not reduce sensitivity to neutralising antibodies. Surface representation of spike homotrimer in open conformation (PDB: 7C2L) with each monomer shown in different shades of grey. On the monomer shown positioned to the right, the exposed loop consisting of residues 69-77 is shown in cyan and the neutralising antibody (4A8) binding NTD epitope in magenta. B. Prediction of conformational change in the spike N-terminal domain due to deletion of residues His69 and Val70. The pre-deletion structure is shown in cyan, except for residues 69 and 70, which are shown in red. The predicted post-deletion structure is shown in green. Residues 66-77 of the pre-deletion structure are shown in stick representation and coloured by atom (nitrogen in blue, oxygen in coral). Yellow lines connect aligned residues 66-77 of the pre- and post-deletion structures and the distance of 6 Å between aligned alpha carbons of Thr73 in the pre- and post-deletion conformation is labelled. C. Neutralisation of spike ΔH69/V70 pseudotyped virus and wild type (D614G background) by convalescent sera from 15 donors. GMT (geometric mean titre) with s.d presented representative of two independent experiments each with two technical repeats. Wilcoxon matched-pairs signed rank test, ns not significant. D. ten example neutralisation curves. Indicated is serum log_{10} inverse dilution against % neutralisation. Data points represent means of technical replicates and error bars represent standard deviation. Curves are representative of two independent experiments. E-G. Kinetics of binding to wildtype and ΔH69/V70 NTD of 12 NTD-specific mAbs. E, Biolayer interferometry analysis of binding to wildtype (WT, black) and WT ΔH69/V70 (red) NTDs by 12 NTD-targeting mAbs. Dotted lines separate association phase from dissociation phase. Shown is 1 representative experiment out of 2 independent experiments. F. Side-by-side comparison of binding to WT (black) and ΔH69/V70 (red) NTDs by 11 NTD-targeting mAbs. Binding is shown as area under the curve (AUC). S2L28 mAb is not shown due to too little response measured (< 0.10 nm). G. Binding to NTD of the 11 mAbs shown in B expressed as fold change of AUC of WT compared to ΔH69/V70. Data are representative of two independent experiments (n=2).

Figure 3. Spike ΔH69/V70 enhances entry and is accompanied by increased spike S2 incorporation into virions. A. Single round infectivity on different cell targets by spike ΔH69/V70 v WT pseudotyped virus produced in 293T cells. Data are representative of three independent experiments. Data are shown with mean and standard error of mean (SEM) and the statistics were performed using unpaired Student t test. B. Infectivity of ΔH69/V70 pseudotyped virus on target HeLa cells transduced with ACE2, expressed as fold change relative to WT. C-E. Western blots and quantification of virions with infectivity shown in B.
and of cell lysates of 293T producer cells following transfection with plasmids expressing lentiviral vectors and SARS-CoV-2 S \( \Delta H69/V70 \) versus WT (all with D614G), probed with antibodies for HIV-1 p24 and SARS-Cov-2 S2. **D.** Quantification of spike:p24 ratio in supernatants for wild type virus with \( \Delta H69/V70 \) deletion versus WT alone across multiple replicate experiments. Mean and SEM are shown; one sample t-test *** p<0.001. **E.** Quantification of cleaved S2 spike: full length spike for wild type virus with \( \Delta H69/V70 \) deletion versus WT alone in virions and cell lysates. Each data point represents a single experiment. **F.** Infectivity of \( \Delta H69/V70 \) pseudotyped virus produced in H1299 lung epithelial cells on target 293T cells transiently expressing ACE2 and TMPRSS2. **G.** Western blots of virions and cell lysates of H1299 lung epithelial producer cells following transfection with plasmids expressing lentiviral vectors and SARS-CoV-2 S \( \Delta H69/V70 \) versus WT (all with D614G). The statistical analysis was performed using unpaired Student t test. **H.** Quantification of S2:FL ratio in purified virions from H1299 lung epithelial producer cells. Data from at least two independent experiments are shown (n=2).

**Figure 4. Route of SARS-CoV-2 S mediated virus entry in cell lines is not altered by \( \Delta H69/V70 \) spike.** **A.** Schematic diagram illustrates spike in producer cells with CMK targeting and blocking furin cleavage (left panel). In target cells camostat inhibits TMPRSS2 and therefore cell fusion at the plasma membrane, and E64D blocks cathepsins and targets the endocytic viral entry (right panel). **B.** Western blots show CMK inhibits spike S1/S2 cleavage in the producer cells transfected with S \( \Delta H69/V70 \) plasmid, and the spikes with altered S1/S2 cleavage are incorporated onto the virions. Antibodies against HIV-1 p24 and Spike S2 were used with anti-GAPDH as a loading control. **C.** The viruses produced from the transfected 293T cells in the presence of CMK were used to transduce target cells. The luciferase reading (RLU) is used as a surrogate for the spike infectivity bearing with various S2/FL ratios. The data shown are the technical quadruplicates from one experiment and statistical analysis was done using unpaired t test. **D.** Comparison of infectivity of spike with polybasic cleavage site deleted (\( \Delta PBCS \)) with and without \( \Delta H69/V70 \). Impact of \( \Delta H69/V70 \) is independent of PBCS. **E.** \( \Delta H69/V70 \) deletion does not alter the virus entry route. S pseudotyped lentiviruses bearing either wt S, \( \Delta H69/V70 \) S or VSV-G was used to transduce 293T-ACE2 or 293T-ACE2/TMPRSS2 cells in the presence of either E64D or camostat at different drug concentrations. The cells were then harvested after 2 days and assayed for luciferase expression, which was then normalised against the non-drug control (set as 100%).
The data shown are technical duplicates and are representative of at least two independent experiments (n=2).

Figure 5: ΔH69/V70 appears after spike N439K and Y453F and compensates for their reduced infectivity. A. Maximum likelihood phylogeny of global sequences carrying Spike mutant A. N439K and B. Y453F. All sequences in the GISAID database containing S:439K or S: Y453F (18th February 2021) were downloaded, realigned to Wuhan-Hu-1 using MAFFT and deduplicated. C. Representation of Spike RBM:ACE2 interface (PDB: 6M0J) with residues N439, Y453 and N501 highlighted as spheres coloured by element. D-F. Spike mutant ΔH69/V70 compensates for infectivity defect of Spike RBD mutations and is associated with increased Spike incorporation into virions. D. Infectivity of spike (D614G) ΔH69/V70 deletion in absence and presence of Spike RBD mutations. Single round infection by luciferase expressing lentivirus pseudotyped with SARS-CoV-2 spike protein on target HeLa cells stably transduced with ACE2. Mean and SEM are shown. E. Representative western blot of purified virions and cell lysates probed with antibodies against HIV-1 p24, SARS-CoV-2 spike S2 and GAPDH. F, G. Densitometric quantification of F. spike:P24 and G. cleaved S2 spike:full length spike ratio for spike (D614G) ΔH69/V70 deletion in absence and presence of Spike RBD mutations across multiple experiments in pelleted virus. RLU – relative light units; U – unit of reverse transcriptase activity (RT). Data are representative of 2 independent experiments (n=2). Student t test *** p<0.001

Figure 6. Spike ΔH69/V70 in B.1.1.7 enhances spike infectivity. A. Surface representation of spike homotrimer in open conformation with one upright RBD overlaid with ribbon representation (PDB: 6ZGG, Wrobel et al., 2020), with different monomers shown in black, pale blue and gold. The deleted residues H69 and V70 and the residues involved in amino acid substitutions (501, 570, 716, 982 and 1118) and the deletion at position 144 are coloured red on each monomer and labelled on the monomer with an upright RBD shown in black. Scissors mark approximate location of an exposed loop (residues 677-688), containing the furin cleavage site, and including residue 681, which is absent from the structure. B. A representative infectivity of B.1.1.7 with replacement of H69 and V70 versus B.1.1.7 containing spike ΔH69/V70 and wild type (D614G) spike is shown. Single round infection by luciferase expressing lentivirus pseudotyped with SARS-CoV-2 Spike protein on HeLa cells transduced with ACE2. The data represent technical quadruplicates. C. Fold change of luciferase expression over the replacement of H69/V70 in ACE2, ACE2 and Tmprss2
transfected 293T cells, A459-ACE2/TMPRSS2, and H1299 cells. The data shown were from three independent experiments with each in technical triplicates (one sample t test). D. A representative western blot analysis following transfection of 293T cells with spike and lentiviral plasmids. Virion loading was normalized for input virus using reverse transcriptase activity. Antibodies against HIV-1 p24 and Spike S2 were used with anti-GAPDH as a loading control. S2 to FL spike was analysed by densitometry and the S2/FL cleavage ratio was calculated for virions (E) and for cell lysates (F). G. Quantification of spike:p24 ratio for B.1.1.7 and B.1.1.7 with H69/V70 replacement across three independent experiments (n=3). ** p<0.01, ****p<0.0001.

Figure 7: ΔH69/V70 significantly accelerates cell-cell fusion activity of B.1.1.7 spike protein. A. Schematic of cell-cell fusion assay (created with biorender.com). B Reconstructed images at 6 hours of 293T cells co-transfected with the indicated Spike mutants and mCherry expressing plasmid mixed with green dye-labelled Vero acceptor cells. Scale bars represent 100 mm. Green colour identifies the acceptor cells while red colour marks donor cells. Merged green-red colours indicate the syncytia. C Quantification of cell-cell fusion kinetics showing percentage of green and red overlap area over time. Mean is plotted with error bars representing SEM. D. Quantification of cell-cell fusion of the indicated Spike mutants at 6 hours post transfection. Mean is plotted with error bars representing SEM. E. Representative western blot of cells transfected with the indicated Spike mutants (detected with anti-S2 antibody). The S2 subunit is indicated with the arrowhead. beta actin is shown as loading control. Data are representative of at least three independent experiments (n=2). *p<0.05 unpaired t test

STAR Methods

RESOURCE AVAILABILITY

Lead Contact
Further information should be directed to and will be fulfilled by the Lead Contact, Ravindra K. Gupta rkg20@cam.ac.uk.

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Raw anonymised data are available from the lead contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The study was primarily a laboratory based study using pseudotyped virus (PV) with mutations generates by site directed mutagenesis. We tested infectivity in cell lines with a range of drug inhibitors and monoclonal antibodies. Sensitivity to antibodies in serum was tested using convalescent sera from recovered individuals collected as part of the Cambridge NIHR Bioresource. We also performed phylogenetic analyses of data available publicly in GISAID.

Ethical approval

Ethical approval for use of serum samples. Controls with COVID-19 were enrolled to the NIHR BioResource Centre Cambridge under ethics review board (17/EE/0025).

METHOD DETAILS

Phylogenetic Analysis

All available full-genome SARS-CoV-2 sequences were downloaded from the GISAID database (http://gisaid.org/) (Shu and McCauley, 2017) on 16th February 2021. Low-quality sequences (>5% N regions) were removed, leaving a dataset of 491,395 sequences with a length of >29,000bp. Sequences were deduplicated and then filtered to find the mutations of interest. All sequences were realigned to the SARS-CoV-2 reference strain MN908947.3, using MAFFT v7.475 with automatic strategy selection and the --keelength --addfragments options (Katoh and Standley, 2013). Major SARS-CoV-2 clade memberships were assigned to all sequences using the Nextclade server v0.13 (https://clades.nextstrain.org/), Pangolin
v2.4.2 (Rambaut et al., 2020) (github.com/cov-lineages/pangolin) and a local instance of the PangoLEARN model, dated 18th April 21:49 (https://github.com/cov-lineages/pangoLEARN).

Maximum likelihood phylogenetic trees were produced using the above curated dataset using IQ-TREE v2.1.2 (Minh et al., 2020). Evolutionary model selection for trees were inferred using ModelFinder (Kalyaanamoorthy et al., 2017) and trees were estimated using the GTR+F+I model with 1000 ultrafast bootstrap replicates (Minh et al., 2013). All trees were visualised with Figtree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and ggtree v2.2.4 rooted on the SARS-CoV-2 reference sequence and nodes arranged in descending order. Nodes with bootstraps values of <50 were collapsed using an in-house script.

To reconstruct a phylogeny for the 69/70 spike region of the 20 Sarbecoviruses examined in Figure 5, Rdp5 (Martin et al., 2015) was used on the codon spike alignment to determine the region between amino acids 1 and 256 as putatively non-recombinant. A tree was reconstructed using the nucleotide alignment of this region under a GTR+Γ substitution model with RAxML-NG (Kozlov et al., 2019). Node support was calculated with 1000 bootstraps. Alignment visualisation was done using BioEdit (Hall et al., 2011).

Structural modelling
The structure of the post-deletion NTD (residues 14-306) was modelled using I-TASSER (Roy et al., 2010), a method involving detection of templates from the protein data bank, fragment structure assembly using replica-exchange Monte Carlo simulation and atomic-level refinement of structure using a fragment-guided molecular dynamics simulation. The structural model generated was aligned with the spike structure possessing the pre-deletion conformation of the 69-77 loop (PDB 7C2L (Chi et al., 2020)) using PyMOL (Schrödinger). Figures prepared with PyMOL using PDBs 7C2L, 6M0J (Lan et al., 2020), 6ZGE28 and 6ZGG (Wrobel et al., 2020).

RNA secondary structure modelling.
2990 nucleotides centred around the spike protein amino acids 69-70 from SARS-CoV2 sequence from an individual12 were aligned in CLUSATL-Omega (nucleotides 20277-23265 of the Wuhan isolate MN908947.3) and a consensus structure was generated using RNAalifold (Bernhart et al., 2008)).
Cells

HEK 293T CRL-3216, Vero CCL-81 were purchased from ATCC and maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100mg/ml streptomycin. All cells are regularly tested and are mycoplasma free. H1299 cells were a kind gift from Sam Cook. Calu-3 cells were a kind gift from Paul Lehner, A549 A2T2(Rihn et al., 2021) cells were a kind gift from Massimo Palmerini.

Pseudotype virus preparation

Plasmids encoding the spike protein of SARS-CoV-2 D614 with a C terminal 19 amino acid deletion with D614G, were used as a template to produce variants lacking amino acids at position H69 and V70, as well as mutations N439K and Y453F. Mutations were introduced using Quickchange Lightning Site-Directed Mutagenesis kit (Agilent) following the manufacturer’s instructions. B.1.1.7 S expressing plasmid preparation was described previously, but in brief was generated by step wise mutagenesis. Viral vectors were prepared by transfection of 293T cells by using Fugene HD transfection reagent (Promega). 293T cells were transfected with a mixture of 11µl of Fugene HD, 1µg of pCDNAΔ19 spike-HA, 1ug of p8.91 HIV-1 gag-pol expression vector and 1.5µg of pCSFLW (expressing the firefly luciferase reporter gene with the HIV-1 packaging signal). Viral supernatant was collected at 48 and 72h after transfection, filtered through 0.45um filter and stored at -80˚C as previously described. Infectivity was measured by luciferase detection in target 293T cells transfected with TMPRSS2 and ACE2.

SARS-CoV-2 D614 (Wuhan) and RaTG13 mutant plasmids and infectivity

Plasmids encoding the full-length spike protein of SARS-CoV-2 D614 (Wuhan) and RaTG13, in frame with a C – terminal Flag tag(Conceicao et al., 2020), were used as a template to produce variants lacking amino acids at position H69 and V70. The deletion was introduced using Quickchange Lightning Site-Directed Mutagenesis kit (Agilent) following the manufacturer’s instructions. Viruses were purified by ultracentrifugation; 25mL of crude preparation being purified on a 20% sucrose cushion at 2300rpm for 2 hrs at 4˚C. After centrifugation, the supernatant was discarded and the viral pellet resuspended in 600 µL DMEM (10% FBS) and stored at -80˚C. Infectivity was examined in HEK293 cells transfected with human ACE2, with RLUs normalised to RT activity present in the
pseudotyped virus preparation by PERT assay. Western blots were performed on purified virus with anti-HIV1 p24, 1:1,000 (Abcam) or anti-FLAG, 1:2,000 (Sigma) antibodies used following SDS-PAGE and transfer.

Standardisation of virus input by SYBR Green-based product-enhanced PCR assay (SG-PERT)

The reverse transcriptase activity of virus preparations was determined by qPCR using a SYBR Green-based product-enhanced PCR assay (SG-PERT) as previously described (Vermeire et al., 2012). Briefly, 10-fold dilutions of virus supernatant were lysed in a 1:1 ratio in a 2x lysis solution (made up of 40% glycerol v/v 0.25% Triton X-100 v/v 100mM KCl, RNase inhibitor 0.8 U/ml, TrisHCL 100mM, buffered to pH7.4) for 10 minutes at room temperature.

12µl of each sample lysate was added to thirteen 13µl of a SYBR Green master mix (containing 0.5µM of MS2-RNA Fwd and Rev primers, 3.5pmol/ml of MS2-RNA, and 0.125U/µl of Ribolock RNAse inhibitor and cycled in a QuantStudio. Relative amounts of reverse transcriptase activity were determined as the rate of transcription of bacteriophage MS2 RNA, with absolute RT activity calculated by comparing the relative amounts of RT to an RT standard of known activity.

Generation of Coronavirus-like particles and western blotting

Plasmids encoding codon-optimised (M)embrane, (E)nvelope and (N)ucleocapsid proteins of SARS-CoV-2 were a kind gift from Nevan Krogan (M, pLVX-EF1alpha-SARS-CoV-2-M-2xStrep-IRES-Puro, Addgene #141386; E, pLVX-EF1alpha-SARS-CoV-2-E-2xStrep-IRES-Puro, Addgene #141385; N, pLVX-EF1alpha-SARS-CoV-2-N-2xStrep-IRES-Puro, Addgene #141391) (Gordon et al., 2020). For expression in 293T cells, M, E and N were each subcloned into a modified pmaxGFP (Lonza) vector as M/E/N-IRES-GFP. Plasmids encoding the (S)pike protein of SARS-CoV-2 with a C-terminal plus/minus the ΔH69/V70 deletion are described above (pseudotype virus preparation).

Coronavirus-like particles were prepared essentially as previously described (Swann et al., 2020; Yurkovetskiy et al., 2020). In brief, 4e6 293T cells in 10 cm dishes were transfected using TransIT-LT1 (Mirus) with a total of 4 µg DNA comprising 1 µg each of plasmids encoding S (WT or ΔH69/V70), M, E and N. Media was replaced after 16 h.
Supernatants containing coronavirus-like particles were harvested after 2 d, spun for 10 min at 2,000g, then passed through a 0.45 µm filter. For each condition, 9 ml supernatant was layered on a 2 ml cushion of 20% sucrose in PBS and spun for 2 h at 100,000g in a Type 70 Ti Beckman Coulter Ultracentrifuge Rotor. The pellet was washed once with PBS, then resuspended in 100 µl 2% SDS in PBS. After aspiration of supernatant, cells were washed twice in PBS, then lysed in 800 µl of 2% SDS in TBS with 500 units of Benzonase (Sigma-Aldrich). Lysates were incubated for 30 m at room temperature, then spun for 10 min at 13,000g.

Resuspended pellets containing concentrated coronavirus-like particles were heated in Laemmli buffer with DTT at 95 °C for 5 min. For each condition, 30 µl was loaded on a 4-20% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad). Cell lysates were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific), then heated in Laemmli buffer with DTT at 95 °C for 5 min. For each condition, 20 µg protein was loaded on an identical gel. Proteins were transferred to 45 nm PDVF membranes and blocked with 5% milk in PBS-Tween 0.2%. The following antibodies were used for immunoblotting: anti-S (Invitrogen, PA1-41165); anti-N (Novus Biologicals, NB100-56683) and anti-β-actin (Sigma, A5316).

**Spike cleavage inhibition experiments**

CMK furin inhibitor experiments: 293T cells were transfected with plasmids expressing Gag/pol, luciferase and spike. Furin inhibitor CMK (Calbiochem) was added at either 5 M or 25 uM concentration three hours post transfection. The supernatants and cell lysates were collected after 48 hours for infectivity measurement on target cells or for western blotting.

E64D and Camostat experiments: ACE2 or ACE2 and TMPRSS2 transfected 293T cells were either E64D (Tocris) or camostat (Sigma-Aldrich) treated for 3 hours at each drug concentration before the addition of a comparable amount of input viruses pseudotyped with WT, 6970 deletion or VSV-G (approx. 1 million RLU). The cells were then left for 48 hours before addition of substrate for luciferase (Promega) and read on a Glomax plate reader (Promega). The RLU was normalised against the no-drug control which was set as 100%.

**Cell-cell fusion assay**

Cell fusion assay was carried out as previously described (Papa et al., 2021). Briefly, Vero cells and 293T cells were seeded at 80% confluence in a 24 multiwell plate. 293T cells were co-transfected with 1.5 µg of spike expression plasmids in pCDNA3 and 0.5 µg mCherry-N1
using Fugene 6 and following the manufacturer’s instructions (Promega). Vero cells were treated with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Thermo Scientific) for 20 minutes. 293T cells were then detached 5 hours post transfection, mixed together with the green-labelled Vero cells, and plated in a 12 multiwell plate. Cell-cell fusion was measured using an Incucyte and determined as the proportion of merged area to green area over time. Data were then analysed using Incucyte software analysis. Data were normalised to cells transfected only with mCherry protein and mixed with green labelled Vero cells. Graphs were generated using Prism 8 software.

**Western blotting**

Cells were lysed and supernatants collected 18 hours post transfection. Purified virions were prepared by harvesting supernatants and passing through a 0.45 µm filter. Clarified supernatants were then loaded onto a thin layer of 8.4% optiprep density gradient medium (Sigma-Aldrich) and placed in a TLA55 rotor (Beckman Coulter) for ultracentrifugation for 2 hours at 20,000 rpm. The pellet was then resuspended for western blotting. Cells were lysed with cell lysis buffer (Cell signalling) or were treated with Benzonase Nuclease (70664 Millipore) and boiled for 5 min. Samples were then run on 4%–12% Bis Tris gels and transferred onto nitrocellulose or PVDF membranes using an iBlot or semidry (Life Technologies and Biorad, respectively).

Membranes were blocked for 1 hour in 5% non-fat milk in PBS + 0.1% Tween-20 (PBST) at room temperature with agitation, incubated in primary antibody (anti-SARS-CoV-2 Spike, Invitrogen, PA1-41165), anti-GAPDH (proteintech) or anti-p24 (NIBSC)) diluted in 5% non-fat milk in PBST for 2 hours at 4°C with agitation, washed four times in PBST for 5 minutes at room temperature with agitation and incubated in secondary antibody (anti-rabbit or anti-mouse HRP conjugate), anti-bactin HRP (sc-47778) diluted in 5% non-fat milk in PBST for 1 hour with agitation at room temperature. Membranes were washed four times in PBST for 5 minutes at room temperature and imaged directly using a ChemiDoc MP imaging system (Bio-Rad).

**Serum pseudotype neutralisation assay**

Spike pseudotype assays have been shown to have similar characteristics as neutralisation testing using fully infectious wild type SARS-CoV-2(Schmidt et al., 2020). Virus neutralisation assays were performed on 293T cell transiently transfected with ACE2 and
TMPRSS2 using SARS-CoV-2 spike pseudotyped virus expressing luciferase (Mlochova et al., 2020a). Pseudotyped virus was incubated with serial dilution of heat inactivated human serum samples or convalescent plasma in duplicate for 1h at 37°C. Virus and cell only controls were also included. Then, freshly trypsinized 293T ACE2/TMPRSS2 expressing cells were added to each well. Following 48h incubation in a 5% CO₂ environment at 37°C, the luminescence was measured using Steady-Glo Luciferase assay system (Promega).

Monoclonal antibody neutralisation of B.1.1.7 or B.1.1.7 H69/V70 pseudotyped viruses

Preparation of B.1.1.7 or B.1.1.7 H69/V70 SARS-CoV-2 S glycoprotein-encoding-plasmid used to produce SARS-CoV-2-MLV based on overlap extension PCR. Briefly, a modification of the overlap extension PCR protocol (Forloni et al., 2018) was used to introduce the 9 or 7 mutations of the B.1.1.7 and B.1.1.7 H69/V70 lineages, respectively. In a first step, 9 DNA fragments with overlap sequences were amplified by PCR from a plasmid (phCMV1, Genlantis) encoding the full-length SARS-CoV-2 S gene (BetaCoV/Wuhan-Hu-1/2019, accession number mn908947). The mutations (del-69/70, del-144, N501Y, A570D, D614G, P681H, S982A, T716I and D1118H or K417N, E484K and N501Y) were introduced by amplification with primers with similar Tm. Deletion of the C-terminal 21 amino acids was introduced to increase surface expression of the recombinant S (Case et al., 2020). Next, 3 contiguous overlapping fragments were fused by a first overlap PCR (step 2) using the utmost external primers of each set, resulting in 3 larger fragments with overlapping sequences. A final overlap PCR (step 3) was performed on the 3 large fragments using the utmost external primers to amplify the full-length S gene and the flanking sequences including the restriction sites KpnI and NotI. This fragment was digested and cloned into the expression plasmid phCMV1. For all PCR reactions the Q5 Hot Start High fidelity DNA polymerase was used (New England Biolabs Inc.), according to the manufacturer’s instructions and adapting the elongation time to the size of the amplicon. After each PCR step the amplified regions were separated on agarose gel and purified using Illustra GFX™ PCR DNA and Gel Band Purification Kit (Merck KGaA).

Ab discovery and recombinant expression

Human mAbs were isolated from plasma cells or memory B cells of SARS-CoV or SARS-CoV-2 immune donors, as previously reported. Recombinant antibodies were expressed in ExpiCHO cells at 37°C and 8% CO₂. Cells were transfected using ExpiFectamine.
Transfected cells were supplemented 1 day after transfection with ExpiCHO Feed and ExpiFectamine CHO Enhancer. Cell culture supernatant was collected eight days after transfection and filtered through a 0.2 µm filter. Recombinant antibodies were affinity purified on an ÄKTA xpress FPLC device using 5 mL HiTrap™ MabSelect™ PrismA columns followed by buffer exchange to Histidine buffer (20 mM Histidine, 8% sucrose, pH 6) using HiPrep 26/10 desalting columns.

**Pseudovirus neutralization assay for testing NTD monoclonal antibodies**

MLV-based SARS-CoV-2 S-glycoprotein-pseudotyped viruses were prepared as previously described (Pinto et al., 2020). HEK293T/17 cells were cotransfected with a WT, B.1.1.7 or B.1.1.7 H69/V70 SARS-CoV-2 spike glycoprotein-encoding-plasmid, an MLV Gag-Pol packaging construct and the MLV transfer vector encoding a luciferase reporter using X-tremeGENE HP transfection reagent (Roche) according to the manufacturer’s instructions. Cells were cultured for 72 h at 37°C with 5% CO₂ before harvesting the supernatant. VeroE6 stably expressing human TMPRSS2 were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (100 I.U. penicillin/mL, 100 µg/mL), 8 µg/mL puromycin and plated into 96-well plates for 16–24 h. Pseudovirus with serial dilution of mAbs was incubated for 1 h at 37°C and then added to the wells after washing 2 times with DMEM. After 2–3 h DMEM containing 20% FBS and 2% penicillin–streptomycin was added to the cells. Following 48–72 h of infection, Bio-Glo (Promega) was added to the cells and incubated in the dark for 15 min before reading luminescence with Synergy H1 microplate reader (BioTek). Measurements were done in duplicate and relative luciferase units were converted to percent neutralization and plotted with a non-linear regression model to determine IC50 values using GraphPad PRISM software (version 9.0.0).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

*Infectivity assays, drug inhibitor assays*

Measurements were done in duplicate and relative luciferase units measured with a Glomax luminometer. Data were analysed using GraphPad PRISM software (version 9.0.0). Statistical tests are described in the figure legends along n, mean and standard deviation/error. Data were normally distributed consistent with statistical methods used.
**Neutralisation assays**

Measurements were done in duplicate and relative luciferase units were converted to percent neutralization against no-drug control which was set as 100%. Data were plotted with a non-linear regression model to determine IC50 values using GraphPad PRISM software (version 9.0.0). The 50% inhibitory dilution (EC50) was defined as the serum dilution at which the relative light units (RLUs) were reduced by 50% compared with the virus control wells (virus + cells) after subtraction of the background RLUs in the control groups with cells only. The EC50 values were calculated with non-linear regression, log (inhibitor) versus normalized response using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). The neutralisation assay was positive if the serum achieved at least 50% inhibition at 1 in 3 dilution of the SARS-CoV-2 spike protein pseudotyped virus in the neutralisation assay. The neutralisation result was negative if it failed to achieve 50% inhibition at 1 in 3 dilution. Statistical tests are described in the figure legends along with the value of n, mean and standard deviation/error. Data were normally distributed consistent with statistical methods used.

**Key Resources Table**

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**Recombinant DNA**

- **Plasmid:** SARS-CoV-2 spike D614-FLAG  
  Biobasic  
  N/A
- **Plasmid:** RaTG13 spike-FLAG  
  Biobasic  
  N/A
- **Plasmid:** human ACE2 receptor  
  Biobasic  
  N/A
- **Plasmid:** TMPRSS2  
  Biobasic  
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- **Plasmid:** p8.91  
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- **Plasmid:** CSFLW  
  This paper  
  N/A
- **Plasmid:** pcDNA3.1  
  Thermo Scientific, Invitrogen  
  Cat#V66020

**Software and algorithms**

- **Pangolin v2.4.2**  
  (Rambaut et al., 2020)  
  https://github.com/cov-lineages/pangolin
- **IQTREE2 v2.1.2**  
  (Minh et al., 2020)  
  http://www.iqtree.org/
- **ModelFinder**  
  (Kalyaanamoorthy et al., 2017)  
  NA
- **Figtree v1.4.4**  
  (Rambaut, 2012)  
  http://tree.bio.ed.ac.uk/software/figtree/
- **RDP5**  
  (Martin et al., 2020)  
  http://web.cbio.uct.ac.za/~darren/rdp.htm1
- **RAXML-NG v1.02**  
  (Kozlov et al., 2019)  
  https://github.com/amkozlov/raxml-ng
- **BioEdit v7.2**  
  (Bhullar et al., 2012)  
  NA
- **RNAalifold**  
  (Bernhart et al., 2008)  
  http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAalifold.cgi
- **I-TASSER**  
  (Roy et al., 2010)
- **Pymol v2.4.0**  
  Schrödinger Inc., New York, USA  
  https://github.com/schrodinger/pymol-open-source

**Other**

- **Sequence data from the GISAID public database**  
  (Shu and McCauley, 2017)  
  http://gisaid.org
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Highlights

- Spike ΔH69/V70 does not confer escape from antibodies
- Spike ΔH69/V70 increases cleaved S2 and spike infectivity
- B.1.1.7 requires ΔH69/V70 for efficient cleaved spike incorporation and infectivity
- B.1.1.7 spike requires ΔH69/V70 for rapid syncytia formation

ETOC blurb

Meng et al. report that the SARS-CoV-2 spike ΔH69/V70 deletion has arisen multiple times. The deletion increases entry efficiency, is associated with increased cleaved spike in virions and can compensate for loss of infectivity. The B.1.1.7 spike requires the ΔH69/V70 deletion for efficient cell entry and cell-cell fusion activity.
Figure 4. Route of SARS-CoV-2 S mediated virus entry in cell lines is not altered by ΔH69/V70 spike. A. Schematic diagram illustrates spike in producer cells with CMK targeting and blocking furin cleavage (left panel). In target cells camostat inhibits TMPRSS2 and therefore cell fusion at the plasma membrane, and E64D blocks cathepsins and targets the endocytic viral entry (right panel). B. Western blots show CMK inhibits spike S1/S2 cleavage in the producer cells transfected with SΔH69/V70 plasmid, and the spikes with altered S1/S2 cleavage are incorporated onto the virions. Antibodies against HIV-1 p24 and Spike S2 were used with anti-GAPDH as a loading control. C. The viruses produced from the transfected 293T cells in the presence of CMK were used to transduce target cells. The luciferase reading (RLU) is used as a surrogate for the spike infectivity bearing with various S2/FL ratios. The data shown are the technical quadruplicates from one experiment and statistical analysis was done using unpaired t test. D. Comparison of infectivity of spike with polybasic cleavage site deleted (ΔPBCS) with and without ΔH69/V70, indicating the impact of ΔH69/V70 is independent of PBCS. E. ΔH69/V70 deletion does not alter the virus entry route. S pseudotyped lentiviruses bearing either wt S, ΔH69/V70 S or VSV-G was used to transduce 293T-ACE2 or 293T-ACE2/TMPRSS2 cells in the presence of either E64D or camostat at different drug concentrations. The cells were then harvested after 2 days and assayed for luciferase expression, which was then normalised against the non-drug control (set as 100%). The data shown are technical duplicates and are representative of at least two independent experiments.
Supplementary Figure 1: Infectivity and cleavage of spike ΔH69/V70 in a background of D614 (Wuhan) in pseudotyped lentivirus. Sucrose purified pseudotypes, as indicated, were used to infect human ACE2 expressing HEK293 cells, with luciferase readings read at 72 hours post infection. Experiments were performed in biological quadruplicate with the mean and standard deviation plotted. Results are representative of experiments performed two times. Statistical significance was assessed using an unpaired t-test (ns; non-significant, ***; <0.005). Western blot of purified pseudotype virus. Spike and HIV pseudotype abundances were assessed using Flag and p24 antibodies, respectively. Relative spike expression was calculated by densitometry using Image J. Briefly, inverted pixel intensities for spike and p24 bands were first normalised to a background region of the gel. Spike protein intensities were then normalised to p24 intensity before mutant protein expression was calculated as a factor of wild-type protein. NE: no envelope/spike.

C. Generation of coronavirus-like particles. Supernatants containing coronavirus-like particles were concentrated by ultracentrifugation, then analysed by immunoblot in parallel with lysates from producer cells. 293T cells were transfected with plasmids encoding the indicated SARS-CoV-2 proteins (S, spike; WT or ΔH69/V70; M, membrane; E, envelope; N, nucleocapsid). Cells transfected with WT or ΔH69/V70 S alone (no M, E or N) were included as controls. Arrow heads indicate cleaved S in coronavirus-like particles. Representative data are shown from three independent experiments. Related to Figure 3.
Two distinct lineages of the ΔH69/V70 were observed to expand in the UK, separately from the 501Y lineage. Prior to expansion of the B.1.1.7 lineage, clusters of infections bearing either N501Y or ΔH69/V70 were observed. Alongside expansion of the B.1.1.7 lineage, is a population in Wales that carries 501Y, but no ΔH69/V70. An intermediary was detected alongside the B.1.1.7 lineage (indicated on phylogeny) which had only a subset of the mutations that make up B.1.1.7 (ΔH69/V70, N501Y, A570D and D1118H). Related to Figure 6.
Supplementary Figure 3: Neutralisation and binding by a panel of NTD-specific mAbs against WT, B.1.1.7 and B.1.1.7 H69/V70 mutant SARS-CoV-2 viruses. A. Neutralisation of WT (black), B.1.1.7 (blue) and B.1.1.7 H69/V70 replacement mutant (red) pseudotyped SARS-CoV-2-MLVs by 6 selected mAbs from one experiment. B. Neutralisation of WT, B.1.1.7 and B.1.1.7 H69/V70 SARS-CoV-2-MLVs by 13 mAbs targeting NTD. Shown are the mean IC50 values (ng/ml) from one experiment. The higher the IC50 the less sensitive the virus to antibodies. C. Neutralisation shown as mean IC50 values (upper panel) and mean fold change of B.1.1.7 (blue) or B.1.1.7 H69/V70 (red) relative to WT (lower panel) of the 13 NTD mAbs tested. Lower panel shows IC50 values from one experiment. Related to Figure 6.
Supplementary Figure 4. Comparison of the H69/V70 deletion site to other Sarbecoviruses. A. phylogeny for the Spike peptide region 1-256 B. protein sequences from 20 Sarbecoviruses, including SARS-CoV-2 (Wuhan-Hu-1) and SARS-CoV (HSZ-Cc), with distinct genotypes at the Spike region around amino acid positions 69 and 70 (highlighted in yellow box). The 69/70 HL insertion in the P2V sequence from the Guangxi pangolin virus cluster and the HV convergent insertion in the RShSTT182/200 bat virus sequences are highlighted. C. The nucleotide alignment between SARS-CoV-2 Wuhan-Hu-1, B.1.1.7, the bat sarbecovirus RaTG13 RShSTT182/200 and the Guangxi pangolin viruses shows the difference between the out-of-frame deletion observed in the former and the in-frame deletion in the latter. D. Single round infection by luciferase expressing lentivirus pseudotyped with RaTG13 Spike protein on 293T cells transduced with ACE2. Experiments were performed in biological quadruplicate with the mean and standard deviation plotted. Results are representative of experiments performed two times. Statistical significance was assessed using an unpaired t-test (ns; non-significant, ***, <0.005). E. Representative western blot of supernatant from virus producer cells. Spike and HIV pseudotype abundances were assessed using Flag and p24 antibodies, respectively. Relative spike expression (total spike:p24) was calculated by densitometry using Image J. NE: no envelope. Relates to Figure 7.
Supplementary Figure 5: The positions of common deletion mutations on the RNA structure of the Spike Δ69-70 region of the gRNA. The optimal secondary structure was generated from a consensus alignment of human SARS-CoV2 RNAs using RNAalifold. Figure shows nucleotides 20277-23265. Base-pair probability, representative of the breadth of the structural ensemble that could be adopted by the RNA, is shown in colour according to the key. Relates to Figure 7.