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Im Labor hergestellt? SARS-CoV-2-Genealogie durch die Linse der Funktionsgewinnforschung

Yuri Deigin
22. April · 64 min gelesen

Mitarbeiter feiern die physische Fertigstellung des Labors im Jahr 2015, Wuhan, China (Quelle)

> *Wenn Sie jemanden behaupten hören "Wir wissen, dass der Virus nicht aus einem Labor stammt", kaufen Sie ihn nicht - er könnte es haben. Labore auf der ganzen Welt entwickeln seit Jahren synthetische Viren wie CoV2. Und nein, sein Genom würde nicht unbedingt Kennzeichen menschlicher Manipulation enthalten: Moderne gentechnische Werkzeuge ermöglichen das spurlose Ausschneiden und Einfügen von Genomfragmenten. Auch das*

> *geht schnell: Ein Schweizer Team brauchte weniger als einen Monat, um einen synthetischen CoV2- Klon zu erstellen.*

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Wie ich gelernt habe , mir

Oh , komm schon. Im Labor l
Reaktion, als gerade die I
Laborleck verursacht wird. Biowaffe?
Flat Earth. Wann immer ich etwas über nicht natürliche Ursprünge von SARS-CoV-2 hörte, wischte ich es unter ähnlichen Gefühlen beiseite. Was ist, wenn es in Wuhan ein Institut für Virologie gibt? Wer weiß, wie viele davon in ganz China verteilt sind.

Irgendwann wurde es notwendig, solche Theorien in begründeter Weise beiseite zu schieben, als ihre Befürworter begannen, ihre Thesen über die mögliche künstliche Natur des Virus mit Argumenten aus der Molekularbiologie zu untermauern, und als ich sie in eine Debatte verwickelte, wollte ich dies tun zerschlagen ihre Verschwörungstheorien mit kalten, harten wissenschaftlichen Fakten. Genau wie dieses Naturpapier (oder so dachte ich).

Um Argumente gegen die Labormasse des Virus zu verfolgen, wurde ich mit dem Virus des Zweifels infiziert. Was war die Quelle meiner Zweifel? Die Tatsache, dass je tiefer Sie in den letzten 15 bis 20 Jahren in die Forschungsaktivitäten von Koronavirologen eintauchen, desto mehr wird Ihnen klar, dass die Herstellung von Chimären wie CoV2 in ihren Labors an der Tagesordnung war. Und**CoV2 ist eine offensichtliche Chimäre (wenn auch nicht unbedingt eine im Labor hergestellte), die auf dem Fledermausstamm RaTG13 basiert, bei dem das Rezeptorbindungsmotiv (RBM) in seinem Spike-Protein durch das RBM eines Pangolin-Stammes ersetzt wird Zusätzlich wird ein kleiner, aber sehr spezieller Abschnitt von 4 Aminosäuren eingefügt, der eine Furin-Spaltstelle erzeugt, die, wie Virologen zuvor festgestellt haben, das „Repertoire“ des Virus hinsichtlich seiner Zellen, in die es eindringen kann, erheblich erweitert.** Höchstwahrscheinlich gelang es der neuen Mutante dank dieser neuen Furinstelle, Arten von ihrem ursprünglichen Wirt auf den Menschen zu springen.

In der Tat haben Virologen, darunter Shi Zhengli , der Leiter der Coronavirus-Forschung am Wuhan-Institut für Virologie, in der Vergangenheit viele ähnliche Dinge getan - sowohl das RBM in einem Virustyp durch ein RBM eines anderen ersetzt als auch eine neue Furinstelle hinzugefügt Dies kann einem speziesspezifischen

Coronavirus die Möglichkeit geben, denselben Rezeptor (z. B. ACE2) bei anderen Spezies zu verwenden. Tatsächlich sch
erst 2017 chimäre Konstrukte , als sie
verschiedenen RBMs schufen. Im Jahr
WIV Teil eines NIH-Zuschusses in Höh
des Risikos der Entstehung von Flederm
Schirmherrschaft hat Shi Zhengli 201
Erforschung synthetischer Viren und
wurden :

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> *Derzeit sind keine klinischen Behandlungen oder Präventionsstrategien für ein menschliches Coronavirus verfügbar. Angesichts der konservierten RBDs von SARS-CoV- und Fledermaus-SARSr-CoVs sollten einige in der Entwicklung befindliche Anti-SARS-CoV-Strategien wie Anti-RBD-Antikörper oder Impfstoffe auf RBD-Basis gegen Fledermaus-SARSr-CoVs getestet werden. Jüngste Studien haben gezeigt, dass Anti-SARS-CoV-Strategien nur gegen WIV1 und nicht gegen SHC014 wirken. Darüber hinaus sind nur wenige Informationen zu HKU3-verwandten Stämmen verfügbar, die eine viel breitere geografische Verteilung aufweisen und in ihrer RBD Kürzungen aufweisen. In ähnlicher Weise konnten Anti-S-Antikörper gegen MERS-CoV nicht vor einer Infektion mit einem Pseudovirus schützen, das das Fledermaus-MERSr-CoV S trägt. Darüber hinaus ist wenig über die Replikation und Pathogenese dieser Fledermausviren bekannt.* ***Zukünftige Arbeiten sollten sich daher auf die biologischen Eigenschaften dieser Viren konzentrieren, indem Virusisolierung, Umkehrgenetik sowie*** **In-vitro-** ***und*** **In-vivo-** ***Infektionstests eingesetzt werden.*** *Die daraus resultierenden Daten würden in Zukunft zur Prävention und Bekämpfung neu auftretender SARS-ähnlicher oder MERS-ähnlicher Krankheiten beitragen.*

Wenn das obige Zitat vage erscheint, was genau „Verwendung der Umkehrgenetik“ bedeuten könnte, formuliert der NIH-Zuschuss selbst Folgendes:

> *Ziel 3.* In-vitro- *und* In-vivo- *Charakterisierung des SARSr-CoV-Spillover-Risikos in Verbindung mit räumlichen und phylogenetischen Analysen zur Identifizierung der Regionen und Viren, die für die öffentliche Gesundheit von Belang sind. Wir werden S-Protein-Sequenzdaten, infektiöse Klontechnologie, In-vitro- und In-vivo-Infektionsexperimente und die Analyse der Rezeptorbindung verwenden, um die Hypothese zu testen, dass% Divergenzschwellen in S-Protein-Sequenzen das Spillover-Potenzial vorhersagen.*

"Infektiöse Klontechnologie" steht für die Erstellung lebender synthetischer viraler Klone. In Anbetracht der Höhe der Be
gentechnische Werkzeuge erreicht ha
CoV2 über die oben beschriebene Met

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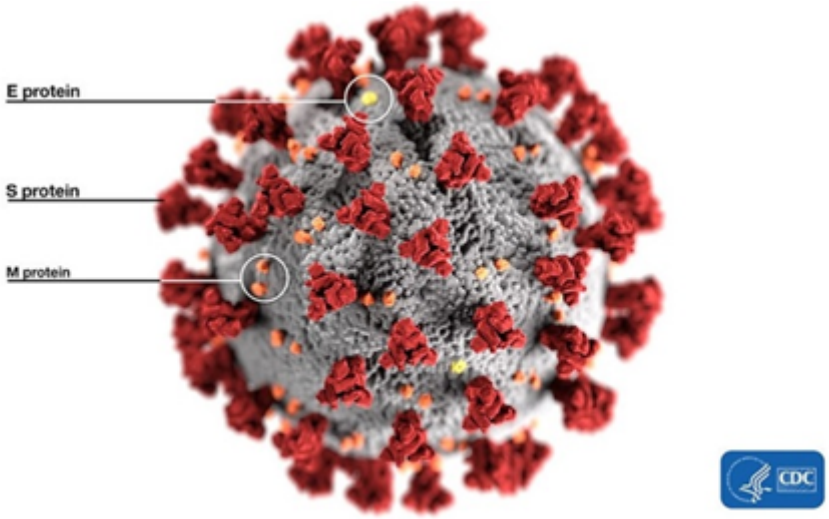
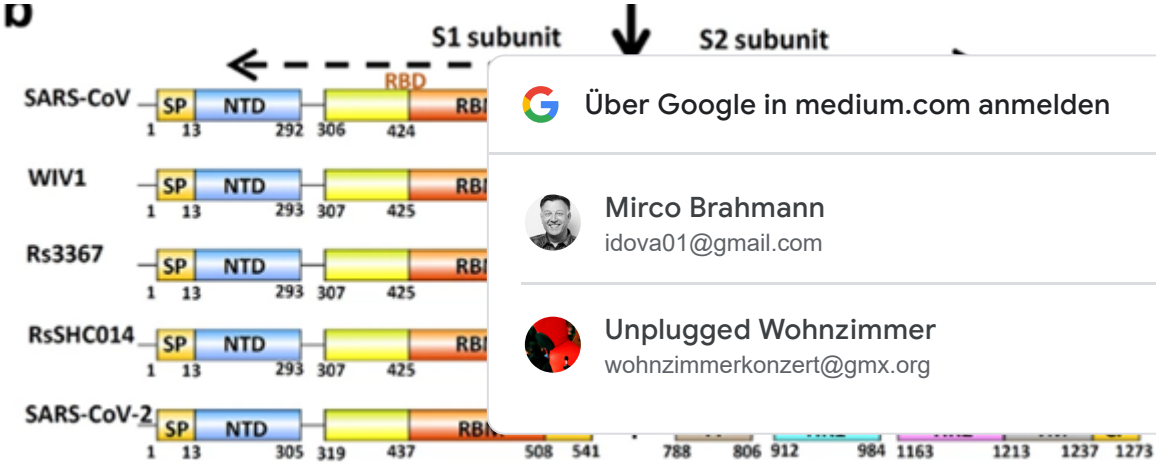
Bevor wir uns jedoch mit den Ursprün
kurz mit seiner Biologie befassen.

Biologie

Ok, fangen wir mit den Grundlagen an. Was ist eine Furinstelle, ein RBM oder ein Spike-Protein? Nehmen Sie mich mit: Wenn Sie einmal durch den Dschungel der Terminologie gestapelt sind, ist konzeptionell alles ziemlich einfach. Zum Beispiel sind Spike-Proteine jene roten Dinge, die aus einem Viruspartikel herausragen - genau aus diesem Grund wurden diese Viren „gekrönt“:



Mit Hilfe dieser Proteine klammert sich das Virion an den Rezeptor der Opferzelle (in unserem Fall ACE2), um dann in das Innere einzudringen. Es ist also ein lebenswichtiger Teil des Virus, da sich Viren nicht replizieren können, ohne in eine Zelle zu gelangen. Das Spike-Protein bestimmt auch, welche Tiere das Virus infizieren kann oder nicht, da ACE2-Rezeptoren (oder andere Ziele für andere Viren) in verschiedenen Spezies in ihrer Struktur unterschiedlich sein können. Gleichzeitig macht das Gen dieses Proteins vom gesamten 30-Kilobasen-Genom (für virale Verhältnisse ziemlich groß) nur 12–13% aus. Das Spike-Protein ist also nur etwa 1300 Aminosäuren lang. Nachfolgend sehen Sie, wie das Spike (S) -Protein in CoV2 und nahen Verwandten strukturiert ist:



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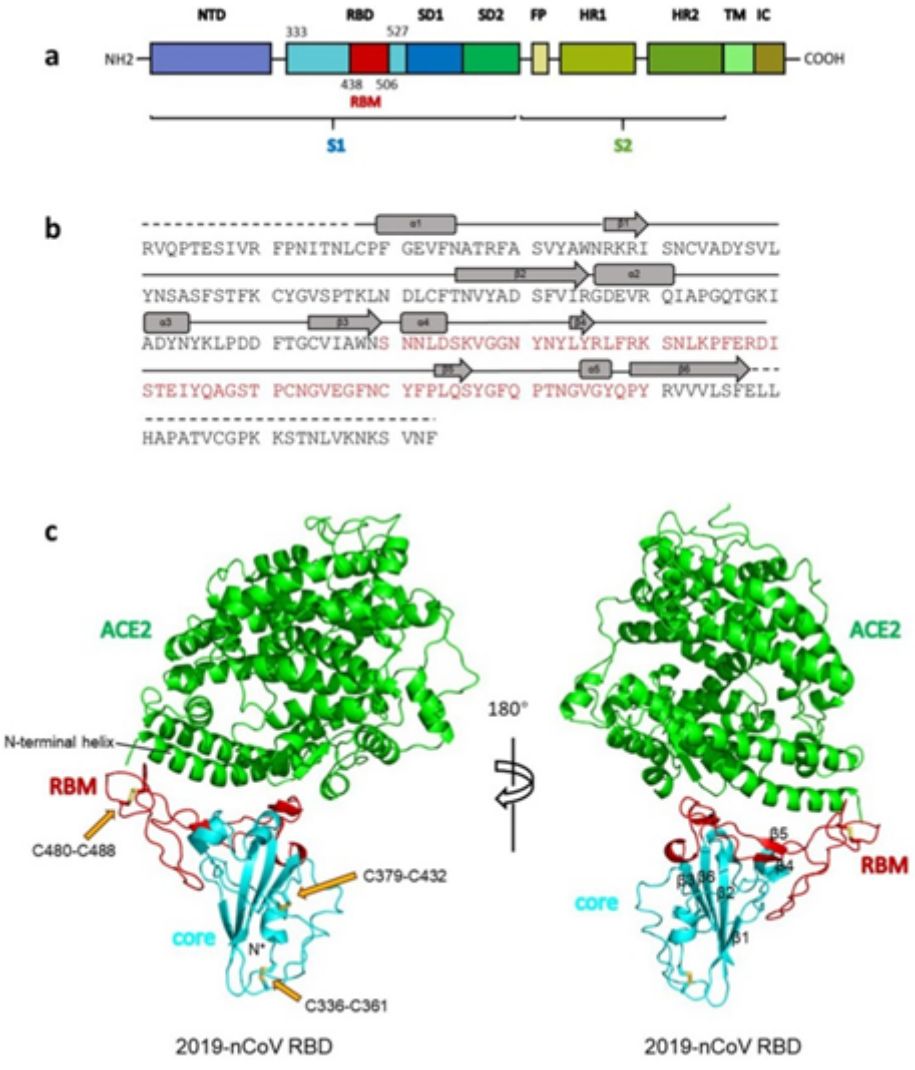
Wie aus der obigen Abbildung ersichtlich ist, besteht das S-Protein aus zwei Untereinheiten: S1 und S2. Es ist S1, das mit dem ACE2-Rezeptor interagiert, und der Ort, an dem S1 dies tut, wird als Rezeptorbindungsdomäne (RBD) bezeichnet, während der Bereich des direkten Kontakts, das Allerheiligste, als Rezeptorbindungsmotiv (RBM) bezeichnet wird. Hier ist eine schöne Illustration aus einer ebenso schönen Arbeit :



Gesamtstruktur der an ACE2 gebundenen RBD 2019-nCoV.

(a) Gesamttopologie des 2019-nCoV-S
Rezeptorbindungsdomäne. RBM, Rezeptorb
Fusionspeptid. HR1, Heptad-Wiederholung 1.
intra
(b) Sequenz und Sekundärstruktur
© Gesamtstruktur der 2019-nCoV-RBD, gebu
Kern ist cyanfarben und der RBM rot gefärb
dargestellt und durch gelbe Pfeile gekennzeic
von

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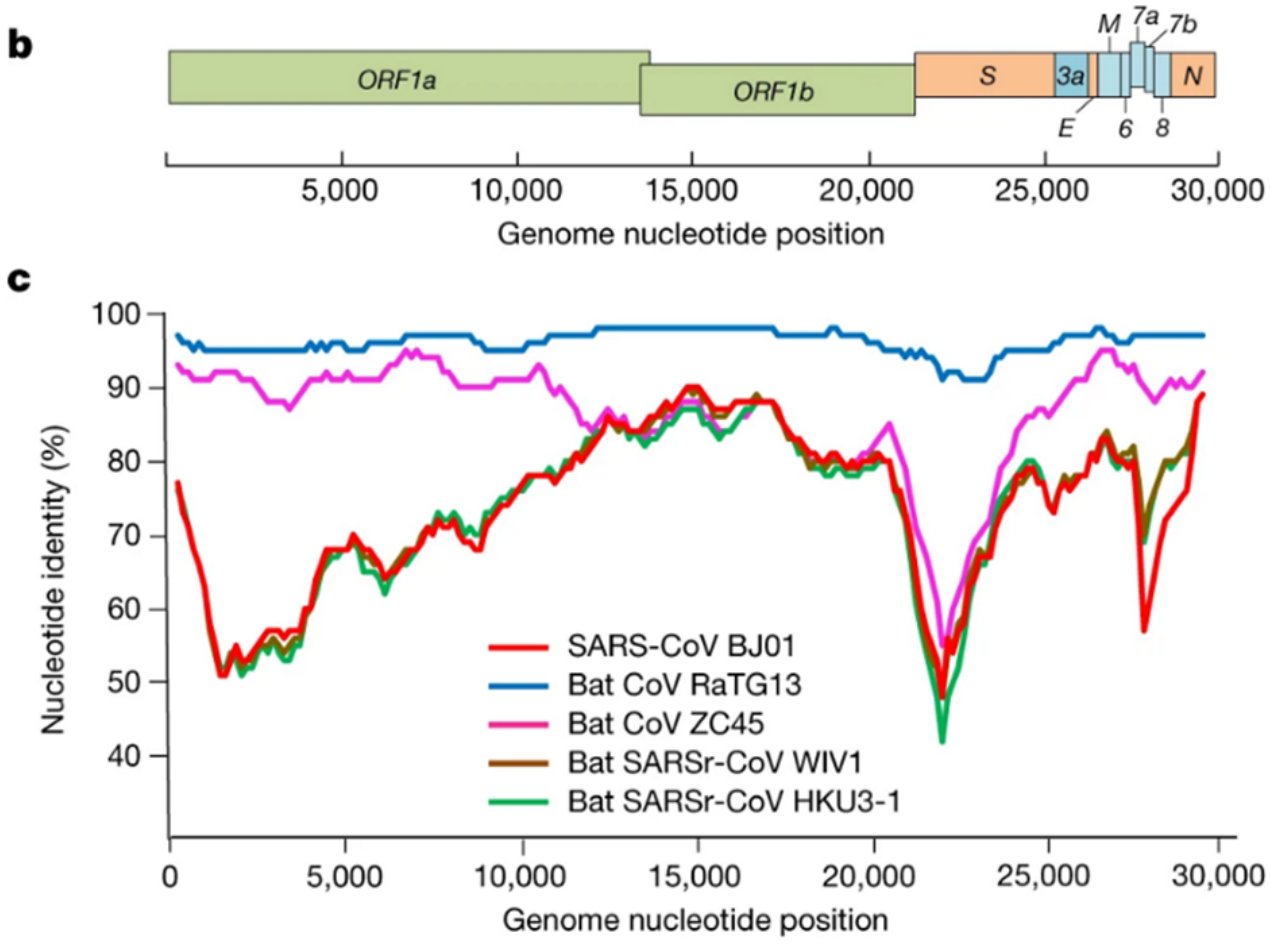
Als das CoV2-Genom am 10. Januar 2020 gerade sequenziert und öffentlich zugänglich gemacht wurde, war dies ein Rätsel, da keine eng verwandten Stämme bekannt waren. Aber ziemlich schnell, am 23. Januar, veröffentlichte Shi Zhengli ein Papier , das darauf hinweist, dass CoV2 zu 96% mit RaTG13 identisch ist, einem Stamm, den ihr Labor 2013 aus Yunnan-Fledermäusen isoliert hatte. Außerhalb ihres Labors wusste jedoch niemand über diesen Stamm Bescheid bis Januar 2020.

Es war sofort klar, dass RaTG13 etwas Besonderes ist. Schauen Sie sich die folgende Abbildung an:



Dies ist ein Genomähnlichkeitsgraph zwischen CoV2 und anderen bekannten Stämmen. Je höher die Kurve ist, dest
Nukleotide. Wie Sie sehen können, be
(zwischen den Nukleotiden 22k und 2
CoV2, während alle anderen Stämme
beide Stämme von anderen Fledermä
ist an sich alles andere als verdächtig
Stämme in den Fledermaushöhlen vo
klar, wie genau das Virus von dort nach Wuhan gelangen könnte, aber hey, mit diesen feuchten Märkten, die man nie kennt.

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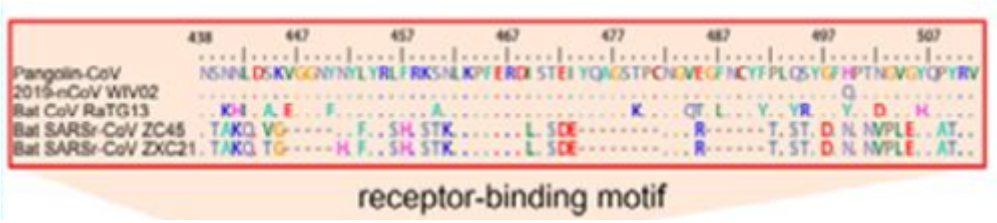
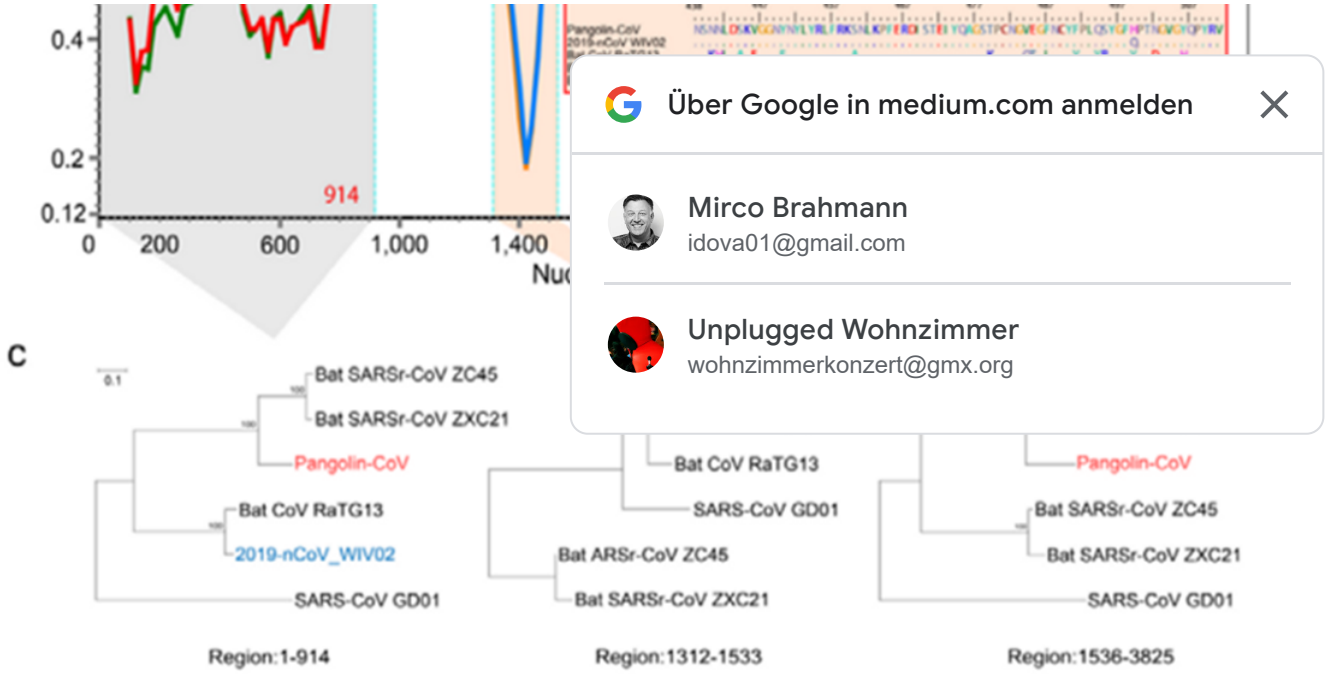
Pangoline

Als nächstes tauchten Pangoline auf: Im Februar entdeckte eine andere Gruppe chinesischer Wissenschaftler einen eigenartigen Stamm des Pangolin-Coronavirus in ihrem Besitz, der in der RBM-Region im Allgemeinen nur zu 90% mit CoV2 vergleichbar war nur ein einziger Aminosäureunterschied (siehe die oberen beiden Sequenzen, Punkte zeigen eine Übereinstimmung mit der oberen Sequenz an):



Überraschenderweise unterscheidet sich der Pangolin-Stamm im ersten Viertel des S-Proteins stark von CoV2, aber nach dem RBM weisen alle drei Stämme (CoV2, Pangolin, RaTG13) einen gemeinsamen hohen Grad an Ähnlichkeit auf. Am auffälligsten ist, dass das RBM von RaTG13 selbst ganz anders ist als das von CoV2, was sich aus dem steilen Abtauchen des grünen RaTG13-Diagramms im Vergleich zum roten CoV2-Diagramm in der RBM-Region (rosa Streifen) im folgenden Diagramm ergibt:



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Diese Beobachtung wird durch die phylogenetische Analyse der drei in der obigen Grafik hervorgehobenen Bereiche bestätigt - im RBM ist der Pangolin-Stamm näher an CoV2 als RaTG13, aber es ist RaTG13, das links und rechts von RBM näher an CoV2 liegt. Es gibt also eine offensichtliche Rekombination, wie die Autoren (und andere Artikel) schließen.

Wie haben die Forscher diese Pangoline erhalten? Das ist wie:

Sie wurden vom chinesischen Zoll von Schmugglern beschlagnahmt und in ein Tierrehabilitationszentrum in Guangdong gebracht, wo sie starben, während sie

schwere Coronavirus-Symptome zeigten. Dies muss natürlich die Aufmerksamkeit lokaler Virologen auf sich gezogen ha

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> *Die in der Studie verwendeten Pangolin*
> *und Forstministerium der Provinz Gua*
> *chinesische Pangoline (Manis pentadac*
> *javanica). Diese Tiere wurden in das W*
> *inaktiv und schluchzten und starben sc*
> *in Gewahrsam. Gewebeproben wurden aus Lunge, Lymphknoten, Leber, Milz, Muskel, Niere und anderen Geweben von Pangolinen entnommen, die gerade für histopathologische und virologische Untersuchungen gestorben waren.*

Diese Pangoline erregten auch die Aufmerksamkeit anderer Virologen. Zum Beispiel erhielt ein Team in Hongkong auch Proben beschlagnahmter Pangoline und veröffentlichte im Februar 2020 ein Papier , in dem deutliche Anzeichen einer Rekombination im CoV2-Spike-Protein festgestellt wurden:

> *Wir erhielten gefrorene Gewebeproben (Lunge, Darm, Blut), die von August 2017 bis Januar 2018 von 18 malaiischen Pangolinen (Manis javanica) entnommen wurden. Diese Pangoline wurden während der Anti-Schmuggel-Operationen von Guangxi Customs erhalten. Bemerkenswerterweise zeigte die Hochdurchsatzsequenzierung ihrer RNA das Vorhandensein von Coronaviren in sechs (zwei Lungen-, zwei Darm-, eine Lungen-Darm-Mischung, ein Blut) von 43 Proben. Mit den gelesenen Sequenzdaten und durch Füllen von Lücken mit Amplikonsequenzierung konnten wir sechs vollständige oder nahezu vollständige Genomsequenzen erhalten - bezeichnet als GX / P1E, GX / P2V, GX / P3B, GX / P4L, GX / P5E und GX / P5L - das in einer phylogenetischen Analyse in die 2019-CoV2-Linie (innerhalb der Gattung Betacoronavirus) fällt (Abbildung 1a).*
> …
> *Bemerkenswerter war jedoch die Beobachtung mutmaßlicher Rekombinationssignale zwischen den Pangolin-Coronaviren, den Fledermaus-Coronaviren RaTG13 und dem menschlichen 2019-CoV2 (Abbildung 1c, d). Insbesondere 2019-CoV2 zeigt eine sehr hohe Sequenzähnlichkeit zu den Guangdong-Pangolin-Coronaviren in der Rezeptorbindungsdomäne (RBD; 97,4% Aminosäureähnlichkeit; angezeigt durch roten Pfeil in 1c und 2a), obwohl es am engsten verwandt ist das Coronavirus RaTG13 im Rest des viralen Genoms zu schlagen. Bat CoV RaTG und das humane 2019-CoV2 weisen bei RBD nur eine Ähnlichkeit von 89,2% Aminosäuren auf.* ***In der Tat besitzen die Guangdong-Pangolin-Coronaviren und 2019-CoV2 identische Aminosäuren an den***

> ***fünf kritischen Resten der RBD, während RaTG13 nur eine Aminosäure mit 2019-CoV2 teilt (Rest 442, humane SARS-***

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Übrigens haben die Autoren dieses Ar
des CoV2-Spike-Proteins hervorgehob

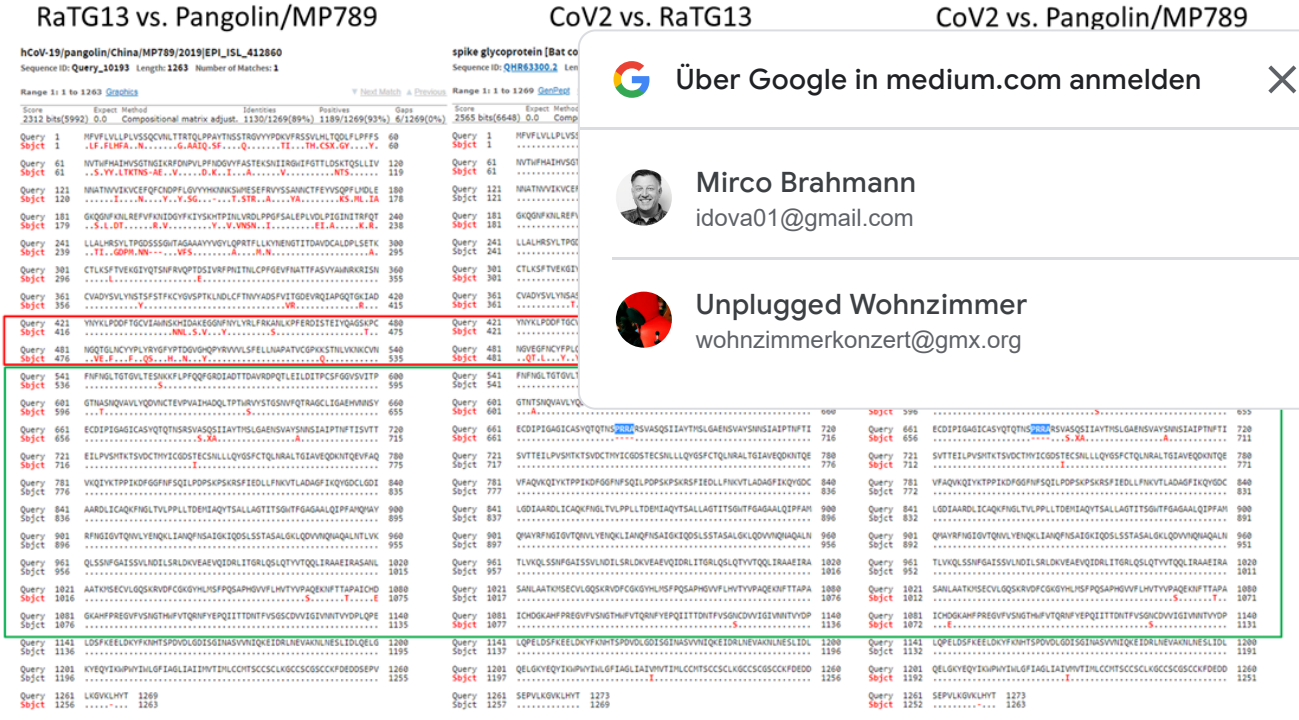
> *Interessanterweise ergab eine phylogen*
> *RBD, dass die phylogenetische Position*
> *viralen Genoms übereinstimmt und nicht der engste Verwandte von 2019-CoV2 ist (Abbildung 2b). Daher ist es möglich, dass die Aminosäureähnlichkeit zwischen der RBD der Guangdong-Pangolin-Coronaviren und 2019-CoV2 eher auf einer selektiv vermittelten konvergenten Evolution als auf einer Rekombination beruht, obwohl es schwierig ist, anhand der aktuellen Daten zwischen diesen Szenarien zu wählen.*

Aus wissenschaftlicher Sicht bedeutet dies, dass wir, wenn wir die gesamte RBD der drei Stämme analysieren, die offensichtlichen Unterschiede (dh nicht synonyme Substitutionen) zwischen ihnen ignorieren, die hauptsächlich im RB **M zu finden sind** (was, wie wir uns erinnern) identisch zwischen CoV2 und Pangolin) und konstruieren einen phylogenetischen Baum für synonyme Substitutionen, ist CoV2 immer noch näher an RaTG13 als an dem Pangolin-Stamm. Was angesichts der Tatsache, dass der Pangolin-Stamm und CoV2 identische RBMs aufweisen (die Segmente innerhalb der RBD sind), ziemlich seltsam ist.

Die Autoren gehen weiter von der Vermutung aus, dass dies das Ergebnis einer konvergenten Evolution sein könnte, mit anderen Worten, dass CoV2 und der Pangolin-Stamm identische RBMs auf ihre eigene Weise und nicht durch Rekombination zwischen gemeinsamen Vorfahren besaßen. Weil es ein ziemlich einzigartiges Rekombinationsereignis erfordert hätte - als hätte jemand ein genaues RBM-Segment aus einem Pangolin-Stamm herausgeschnitten und es verwendet, um das RBM in RaTG13 zu ersetzen. Sprechen Sie über intelligentes Design!

Königliche Genealogie

Um die Herkunft von CoV2 besser zu verstehen, werfen wir einen Blick auf die Spike-Proteinsequenzen unserer unheiligen Dreifaltigkeit: CoV2, RaTG13 und MP789 (Pangolin-2019). Vergleichen wir die paarweisen Unterschiede zwischen ihnen (identische Aminosäuren sind mit Punkten markiert, rote Buchstaben kennzeichnen Unterschiede und Striche zeigen gelöschte / eingefügte Aminosäuren an):



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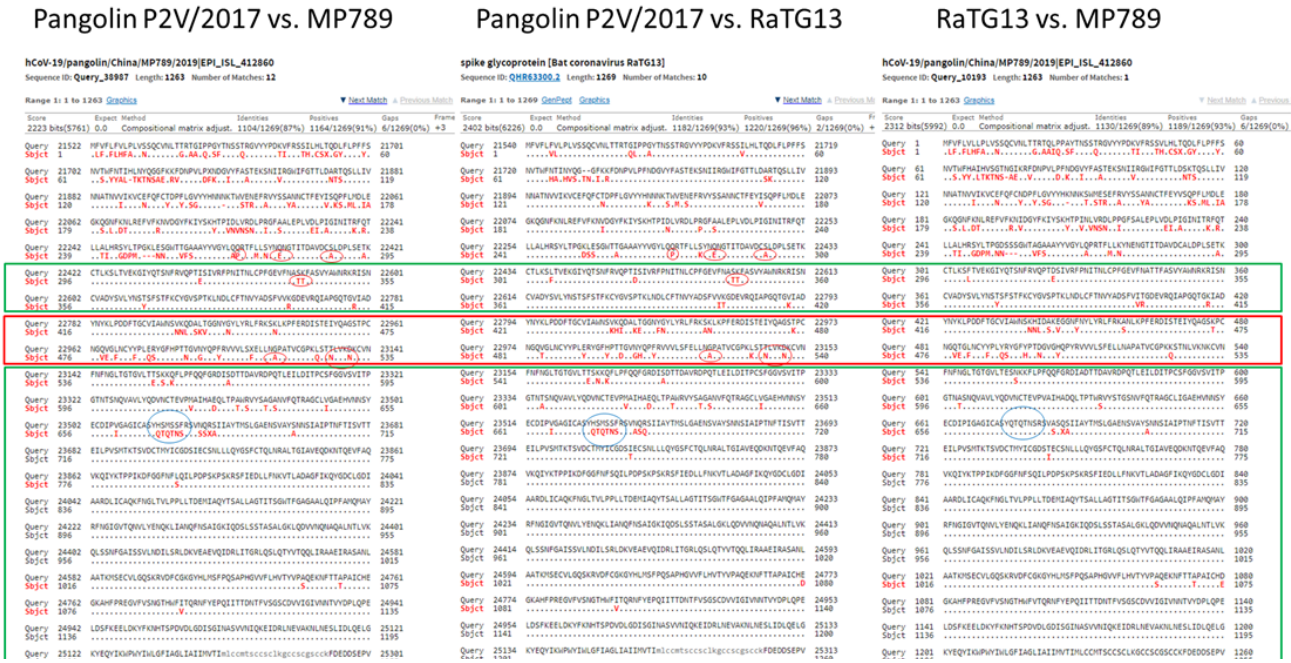
Die Vergleiche veranschaulichen, was zuvor zitierte Arbeiten festgestellt haben: Im ersten Viertel der Sequenz ist der Pangolin-Stamm weit von CoV2 und RaTG1 entfernt, und ohne die RBM-Region (rotes Rechteck) wäre RaTG13 sehr nahe gewesen zu CoV2. Aber wie ich bereits sagte, ist das RBM in CoV2 dem des Pangolin-Stammes am nächsten.

Was ist mit anderen Pangolin-Stämmen? Bisher haben wir nur den MP789-Stamm analysiert, der 2019 aus vom Zoll beschlagnahmten Pangolinen isoliert wurde. 2017 wurde jedoch eine weitere Charge von Pangolinen beschlagnahmt, und es wurde auch ein ähnlicher Coronavirus-Stamm isoliert. Vergleichen wir es mit RaTG13 und MP789:



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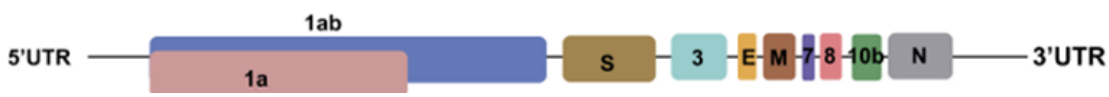
Im ersten Quartal des S-Proteins sind
(und CoV2) als ihr Pangolin-Gegenstü
den durch grüne Rechtecke gekennze
gemeinsamen Vorfahren, und in diese
(MP789) näher beieinander als Pango
Mutationen aufweisen (markiert durch rote und blaue Ellipsen), die in Pangolin-2017 fehlen. Aber das RBM für alle drei ist unterschiedlich und in ungefähr demselben Verhältnis und an ähnlichen Orten unterschiedlich.

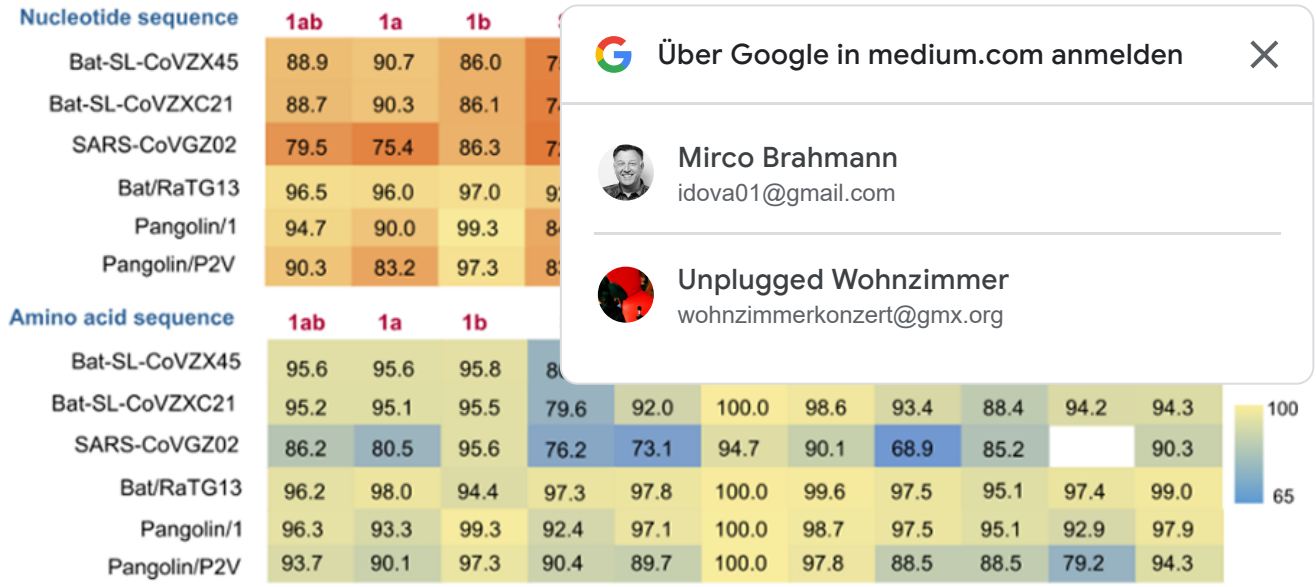
Nachdem die Vorfahren von RaTG13 und MP789 auseinander gegangen waren, wurde beim MP789-Vorfahren möglicherweise das erste Viertel seines Proteins ersetzt (was in RaTG13 oder Pangolin-2017 nicht vorkam), und der Rest des Proteins blieb für alle drei Stämme gemeinsam. Später kreuzten sich die Wege der RaTG13- und MP789-Genpools erneut und produzierten CoV2. Es ist auch möglich, dass der Vorfahr von RaTG13 als Ergebnis der Rekombination von Pangolin-Stämmen der Vorfahren entstanden ist.

Es ist auch interessant, eine ziemlich einzigartige identische Mutation (QTQTNS) in RaTG13 und Pangolin-2019 direkt vor der Stelle zu sehen, an der CoV2 eine neue Furin-Spaltstelle aufweist. Diese Furinstelle entstand, wie bereits erwähnt, durch Insertion von 4 neuen Aminosäuren (PRRA). Wenn wir uns die Nukleotidsequenz um diese Insertion ansehen, können wir sehen, dass RaTG13 und CoV2 in diesem Bereich näher beieinander liegen als zu Pangolin-2019, da sie mehrere häufige Mutationen besitzen (blau hervorgehoben):

```
Pangolin M789:     G  A  G  I  C  A  S  Y  Q  T  Q  T  N  S  .  .  .  .  R  S  V  S  S  X  A  I  I
                   ggt gca gga ata tgt gcc agt tat cag act caa act aat tca ... ... ... ... cgt agt gtt tca agt cna gct att att

RaTG13:            G  A  G  I  C  A  S  Y  Q  T  Q  T  N  S  .  .  .  .  R  S  V  A  S  Q  S  I  I
                   ggt gca gga ata tgc gcc agt tat cag act caa act aat tca ... ... ... ... cgt agt gtg gcc agt caa tct att att

Cov2:              G  A  G  I  C  A  S  Y  Q  T  Q  T  N  S  P  R  R  A  R  S  V  A  S  Q  S  I  I
                   ggt gca ggt ata tgc gct agt tat cag act cag act aat tct cct cgg cgg gca cgt agt gta gct agt caa tcc atc att

      Black  = common for all 3
      Red    = unique to Cov2
      Green  = unique to RaTG13
      Blue   = common difference of RaTG13 and CoV2 from M789
```

Übrigens ist Orf1ab auch ein phylogenetisches Durcheinander in CoV2: 1a ist näher an RaTG13, aber 1b ist näher an Pangolin-2019:





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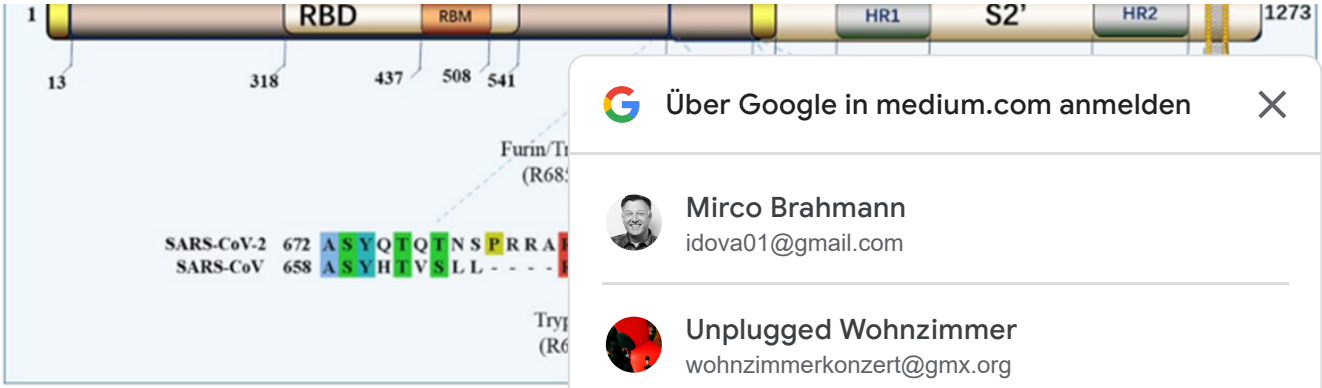
(Bildquelle)

Bedeutet dies, dass der Vorfahr von CoV2 mindestens zweimal mit dem gemeinsamen Vorfahren von Pangolin-19 gekreuzt hat? Erstens, als es (zusammen mit einem gemeinsamen Vorfahren von RaTG13) Orf1ab und die zweite Hälfte des Spike-Proteins mit der QTQTNS-Mutation erbte, und zweitens, als es 1b und RBM erwarb, die sich von RaTG13 unterscheiden. All dies ist in der Natur durchaus möglich - schließlich mutieren diese Viren und rekombinieren ständig. Eine andere Frage ist, wo genau Fledermaus- und Pangolin-Viren bei solchen Orgien am wahrscheinlichsten aufeinander treffen - in Berghöhlen, auf „feuchten Märkten“, in Tierheimen für beschlagnahmte Tiere oder sogar in Labors. Aber lassen Sie uns diese Fragen vorerst beiseite legen. Lassen Sie uns zunächst diskutieren, was der wohl auffälligste Aspekt des neuen Virus ist - eine 4-Aminosäuren-Insertion, die es zu einem natürlichen Killer gemacht hat.

Ein Killer-Intro

Es ist unmöglich, die Einführung eines PRRA-Einsatzes zwischen S1 und S2 zu ignorieren: Er ragt wie ein Splitter heraus. Dieser Einsatz erzeugt die Furin-Spaltstelle , die ich ganz am Anfang erwähnt habe. Lassen Sie mich erklären, was eine Furin-Site ist. Erinnern Sie sich an die Struktur unseres Spike-Proteins? Hier ist ein detailliertes Diagramm:



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Das Protein besteht aus zwei Teilen, S1 und S2, von denen S1 für den primären Kontakt mit dem Rezeptor verantwortlich ist (siehe Rezeptorbindungsdomäne / -motiv) und S2 für die Fusion mit der Zellmembran und das Eindringen in die Zelle verantwortlich ist. Der Fusionsprozess wird durch das gelb markierte Fusionspeptid gestartet. Damit es jedoch an seiner schmutzigen Tat teilnehmen kann, muss jemand das S-Protein an einer der im obigen Diagramm mit Diamanten markierten Stellen schneiden. Das Virus hat keine eigenen solchen „Cutter“, daher ist es auf verschiedene Proteasen seiner Opfer angewiesen. Es gibt verschiedene Arten solcher Proteasen, die aus der Fülle der Farben dieser Diamanten abgeleitet werden können. Aber nicht alle Proteasen sind gleich und nicht alle Zelltypen haben Proteasen, die vom Virus benötigt werden. Furin ist eines der wirksamsten und befindet sich nicht nur auf der Oberfläche von Zellen, sondern auch im Inneren.

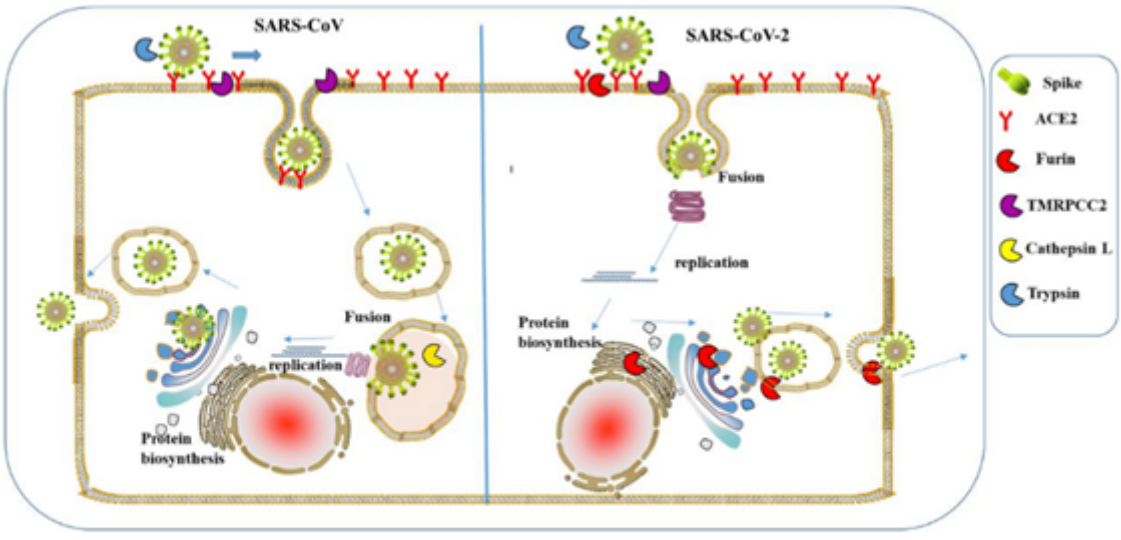

Figure 3.A schematic diagram of the process of SARS-CoV and SARS-CoV-2 infecting host cells. Those protease are presented by sector in different colors. Furin can cleaveSpike in the process of viral maturation.

Wie aus dem Diagramm ersichtlich ist, können im Fall von CoV2 dank der Furinstelle nicht zwei, sondern drei Klassen von Proteasen (drei farbige PacMans) sein S-Protein außerhalb der Zelle schneiden. Aber vielleicht ist der wichtigste Unterschied, dass

Furin auch **in** der Zelle vorhanden ist, so dass es das S-Protein unmittelbar nach dem Zusammenbau der Virionen schneide
erhalten, sich sofort mit neuen Zellen

Die Bedeutung der neuen Furinstelle i
durch eine Studie bei Hamstern , wo d
Mutation) stark mutiert COV2 der Pat

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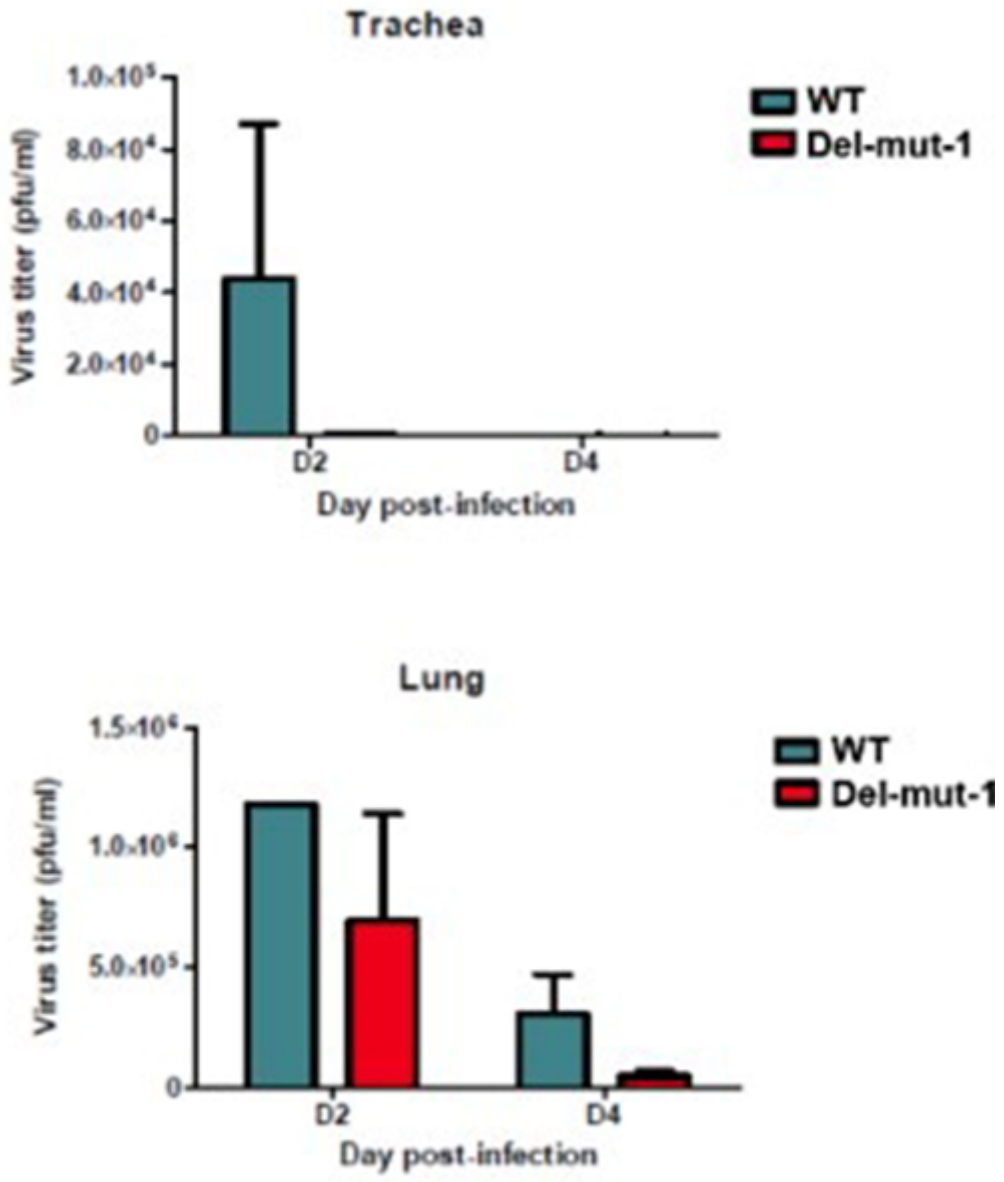
> *Die Infektion von Hamstern zeigt, dass eine der Varianten (Del-mut-1), die **die** Deletion von 10 Aminosäuren (30 bp) trägt, keinen Körpergewichtsverlust oder schwerwiegendere pathologische Veränderungen in der Lunge verursacht, die mit einer Wildtyp-Virusinfektion verbunden sind .*



Virusreplikation im Lungengewebe von Hamstern, die entweder mit WT- oder Del-mut-1-SARS-CoV-2-Virus infiziert sind. Virustitration durch Plaque-Assay von Lungen- und Trachealgeweben, die am Tag 2 und 4 nach der Infektion entnommen wurden

Die gute Nachricht ist, dass es bereits verschiedene Furin- und andere Proteaseinhibitoren gibt und einige vo
bereits klinisch gegen CoV2 getestet w

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Übrigens ist es möglich, dass die neue
altersabhängige Morbidität und Morta

> *Patienten mit Bluthochdruck, Diabetes
> Erkrankung, chronisch obstruktiver Lungenerkrankung und Nierenfunktionsstörung haben aus unbekannten Gründen schlechtere klinische Ergebnisse, wenn sie mit SARS-CoV-2 infiziert werden. Der Zweck dieser Übersicht ist es, die Beweise für das Vorhandensein von erhöhtem Plasmin (ogen) bei COVID-19-Patienten mit diesen komorbiden Zuständen zusammenzufassen. Plasmin und andere Proteasen können extrazellulär eine neu inserierte Furinstelle im S-Protein von SARS-CoV-2 spalten, was dessen Infektiosität und Virulenz erhöht.*

Furin schneidet Proteine an genau definierten Stellen, nämlich nach einer RxxR-Sequenz (dh Arg-XX-Arg, wobei X eine beliebige Aminosäure sein kann). Befindet sich Arginin ebenfalls an zweiter oder dritter Stelle (dh RRxR oder RxRR), ist die Spaltungseffizienz signifikant erhöht.

Daher wurde das Auftreten einer neuen Furin-Spaltstelle sofort bemerkt , da keiner der nächsten oder sogar entfernten Verwandten von Cov2 eine solche Stelle hat - jene Coronaviren, die dies tun, teilen nur 40% ihres Genoms mit Cov2:

> *Es wurde gefunden, dass alle Spike mit einer SARS-CoV-2-Spike-Sequenzhomologie von mehr als 40% keine Furinspaltungsstelle hatten (1, Tabelle 1), einschließlich Bat-CoV RaTG13 und SARS-CoV (mit einer Sequenzidentität von 97,4) % bzw. 78,6%).* ***Die Furinspaltungsstelle „RRAR“ in SARS-CoV-2 ist in ihrer Familie einzigartig und wird durch die einzigartige Einfügung von „PRRA“ wiedergegeben. Es ist unwahrscheinlich, dass sich die Furinspaltungsstelle von SARS-CoV-2 aus MERS, HCoV-HKU1 usw. entwickelt hat.*** *Aus den derzeit verfügbaren Sequenzen in Datenbanken ist es für uns schwierig, die Quelle zu finden. Vielleicht warten noch viele evolutionäre Zwischensequenzen darauf, entdeckt zu werden.*

Hier ist eine großartige Illustration aus dem Quellartikel des obigen Zitats. Coronaviren mit einer Furinstelle sind rosa markiert, 3 verschiedene Cov2-Stämme sind um 10 Uhr gezeigt:

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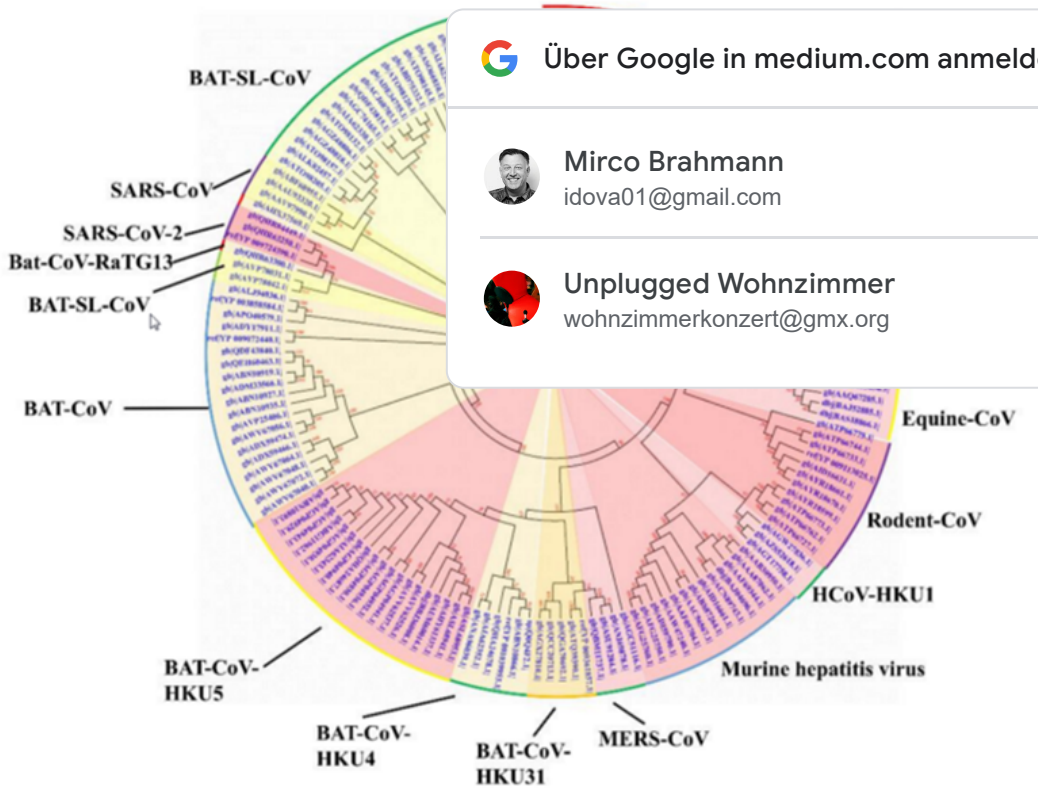


Der engste Verwandte mit einer Furinstelle ist der HKU5-Stamm, der 2014 vom Shi Zhengli-Team in Guangzhou aus Fledermäusen der Gattung *Pipistrellus* isoliert wurde (2018 zur GenBank hinzugefügt). Aber es ist ein sehr entfernter Verwandter - ihre Spike-Proteine teilen nur 36%.

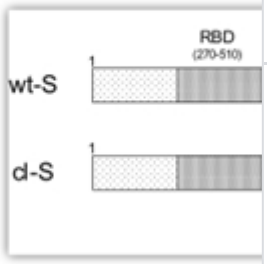
Die Virologen sind also verwirrt. Woher kam dieses 12-Nucleotid-Insert? Könnte es im Labor hergestellt werden? Nun, Virologen haben jahrzehntelang Furinstellen in Coronaviren untersucht und viele künstliche in einem Labor eingeführt. Zum Beispiel hatte ein amerikanisches Team RRSRR in das Spike - Protein des ersten SARS-CoV eingeführt im Jahr 2006 :

> *Um zu untersuchen, ob eine proteolytische Spaltung an den basischen Aminosäureresten, falls sie auftreten sollte, die Zell-Zell-Fusionsaktivität erleichtern könnte, mutierten wir das Wildtyp-SARS-CoV-Glykoprotein, um an beiden Positionen eine prototypische Furin-Erkennungsstelle (RRSRR) zu konstruieren.*

```
                                  ♦667     ♦672
wild-type        PIGAGICASYHTVSLLR   STSQKSIVAYTMSLGADSSIAYSNNTIAIPTNFSISITTEVMPVS
HTR              PIGAGICASYHTRRSRR   STSQKSIVAYTMSLGADSSIAYSNNTIAIPTNFSISITTEVMPVS
HTVR             PIGAGICASYHTVRRSRR  STSQKSIVAYTMSLGADSSIAYSNNTIAIPTNFSISITTEVMPVS
SLLR             PIGAGICASYHTVSLLRRSRR    SIVAYTMSLGADSSIAYSNNTIAIPTNFSISITTEVMPVS
```

Und die Japaner haben 2008 eine ähnliche Stelle (RRKR) in das SARS-CoV-Protein eingefügt , allerdings etwas stromabwärts als in CoV2:

FIG. 1.



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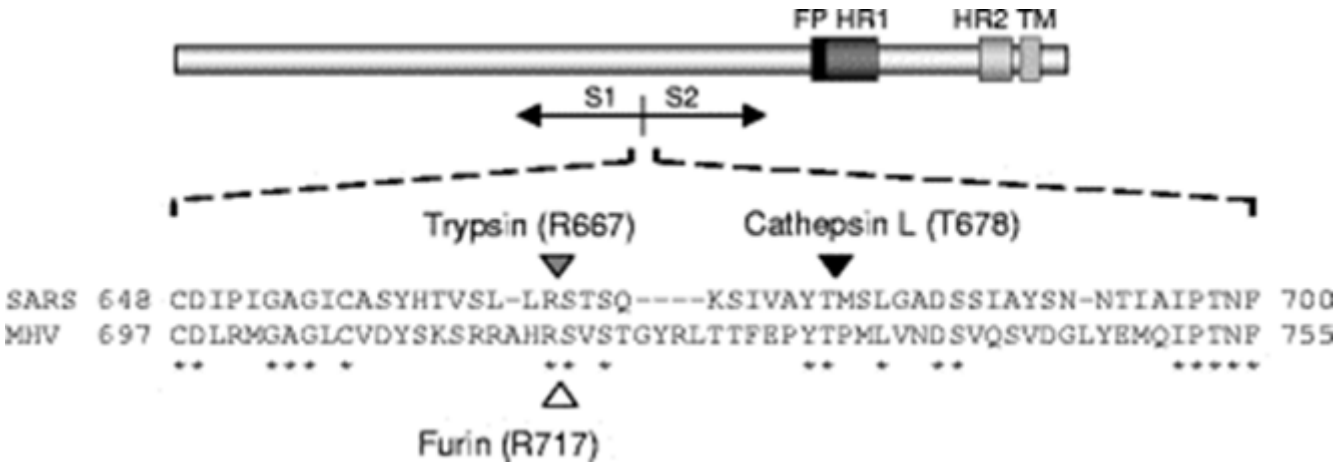
Schematic illustration of SARS proteins are shown in the box, in which the RBD, putative fusion peptide (FP), two HRs, and transmembrane region (TM) are indicated. Cleavage sites by trypsin (Try-CS) and CPL (CPL-CS) are also shown. Amino acid positions 798 and 799 are changed into arginine to make the recognition sequence of furin-like protease, KRRKR. Nineteen C-terminal amino acids (aa) are deleted for the efficient pseudotype formation of VSV.

Im selben Jahr 2008 untersuchten ihre niederländischen Kollegen auch diese Protease-Stellen von SARS-CoV und verglichen sie mit dem murinen Coronavirus MHV, das ebenfalls eine solche Stelle (SRRAHR | SV) aufweist, die der Stelle von CoV2 ziemlich ähnlich ist (SPRRAR | SV):



In 2009, another American group also worked on “improving” SARS-CoV and, continuing the American tradition of not penny-pinching on arginines, they inserted as many as 4 of them (RRSRR):

> *To examine the potential use of the SARS-CoV S1–S2 and S2′ positions as sites for proteolytic cleavage, we first introduced furin cleavage recognition sites at these locations by making the following mutations 664-SLLRSTSQSI — SLL**RRSRRS**I-671 (S1–S2) and 792-LKPTKRSF — LKRTKRSF-799 (S2′).*

Beijing 2019

But the most recent work of this kind
from several Beijing labs, where the n
some pseudovirus, but into an actual l
virus (IBV):

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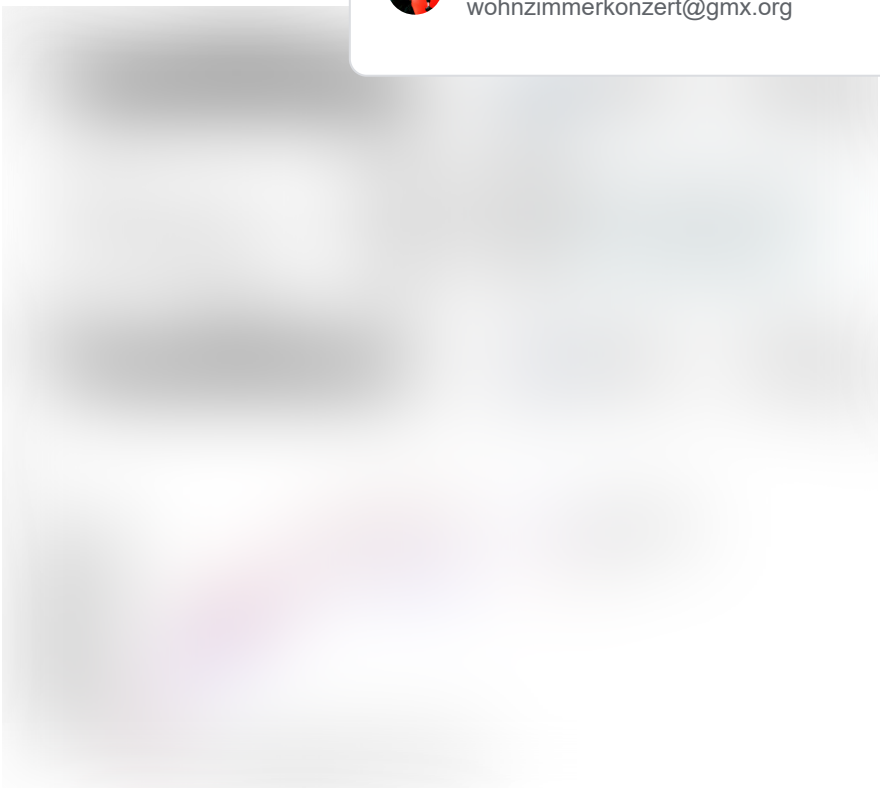

An interesting side note is that, as the authors point out, the addition of a furin site allows the mutant virus to infect nerve cells. Perhaps the CoV2 furin site is the reason why some patients with CoV2 exhibit neurological symptoms, including loss of smell:

> *Mutation of the S2′ site of QX genotype (QX-type) spike protein (S) in a recombinant virus background results in higher pathogenicity, pronounced neural symptoms and neurotropism when compared with conditions in wild-type IBV (WT-IBV) infected chickens. In this study, we present evidence suggesting that recombinant IBV with a mutant S2′ site (furin-S2′ site) leads to higher mortality. Infection with mutant IBV induces severe encephalitis and breaks the blood–brain barrier.*
>
> …
>
> *In summary, our results demonstrate that the furin cleavage site upstream of the FP in S protein is an important site for CoV, modulating entry, cell–virus fusion, adaptation to its host cell, cell tropism and pathogenicity, but not antigenicity.*

To be clear, many coronaviruses have naturally occurring furin sites, and they are very diverse. Obviously, they can appear as
happened in the case of MERS, as was
authors, including Shi Zhengli and Ra
coronavirusology. We will come back
about that article. In it the authors ha
to jump from bats to humans, and one
it was not an insertion of new amino a
in red on the left below):

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The authors did not just show this, but actually introduced these mutations back into the original bat strain: they created the same furin site and showed that it enables the bat strain to infect 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.*
>
> …
>
> *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 hu*
> ***hECP motifs enabled HKU4 spike to***
> ***and thereby allowed HKU4 pseudov***
> ***proteases to enter human cells. Thes***
> ***single mutations at the S1/S2 boun***
> ***entry into human cells.***

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By the way, *how* they did it might frighten those who aren't familiar with modern biotechnology — because the authors inserted this coronavirus spike-like protein into inactivated HIV:

> *Briefly, MERS-CoV-spike-pseudotyped retroviruses expressing a luciferase reporter gene were prepared by cotransfecting HEK293T cells with a plasmid carrying Env-defective, luciferase-expressing HIV-1 genome (pNL4–3.luc.R-E-) and a plasmid encoding MERS-CoV spike protein.*

Perhaps this is what prompted Indian researchers to look for sequences similar to HIV in the CoV2 genome (but their preprint was quickly criticized for bad methodology and erroneous conclusions). In fact, experts use such pseudoviruses regularly, and in general, one should not be scared of retroviruses as a class — their subspecies lentiviruses have been used for gene therapy for many years.

Where Did RaTG13 Come From?

RaTG13 is a very unusual strain. Odd to see that Shi Zhengli's group was silent about it for all these years. After all, it is very different from its SARS-like siblings, especially in the spike protein, which is precisely what determines which types of cells (and in which animals) this virus can infect. Here is a genome similarity graph of CoV2 compared to other bat coronaviruses (panel B):

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The red curve represents RaTG13 while the blue curve is for the strains closest to RaTG13 (ZXC21 and ZC45). These strains were isolated from Chinese horseshoe bats (*Rhinolophus sinicus*) in Zhoushan in 2015 (ZXC21) and 2017 (ZC45). As can be seen from the above graph, even they differ in their S proteins from RaTG13. A direct sequence comparison illustrates this difference best:

As we can see, the spike proteins of ZXC21 and ZC45 are not only 23–24 amino acid residues shorter than the RaTG13 protein, but they are shorter in the most important place — in the RBM (note the deletions in the red box marked with red dashes).

So where did RaTG13 come from? As I already mentioned, in 2020 Shi Zhengli reported that she isolated it in 2013 fr
affinis, not the usual suspects *R. sinic*
was not known, and here is how Shi Z
RaTG13's similarity to CoV2:

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> *We then found that a short region of RN*
> *coronavirus (BatCoV RaTG13) — whic*
> *from Yunnan province — showed high sequence identity to 2019-CoV2. We carried out full-length sequencing on this RNA sample (GISAID accession number EPI_ISL_402131). Simplot analysis showed that 2019-CoV2 was highly similar throughout the genome to RaTG13 (Fig. 1c), with an overall genome sequence identity of 96.2%.*

Not much detail: *previously detected*, and that is that. Moreover, the quote seems to imply that until 2020, they only sequenced a part of its genome, the RdRp gene (which is part of Orf1b that precedes the spike protein gene). Ok, but where exactly in Yunnan was it obtained? The paper doesn't mention it, and neither does GenBank. However, the GISAID entry seems to have a bit more info: collected in Pu'er City from a male bat's fecal swab:

This rang a bell, as in my wanderings around Pubmed, I had already encountered an expedition to Pu'er in the summer of 2013:

> *Bats were captured from various locations in five counties of four prefectures of Yunnan Province, China, from May to July 201*

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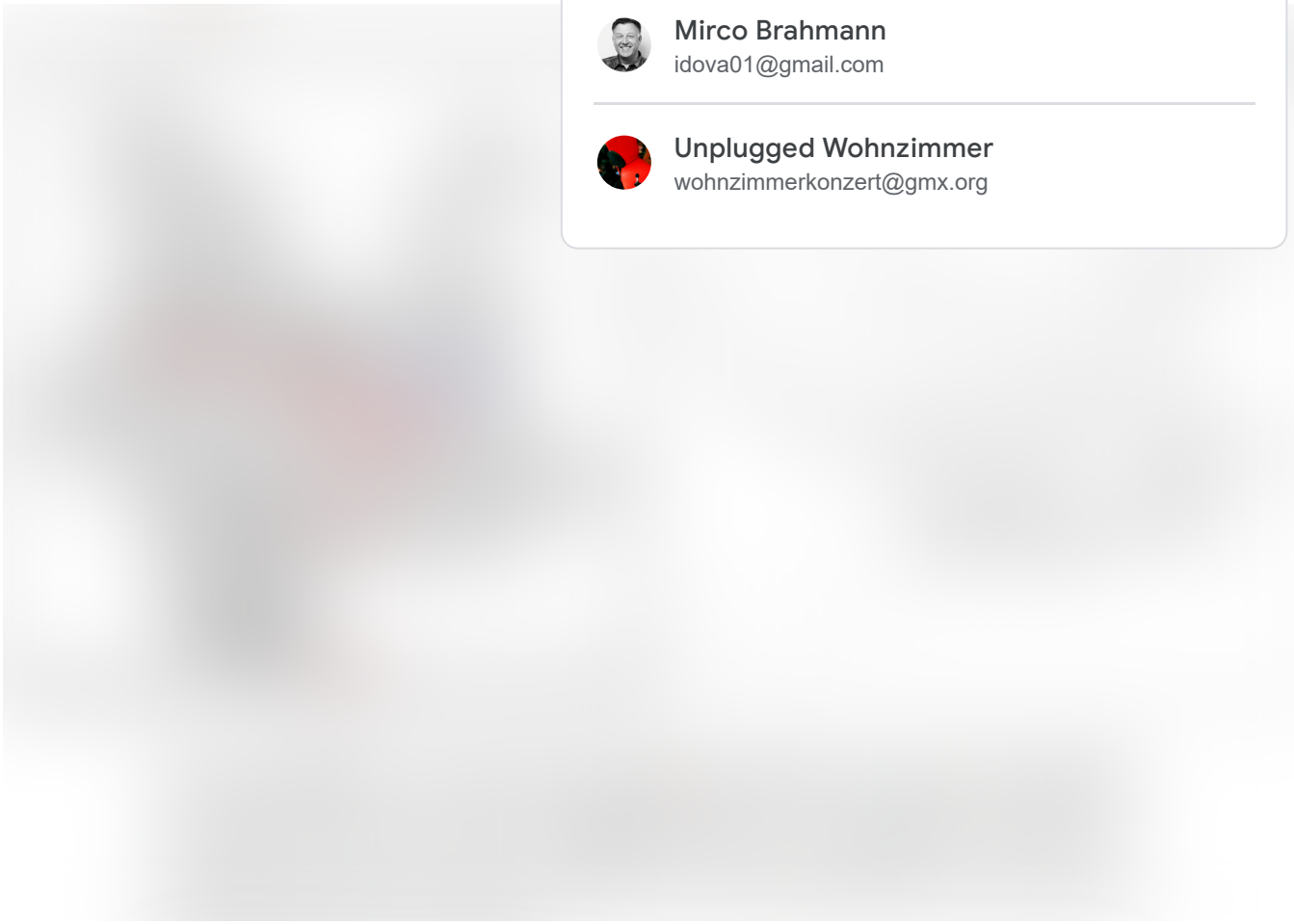

Researchers did not report anything particularly interesting for us from that expedition, but maybe it was then that Shi Zhengli or someone from her group obtained the RaTG13 sample? Which they sequenced only partially, and for some reason decided not to publish, although it was very different from everything known before.

By the way, Shi Zhengli could well have personally participated in that expedition, as she expressed great fondness when describing them — for example, in her TED-like talk in 2018, where she showed personal photos from such expeditions:

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Moreover, it was a series of exactly such expeditions that brought Shi Zhengli worldwide fame and a “Batwoman” moniker: in a 2013 Nature paper, her group triumphantly announced that in Yunnan caves they had discovered carrier bats of the RsSHC014 and Rs3367 strains that coincided with the first SARS-CoV by 85% and 96%, respectively.

It is quite a coincidence that around the same time in Yunnan, Shi Zhengli’s group also discovered RaTG13, the closest strain to CoV2, and the two also share 96% of their genomes.

UPD: Is RaTG13 the same as RaBtCoV/4991?

[UPDATED] After I had published this post, I was pointed to this preprint that alleges that RaTG13 is, in fact, RaBtCoV/4991 (KP876546), which Shi Zhengli had previously reported discovering in an abandoned mineshaft in Yunnan in 2013. There indeed are several reasons to think so. First and foremost, the only published sequence for RaBtCoV/4991 is 100% identical to that of RaTG13 at the **nucleotide** level, albeit being just a 370-bp stretch of the RdRp gene:

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Second, the collection details of the tw
collected in July 2013 from a fecal swa

RaBtCoV/4991 was collected in a mineshaft located in the Mojiang county, which is under the jurisdiction of Pu'er City:

> *Mojiang Hani Autonomous County is an autonomous county under the jurisdiction of Pu'er City, in the south of Yunnan Province, China.*
> *Wikipedia*

And Pu'er City is listed as the collection location of RaTG13 at the GISAID database, which could well be an approximation for the Mojiang mineshaft.

It is odd that in her 2020 paper on RaTG13 Shi Zhengli fails to mention RaBtCoV/4991 or cite her 2016 paper about its discovery, for which she is listed as the one who "designed and coordinated the study". It is not like RaBtCoV/4991 was forgotten by her group, as it is mentioned in their 2019 paper, where it is included in a phylogenetic tree of other coronaviruses:

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I doubt that RaBtCoV/4991's place in that tree was determined based solely on a 370-bp fragment, so I would think that by early 2019, Shi Zhengli's group would have sequenced its full genome.

Intriguingly, both pangolin-2017 and pangolin-2019 genomes are also very close in this stretch of the RdRp gene, and CoV2 and pangolin-2019 share a few common mutations not found in RaTG13:

But let's put this topic aside for now and get back to the story of Shi Zhengli's famous 2013 Nature paper.

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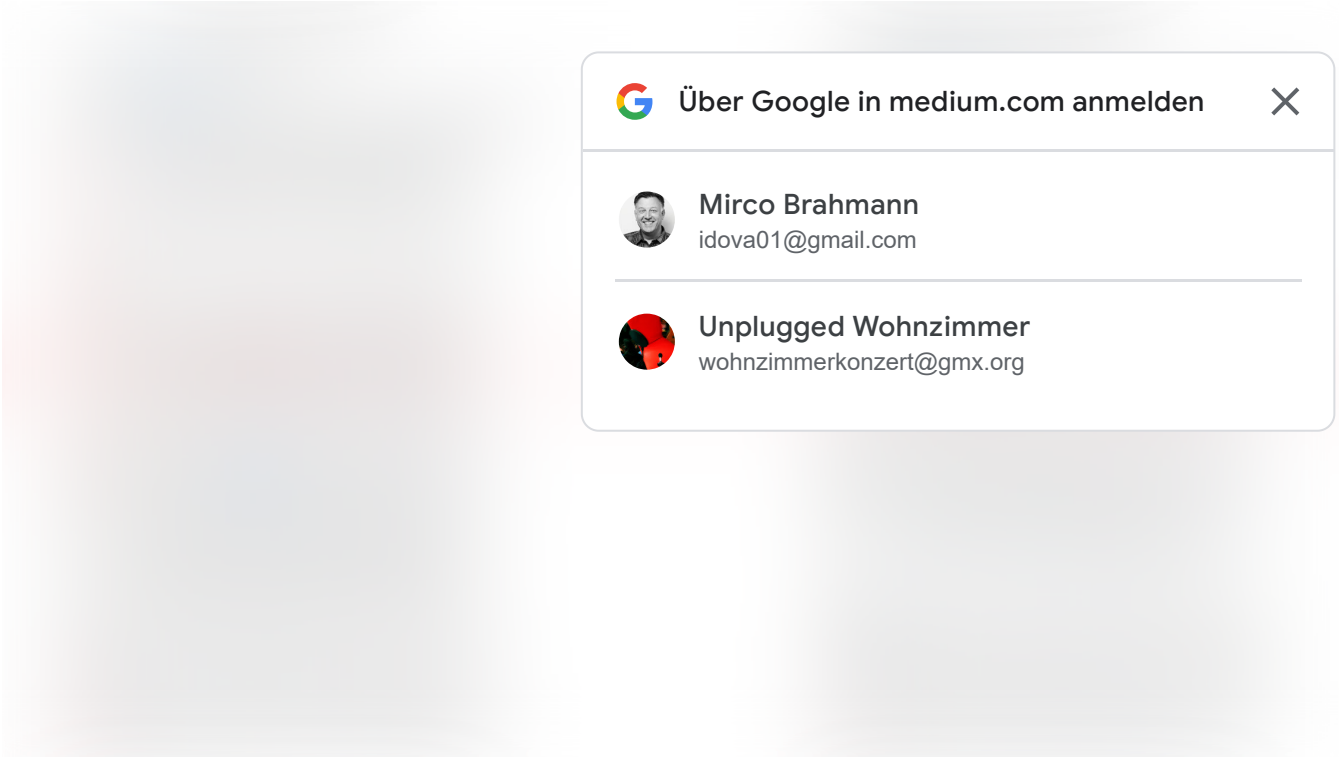
"Wuhan-1"

In that paper, Shi Zhengli's group alsc
in monkey Vero cells, they managed t
the Rs3367 strain. The authors name
Wuhan Institute of Virology):

> *Most importantly, we report the first recorded isolation of a live SL-CoV (bat SL-CoV-WIV1) from bat faecal samples in Vero E6 cells, which has typical coronavirus morphology, 99.9% sequence identity to Rs3367 and uses ACE2 from humans, civets and Chinese horseshoe bats for cell entry. Preliminary in vitro testing indicates that WIV1 also has a broad species tropism.*

Let's compare RaTG13 with Rs3367 and RsSHC014:

As we can see, the spike proteins of these strains are not only 13 amino acids shorter than that of RaTG13, but they also differ in the first quarter of the protein quite substantially. By the way, it is curious that the spike proteins of Rs3367 (aka WIV1) and RsSCH014 are almost identical, and differ only in the RBD region (right sequence below). Almost like CoV2 and RaTG13 (not counting the furin insert):

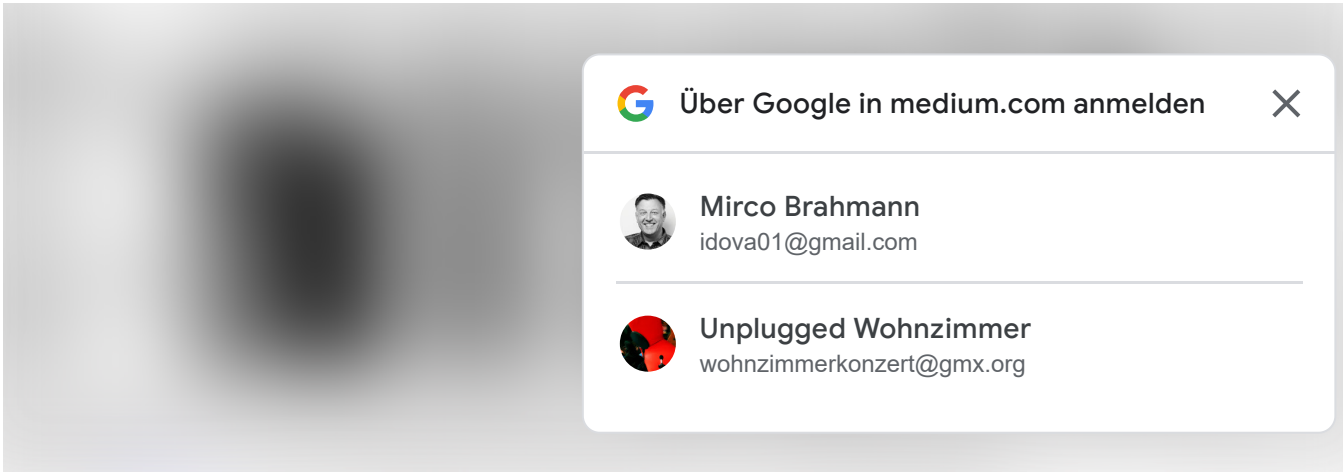


Could researchers, having received coronavirus samples from pangolins that were intercepted by customs in March 2019, then want to check whether the RBM in pangolin strains can bind to the human ACE2 receptor? And could such researchers also decide to throw an extra furin site in the mix?

Theoretically, of course, they could. From a technical standpoint, it is almost routine for virologists to conduct such experiments. A reasonable question might be: why use RaTG13 as a backbone, and not, say, the tried and true WIV1? Well, it doesn't have to be either-or: maybe a chimera with WIV1 was also tested. But in parallel, they might have decided to simulate recombination of the pangolin virus with the bat strain closest to it — after all, RaTG13 is much closer to the pangolin strains than WIV1: its spike protein is closer to them both phylogenetically and structurally — it even matches them in length, while the proteins of WIV1/Rs3367 and RsSHC014 are 13 amino acids shorter. Also, the QTQTNS mutation common to RaTG13 and pangolin-2019 (MP789) just before the protease site could not have gone unnoticed by coronavirus experts.

Other Yunnan Strains

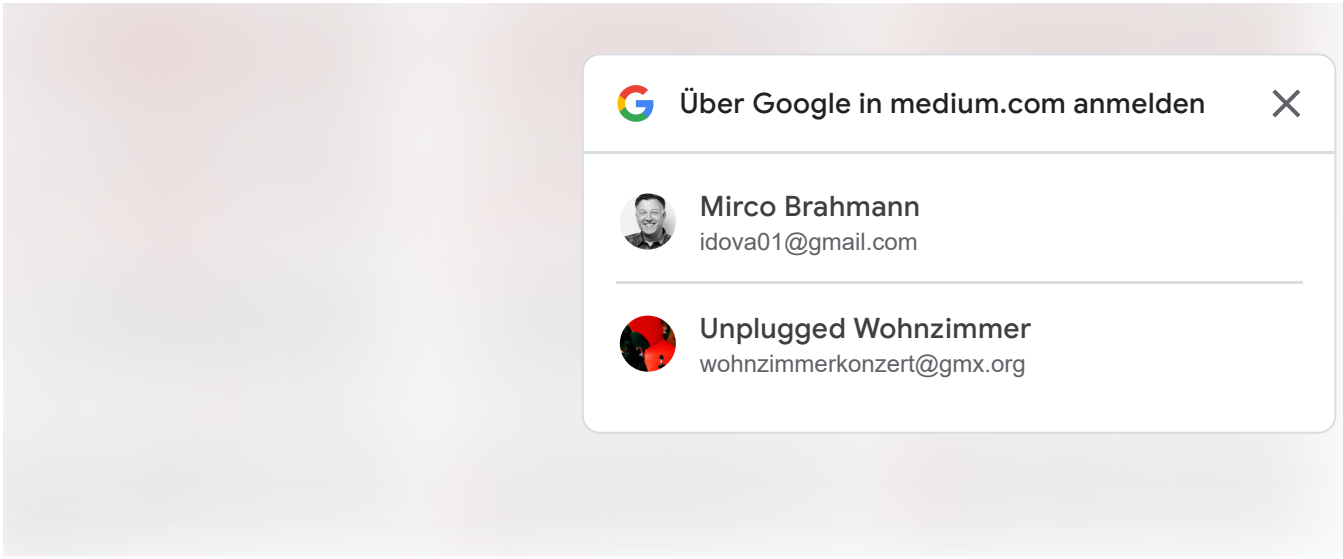
In 2011, other researchers had also found samples of coronaviruses from the Yunnan *Rhinolophus* ***affinis***. The strain LYRa11 seemed to me the most interesting:



But it is also quite distant from RaTG13, and much closer to Rs3367 (that's the strain that shares 96% with the first SARS-CoV):

But RaTG13, isolated from the same *Rhinolophus* ***affinis*** bats as LYRa11, looks the least like it (left sequence comparison).

Finally, another Yunnan strain (ingenuously named Yunnan2011), isolated in 2011 from another subspecies of horseshoe bats, *Rhinolophus pusillus*, is even less similar to RaTG13 than LYRa11:



Between themselves, Yunnan2011 and LYRa11 (the right sequence above) are not particularly similar, apart from the highly conserved S2 region. By the way, what's up with the differing naming conventions for these strains? Sometimes they fully spell out the year, sometimes partially, yet other times not at all (Rs3367). The carrier species sometimes leads (***Ra***TG13), sometimes follows (LY***Ra***11). And what do TG, LY or SHC stand for? Initials of the person sequencing the genome?

Anyways, let's move on from viral archeology to viral engineering, namely transplanting key areas of the spike protein between species and other gain-of-function (GOF) experiments.

1999: First Chimeric Coronavirus

If you think that all of the gain-of-function coronavirus research into what exactly allows coronaviruses to jump from one species to another began in response to the first SARS outbreak in 2002, you'd be mistaken. Virologists experimented with chimeric coronaviruses long before that. Here, for example, is a 1999 paper from the Dutch group of Peter Rottier from Utrecht University with a revealing title *Retargeting of Coronavirus by Substitution of the Spike Glycoprotein Ectodomain: Crossing the Host Cell Species Barrier*:

> *Using targeted RNA recombination, we constructed a mutant of the coronavirus mouse hepatitis virus (MHV) in which the ectodomain of the spike glycoprotein (S) was replaced with the highly divergent ectodomain of the S protein of feline infectious peritonitis virus. The resulting chimeric virus, designated fMHV, acquired the ability to infect feline cells and simultaneously lost the ability to infect murine cells in tissue culture.*

By the way, Shi Zhengli seems to have worked under the supervision of Peter Rottier in Utrecht for a time. At least in 2005, sh
listed as her affiliation (but her currer
article itself is quite curious — in it th
viruses to expand their species tropisr

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> *Only a relatively few mutations in its sp*
> *from a murine-restricted tropism to an*
> *One such virus that we studied had acquired two putative heparan sulfate-binding sites* ***while preserving another site in the furin-cleavage motif****. The adaptation of the virus through the use of heparan sulfate as an attachment/entry receptor was demonstrated by increased heparin binding as well as by inhibition of infection through treatment of cells and the virus with heparinase and heparin, respectively.*

It is interesting that the furin site in that virus (SRRAHR | SV) is similar to the site in CoV2 (SPRRAR | SV), although in CoV2 it is cut more efficiently due to dual arginines (this is what makes it a **poly**basic site, i.e. it has **multiple basic** amino acids in a row in the RxxR sequence):

But what is especially curious is that the mutations that allowed the virus to “expand its horizons” occurred not in animals, but *in vitro*. Moreover, it seems, they happened pretty quickly:

> *MHV/pi23, a virus obtained after 23 of the 600 passages that resulted in MHV/BHK, also contains a putative HS-binding site in t*
> *MHV/BHK, albeit as a smaller insertio*
> *immediately upstream of the fusion pep*
> *some extent but much less efficiently th*
> *binding sites, however, mutations foun*
> *domain and the putative fusion peptide*
> *entry into nonmurine cells. We are curr*
> *mutations that are required for the extended host range phenotype.*

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Skipping ahead, I'll just mention that there were other groups that used *in vitro* mutagenesis to increase the virulence of coronaviruses, for example, MERS:

> *To better understand the species adaptability of MERS-CoV, we identified a suboptimal species-derived variant of DPP4 to study viral adaption. Passaging virus on cells expressing this DPP4 variant led to accumulation of mutations in the viral spike which increased replication.*

Moreover, their mutations arose after just several passages (rounds of cell culture reproduction):

(F) Schematic of single and double mutation emergence in MERS-CoV spike over different passages. (G) Location of mutations within MERS-CoV spike.

But those experiments occurred much later. In the meantime, let's go back to 2002 — BEFORE the outbreak of the first SARS-CoV.

Ralph "Trailblazer" Baric

Ralph Baric is a legend in coronavirology. He is a trailblazer of synthetic genomic manipulation techniques. Back in 200 marked a milestone in both the study gain-of-function research. In their pap synthetic clone of a natural murine co

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> *A novel method was developed to assem coronavirus mouse hepatitis virus strai that spanned the 31.5-kb MHV genome were isolated. The ends of the cDNAs were engineered with unique junctions and assembled with only the adjacent cDNA subclones, resulting in an intact MHV-A59 cDNA construct of ~31.5 kb in length. The interconnecting restriction site junctions that are located at the ends of each cDNA are systematically removed during the assembly of the complete full-length cDNA product, allowing reassembly without the introduction of nucleotide changes… The method has the potential to be used to construct viral, microbial, or eukaryotic genomes approaching several million base pairs in length and used to insert restriction sites at any given nucleotide in a microbial genome.*

In essense, the authors have “translated” the RNA virus into the language of DNA (using reverse transcriptase), which enabled them to manipulate its genome with the help of existing genetic engineering tools. Having created 7 such cDNA provirus segments, the authors then stitched them together “seamlessly” (i.e. without introducing any new, even silent mutations, including new restrictase sites), after which they transcribed their construct back into RNA, which was then translated into virus particles in other cells.

SARS-2003

Just a few weeks after the publication of the above work, the first SARS-CoV epidemic broke out. The Baric group sprang int
submitted a paper on synthetically rec

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> *Using a panel of contiguous cDNAs tha*
> *length cDNA of the SARS-CoV Urbani s*
> *viruses (infectious clone SARS-CoV) th*
> *inserted into the component clones. Rec*
> *virus and both were inhibited by treatment with the cysteine proteinase inhibitor…*
> *Availability of a SARS-CoV full-length cDNA provides a template for manipulation of the viral genome, allowing for the rapid and rational development and testing of candidate vaccines and therapeutics against this important human pathogen.*

The speed of the Baric group illustrates how quickly a qualified team of virologists can create a synthetic clone from a natural virus, and therefore make genetic modifications to it. Moreover, that was back in 2003. Today, a qualified laboratory can repeat those steps in a matter of weeks.

In fact, two just did: the Swiss have created a synthetic clone of CoV2 in under a month, while it took the Galveston BS

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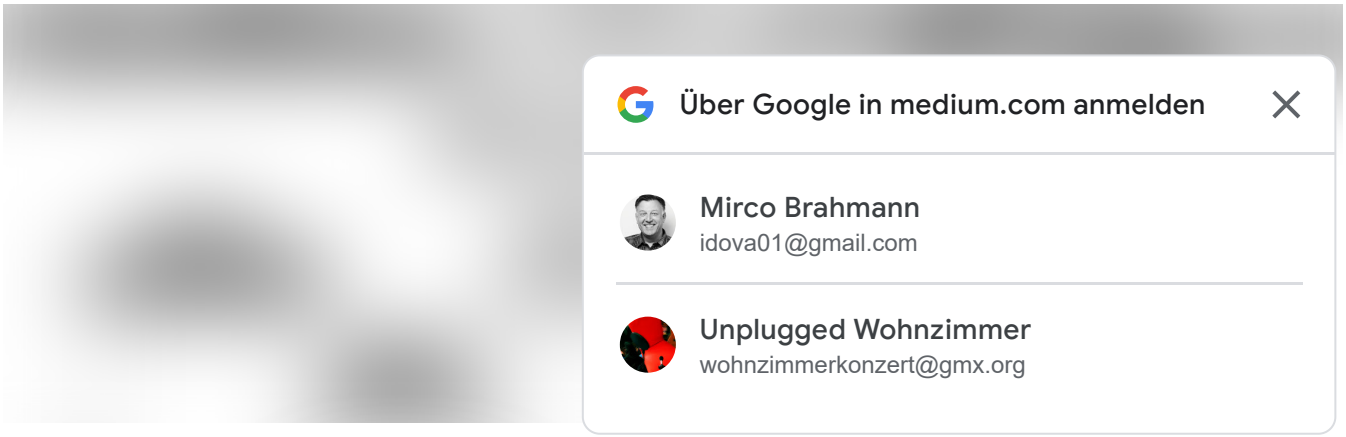
SARS-2006

Baric was the first, but far from the las
bounds, creating newer and better toc
virology techniques. For example, in 2
footsteps, also creating a synthetic SARS clone, but using an alternative approach (bacterial artificial chromosome):

> *The engineering of a full-length infectious cDNA clone and a functional replicon of the severe acute respiratory syndrome coronavirus (SARS-CoV) Urbani strain as bacterial artificial chromosomes (BACs) is described in this study. In this system, the viral RNA was expressed in the cell nucleus under the control of the cytomegalovirus promoter and further amplified in the cytoplasm by the viral replicase. Both the infectious clone and the replicon were fully stable in Escherichia coli.*
>
> …
>
> *The assembled SARS-CoV infectious cDNA clone was fully stable during its propagation in E. coli DH10B cells for more than 200 generations, considerably facilitating the genetic manipulation of the viral genome (data not shown). The detailed cloning strategy, plasmid maps, and sequences are available upon request.*



Strategy to assemble a SARS-CoV infectious cDNA clone as a BAC.
(A) Genetic structure of the SARS-CoV Urbani strain genome. Relevant restriction sites used for the assembly of the full-length cDNA clone are indicated. Numbers in parentheses indicate the genomic positions of the first nucleotide of the restriction endonuclease recognition sequence. Letters and numbers indicate the viral genes. L, leader sequence; UTR, untranslated region; An, poly(A) tail. (B) Construction of pBAC-SARS-CoV 5′-3′. After the selection of appropriate restriction sites, the intermediate plasmid pBAC-SARS-CoV 5′-3′ was constructed as the backbone for assembling the infectious cDNA clone. This plasmid includes the first 681 nt of the genome under the control of the CMV promoter, a multiple-cloning site containing the restriction sites selected for the final assembly of the infectious clone, and the last 975 nt of the genome, followed by a synthetic poly(A) tail (pA), the hepatitis delta virus ribozyme (Rz), and the bovine growth hormone termination and polyadenylation sequences (BGH). All these elements were precisely joined by overlapping PCR. The CMV promoter transcription start and the ribozyme cleavage site are shown. © Schematic diagram showing the five-step cloning strategy used for the assembly of the SARS-CoV full-length cDNA clone. The five overlapping cDNA fragments, named SARS 1 to SARS 5, were sequentially cloned into the plasmid pBAC-SARS-CoV 5′-3′ to generate the plasmid pBAC-SARS-CoVFL. Relevant restriction sites are indicated. The labels are as described for panel A.

True, they didn't do it as elegantly as Baric, as their final assembly of the synthetic virus included their added restriction enzyme sites, while Baric learned to combine fragments "seamlessly". But this is a minor point, the Spanish approach is just as robust — in 2013, with its help, the same authors had created a synthetic clone of MERS, and in 2015 their technique was included in a coronavirus textbook (chapter 13).

Wuhan 2007

Let's get back to 2007. That is when the Shi Zhengli group joined the synthetic virology race with a study of the spike protein of human and bat coronaviruses, trying to determine what exactly is responsible for the ability to skip from one species to another:

> *A series of S chimeras was constructed by inserting different sequences of the SARS-CoV S into the SL-CoV S backbone.*

That is, the authors inserted different segments from the human SARS-CoV spike protein into the spike protein of the ba

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> *From these results, it was deduced that*
> *necessary and sufficient to convert Rp3*

At the same time, they tried to replace

> ***For introduction of the RBM of SARS-CoV S into the SL-CoV S, the coding region from aa 424 to 494 of BJ01-S was used to replace the corresponding regions of Rp3-S, resulting in a chimeric S (CS) gene designated CS424–494.***

Given that the above was written in 2007, I think today it will not be difficult for even a novice virologist to replace the RBM of one virus by an RBM from another.

Chimera-2015

In light of the above experiments, it is not very clear what caused the uproar that followed probably the most famous gain-of-function virology paper. I am referring to the joint 2015 work of Shi Zhengli and Ralph Baric, in which they created a synthetic chimeric virus:

> *Using the SARS-CoV reverse genetics system, we generated and characterized a chimeric virus expressing the spike of bat coronavirus SHC014 in a mouse-adapted SARS-CoV backbone. The results indicate that group 2b viruses encoding the SHC014 spike in a wild-type backbone can efficiently use multiple orthologs of the SARS receptor human angiotensin converting enzyme II (ACE2), replicate efficiently in primary human airway cells and achieve in vitro titers equivalent to epidemic strains of SARS-CoV. Additionally, in vivo experiments demonstrate replication of the chimeric virus in mouse lung with notable pathogenesis. Evaluation of available SARS-based immune-therapeutic and prophylactic modalities revealed poor efficacy; both monoclonal antibody and vaccine approaches failed to neutralize and protect from infection with CoVs using the novel spike protein. On the basis of these findings, we synthetically re-derived an infectious full-length SHC014 recombinant virus and demonstrate robust viral replication both in vitro and in vivo.*

To me, the authors followed a familiar path: they took the spike-like protein from RsSHC014, which Shi Zhengli isolated from Yunnan bats in 2011, and inserted it into a murine-adapted variant of SARS-CoV for subsequent *in vivo* experiments. They also

tested it in human cells, and almost as an aside created a recombinant clone of the same RsSHC014 strain:

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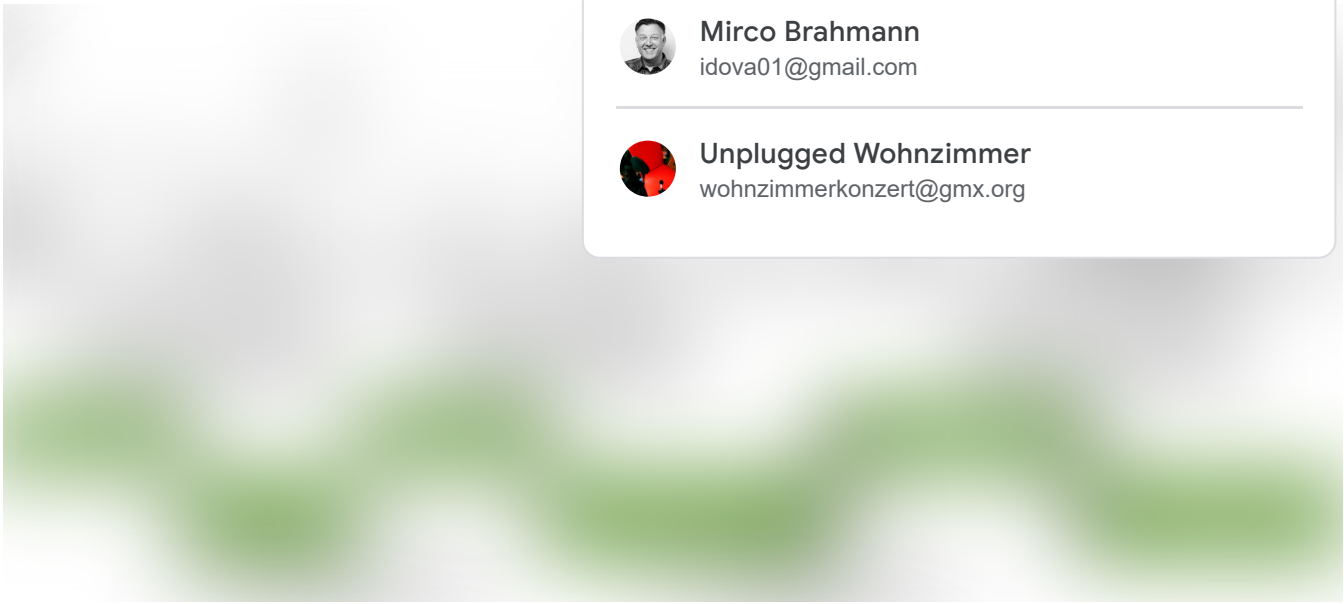


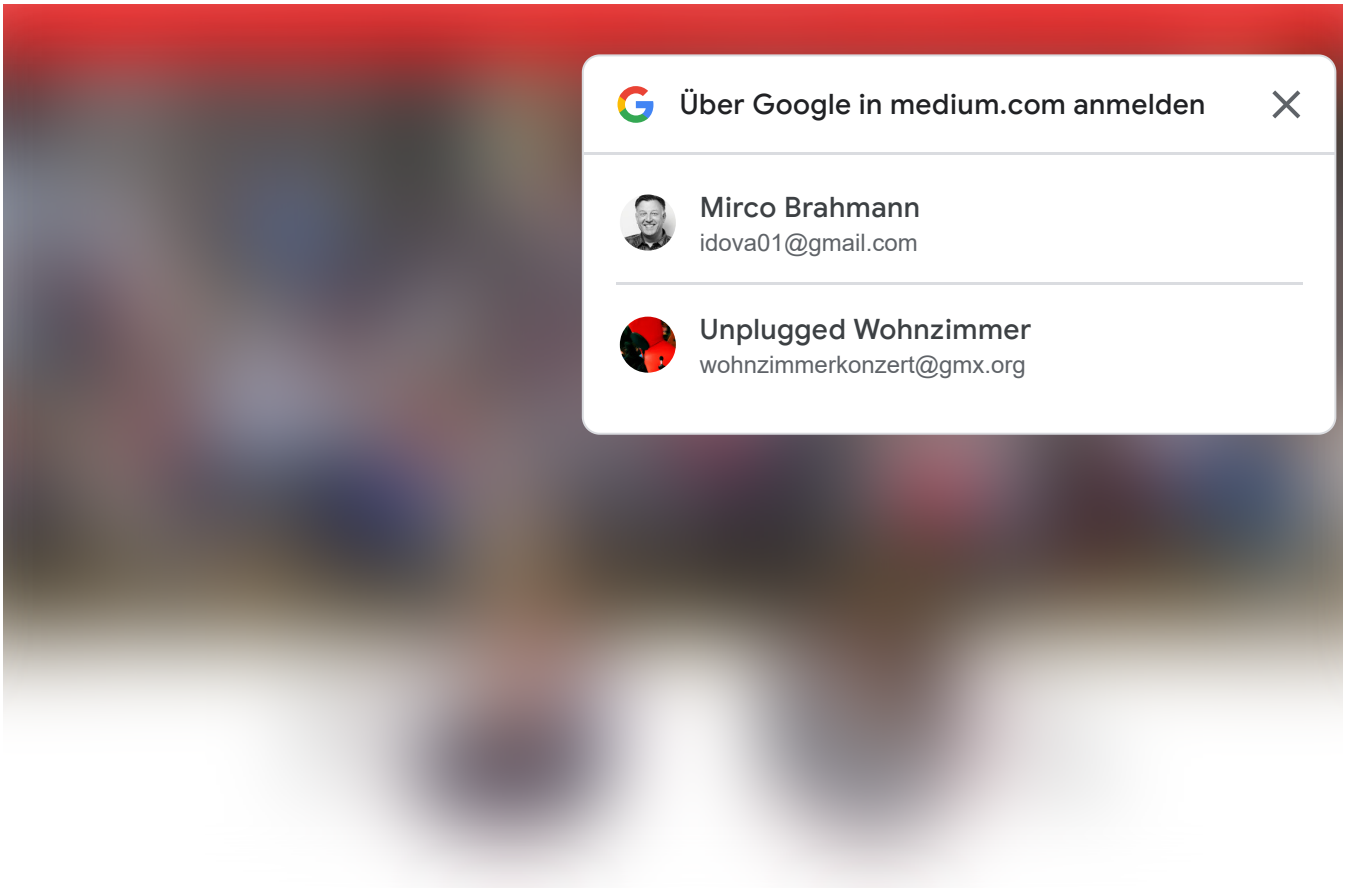
(a) Schematic of the SHC014-CoV molecular clone, which was synthesized as six contiguous cDNAs (designated SHC014A, SHC014B, SHC014C, SHC014D, SHC014E and SHC014F) flanked by unique BglI sites that allowed for directed assembly of the full-length cDNA expressing open reading frames (for 1a, 1b, spike, 3, envelope, matrix, 6–8 and nucleocapsid). Underlined nucleotides represent the overhang sequences formed after restriction enzyme cleavage.

The researchers also uncovered that it was not only the binding of spike protein to the receptor that determined the virus's potential for transition from one animal species to another, because the SHC014-MA15 chimera was more virulent than SHC014 itself, even in human cells:

> *Notably, differential tropism in the lung as compared to that with SARS-MA15 and attenuation of full-length SHC014-CoV in [human epithelial airway cell] cultures relative to SARS-CoV Urbani suggest that factors beyond ACE2 binding — **including spike processivity**, receptor bio-availability or antagonism of the host immune responses — may contribute to emergence.*

I especially want to highlight the spike processivity in the quote, because this is not the first time that virologists have mentioned that the ability of a spike protein to be cleaved by proteases (including furin) can have an impact on virulence.

That's all I have to say about that paper. As a curiosity here is a common photo of its key authors, which was taken in Wuhan, in October 2018. Fittingly, Ralph Baric and Shi Zhengli are front and center. I call this photo "The Wuhan Clan". (Sorry, couldn't resist).



Murine SARS-2007

One quick aside regarding the “murine virus MA15” from the above paper. That was not some kind of natural murine coronavirus, as one might think. It was a laboratory-modified human SARS-CoV, which back in 2007 the Baric group — possibly in competition with the Shi Zhengli group (remember their article from 2007) — turned into a real beast. To do this, they first iteratively “improved” it in mice, and when after several iterations it became maximally “effective”, they reproduced the observed mutations in a synthetic clone, and once again checked that it really does have increased virulence and lethality:

> *We adapted the SARS-CoV (Urbani strain) by serial passage in the respiratory tract of young BALB/c mice. Fifteen passages resulted in a virus (MA15) that is lethal for mice following intranasal inoculation. Lethality is preceded by rapid and high titer viral replication in lungs, viremia, and dissemination of virus to extrapulmonary sites accompanied by lymphopenia, neutrophilia, and pathological changes in the lungs. Abundant viral antigen is extensively distributed in bronchial epithelial cells and alveolar pneumocytes, and necrotic cellular debris is present in airways and alveoli, with only mild and focal pneumonitis. These observations suggest that mice infected with MA15 die from an overwhelming viral infection with extensive, virally mediated destruction of pneumocytes and ciliated epithelial cells. The MA15 virus has six coding mutations*

> *associated with adaptation and increased virulence;* ***when introduced into a recombinant SARS-CoV, these mutat***
> ***(rMA15)****, duplicating the phenotype o*
> *inoculation with MA15 reproduces ma*
> *SARS.*

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Baric-2008

Here is another example of the potential scientific rivalry between the Baric and Shi Zhengli groups. In 2008, the Baric group took the Bat-SCoV strain and replaced its RBD with an RBD from human SARS. That is, they essentially reproduced the work of Shi Zhengli's group from 2007, except they didn't limit themselves to pseudo-viruses, but created a real chimeric virus:

> *Here, we report the design, synthesis, and recovery of the largest synthetic replicating life form, a 29.7-kb bat severe acute respiratory syndrome (SARS)-like coronavirus (Bat-SCoV), a likely progenitor to the SARS-CoV epidemic.*
> …
> *To test whether the RBDs of Bat-SCoV and SARS-CoV were interchangeable, we replaced the Bat-SCoV RBD (amino acid 323–505) with the SARS-CoV RBD (amino acid 319–518) (27, 28) (GenBank accession no. FJ211860), simulating a theoretical recombination event that might occur during mixed infection in vivo (Fig. 1B).*

(B) Schematic representation showing organization of the SARS-CoV and Bat-SCoV Spike proteins. The engineered Spike proteins are pictured below with the virus name to the left. Bat-SRBD includes all of the Bat-SCoV Spike sequence except that the Bat-SCoV RBD (Bat-SCoV amino acid 323–505) is replaced with the SARS-CoV RBD (amino acid 319–518) (GenBank accession no. FJ211860). Bat-SRBD-MA includes the MA15 Spike RBD change at SARS-CoV aa Y436H. Bat-SRBM includes the minimal 13 SARS-CoV residues critical for ACE2 contact, resulting in a chimeric RBD of Bat-SCoV amino acid 323I-429T and SARS-CoV

amino acid 426R-518D. Bat-Hinge is Bat-SRBM sequence, with Bat-SCoV amino acid 392L-397E replaced with SARS-CoV amino acid 388V-393D. Bat-F
with the remaining 3′ sequence from Bat-SCo
of transcript activity and approximate sto
infectious viru

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Baric-2016

The Baric group does seem to have its
they essentially repeated their collaboration with Shi Zhengli from 2015 to create a chimeric virus, only this time they inserted a spike protein segment into their mouse-adapted SARS not from RsSCH014, but from another strain Shi Zhengli found in Yunnan — its close relative Rs3367. Or, to be exact, from WIV1 — the laboratory clone of Rs3367 isolated at the Wuhan Institute of Virology in 2013:

> *Using the SARS-CoV infectious clone as a template (7), we designed and synthesized a full-length infectious clone of WIV1-CoV consisting of six plasmids that could be enzymatically cut, ligated together, and electroporated into cells to rescue replication competent progeny virions (Fig. S1A). In addition to the full-length clone, we also produced WIV1-CoV chimeric virus that replaced the SARS spike with the WIV1 spike within the mouse-adapted backbone (WIV1-MA15, Fig. S1B). … To confirm growth kinetics and replication, Vero cells were infected with SARS-CoV Urbani, WIV1-MA15, and WIV1-CoV.*

To me, the 2016 paper looks a lot like the 2015 one. Moreover, its rationale is not very clear to me: after all, WIV1/Rs3367 al
CoV. So I am not sure why one would
relative back into SARS-CoV. Maybe ju
the article acquires a certain duality:

Oh, and I am not sure how in 2015 Ba
"chimeric coronavirus spike proteins",
disclosed in their papers long before 2015.

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Baric-1990

Just so you appreciate how long Ralph Baric has been at this game — he was designing recombinant coronaviruses way before there were any DNA sequencing machines or other modern tools of genetic engineering. Here is his paper on the creation of "temperature mutants" from mouse coronavirus from 1990:

> *The A59 strain of mouse hepatitis virus (MHV-A59) was used throughout the course of this study. Virus was propagated and cloned three times in the continuous murine astrocytoma cell line (DBT).*
> …
> *Various combinations of [temperature sensitive] mutants were mixed and inoculated onto cells at a multiplicity of infection of 10 each.*

So Dr. Baric has been creating mutant viruses for over 30 years.

Wuhan-2017

The Shi Zhengli group has also not been idle since the famous 2015 paper. In 2017, they published a paper where they reported creating not one but 8 chimeric viruses — all made using transplanted RBDs from bat SARS-like viruses which they collected over a span of 5 years from the very cave around Kunming, Yunnan Province, where Shi Zhengli originally found Rs3367 and RsSCH014.

> *Using the reverse genetics technique we previously developed for WIV1 [23], we constructed a group of infectious bacterial artificial chromosome (BAC) clones* ***with the backbone of WIV1 and variants of S genes from 8 different bat SARSr-CoVs****. Only the infectious clones for Rs4231 and Rs7327 led to cytopathic effects in Vero E6 cells after transfection (S7 Fig). The other six strains with deletions in the RBD region, Rf4075,*

> *Rs4081, Rs4085, Rs4235, As6526 and Rp3 (S1 Fig) failed to be rescued, as no cytopathic effects was observed and viral replicati*
> *in Vero E6 cells (S7 Fig). In contrast, w*
> *two successfully rescued chimeric SARS*
> *the newly isolated Rs4874, efficient vir*

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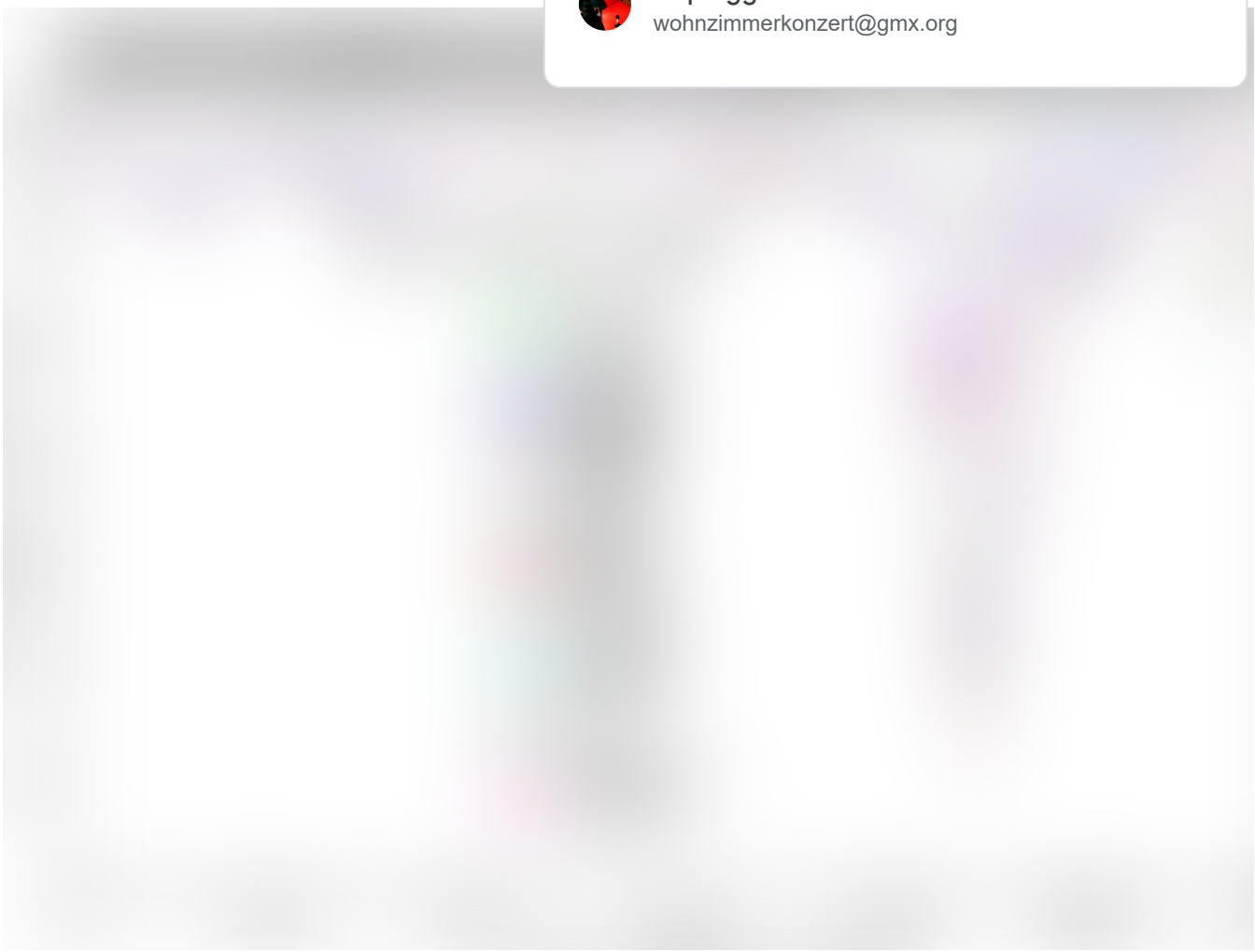

Similarity plot based on the full-length genome sequence of civet SARS CoV SZ3.
Full-length genome sequences of all SARSr-CoV detected in bats from the cave investigated in this study were used as reference sequences. The analysis was performed with the Kimura model, a window size of 1500 base pairs and a step size of 150 base pairs.

The authors then checked if their chimeras can infect human cells, and this time they used a live synthetic virus, rather than not pseudo-typed HIV constructs as before:

> *To assess whether the three novel SARSr-CoVs can use human ACE2 as a cellular entry receptor, we conducted virus infectivity studies using HeLa cells with or without the expression of human ACE2. All viruses replicated efficiently in the human ACE2-expressing cells. The results were further confirmed by quantification of viral RNA using real-time RT-PCR (Fig 8).*

Baric-2019

Ralph Baric also showed no signs of sl
group submitted for publication anoth
protease cleavage (remember the furi
infection" by coronaviruses:

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> *Together, these results demonstrate tha*
> *infection of Vero cells with HKU5-CoV.*
> *cleavage at S1/S2 border, S2', and the endosomal cysteine protease site across MERS, PDF2180, and HKU5 spikes (Fig. 6D) (26). For the S1/S2 site, MERS, Uganda, and HKU5 maintain the RXXR cleavage motif, although the different interior amino acids may alter efficiency. For the S2' sequence, MERS and HKU5 also retain the RXXR motif; however, the Uganda spike lacks the first arginine (SNAR), potentially impacting cleavage.*

As I recall the spirit of scientific competition between the groups of Baric and Shi Zhengli, I can't help but wonder whether someone was conducting similar research in the Wuhan lab in 2019.

Gain-of-Function: Risky Business

Many people who first learn about the above research ask a very valid question: "But why?" Why do scientists create chimeric killer viruses? The politically correct answer is to develop preventive measures (drugs or vaccines) from possible natural chimeras and to understand the risks of their occurrence. Here, in fact, is what Baric, Shi Zhengli, and co-authors themselves wrote on this subject in their famous 2015 paper:

> *In addition to offering preparation against future emerging viruses, this approach must be considered in the context of the US government–mandated pause on gain-of-function (GOF) studies. On the basis of previous models of emergence (Fig. 4a,b), the creation of chimeric viruses such as SHC014-MA15 was not expected to increase pathogenicity. Although SHC014-MA15 is attenuated relative to its parental mouse-adapted SARS-CoV, similar studies examining the pathogenicity of CoVs with the wild-type Urbani spike within the MA15 backbone showed no weight loss in mice and reduced viral replication. Thus, relative to the Urbani spike–MA15 CoV, SHC014-MA15 shows a gain in pathogenesis (Fig. 1). On the basis of these findings, scientific review panels may deem similar studies building chimeric viruses based on circulating strains too risky to pursue, as increased pathogenicity in mammalian models cannot be excluded. Coupled with restrictions on mouse-adapted strains and the development of monoclonal antibodies*

> *using escape mutants, research into CoV emergence and therapeutic efficacy may be severely limited moving forward. Toget*
> *crossroads of GOF research concerns; th*
> *outbreaks must be weighed against the*
> *developing policies moving forward, it*
> *generated by these studies and whether*
> *further investigation versus the inheren*

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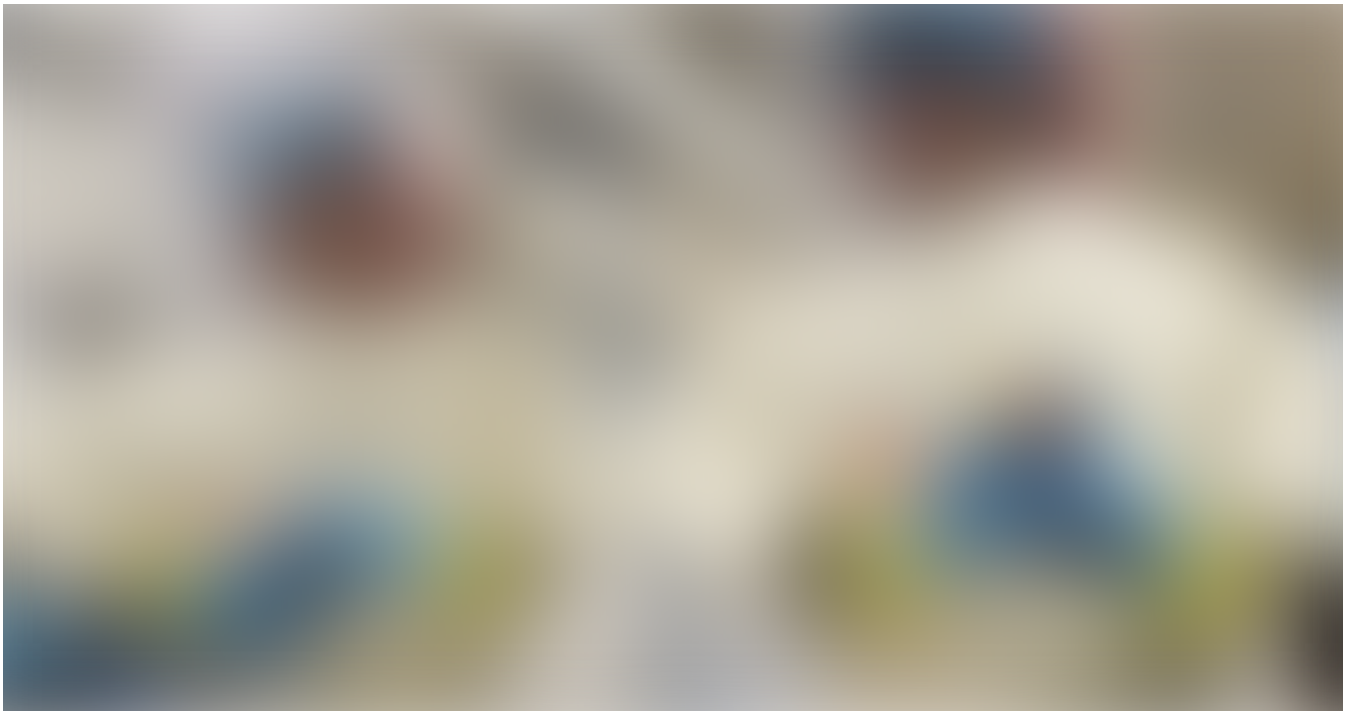
Were these words prophetic? At the end of 2014, the United States introduced a moratorium on state financing of such gain-of-function studies, but it was shortly canceled (in 2017). In China, no moratorium on such studies was introduced, on the contrary, they went full steam ahead with creating new “super labs” of the highest biosafety level (BSL-4), as in 2017 in Wuhan:



To be clear, the Wuhan lab was allowed to work with coronaviruses even before 2017, as these viruses only required a BSL-3 rating which the Wuhan Institute of Virology had. But their aspirations to obtain BSL-4 made a lot of people uneasy, including fellow researchers:

> *Future plans include studying the pathogen that causes SARS, which also doesn’t require a BSL-4 lab, before moving on to Ebola and the West African Lassa virus, which do. Some one million Chinese people work in Africa; the country needs to be ready for any eventuality, says Yuan. “Viruses don’t know borders.”*
> …

> *The plan to expand into a network heightens such concerns. One BSL-4 lab in Harbin is already awaiting accreditation; the nex*
> ***the latter focused on using monkey***
>
> *Lina says that China's size justifies this*
> *research with an abundance of researc*
> *than those in the West when it comes to*
> *want to test vaccines or antivirals, you*
>
> *But Ebright is not convinced of the need for more than one BSL-4 lab in mainland China. He suspects that the expansion there is a reaction to the networks in the United States and Europe, which he says are also unwarranted. He adds that governments will assume that such excess capacity is for the potential development of bioweapons.*
>
> *"These facilities are inherently dual use," he says. The prospect of ramping up opportunities to inject monkeys with pathogens also worries, rather than excites, him: "They can run, they can scratch, they can bite."*
>
> *Trevan says China's investment in a BSL-4 lab may, above all, be a way to prove to the world that the nation is competitive. "It is a big status symbol in biology," he says, "whether it's a need or not."*

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Interestingly, in addition to Wuhan, the Chinese government planned to open a new BSL-4 lab in Kunming, with an eye to testing vaccines on primates. As you might recall, Kunming is not only the capital of Yunnan, but it is also where Shi Zhengli found the strains Rs3367 and RsSHC014 in nearby caves. By the way, primate testing was mentioned by Baric and Shi Zhengli as possible next steps for the development of preventive vaccines against potential future outbreaks of coronaviruses in their famous 2015 paper:

> *However, further testing in nonhuman primates is required to translate these finding into pathogenic potential in humans. Importantly, the failure of available therapeutics defines a critical need for further study and for the development of treatments. With this knowledge, surveillance programs, diagnostic reagents and effective treatments can be produced that are protective against the emergence of group 2b–specific CoVs, such as SHC014, and these can be applied to other CoV branches that maintain similarly heterogeneous pools.*

Maybe by 2019 the creation and testing of potential vaccines against various SARS-like coronaviruses was already in full swin

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Beware of Lab

Let's now take a look at the lab leak hy
context, including previous confirmed
First and foremost, lab leaks of the firs
Singapore, then in December 2003 in Taiwan, and in the spring of 2004 twice in Beijing.

There were close calls in Europe and the USA, although thankfully no infections occurred there. For example, a French lab once lost vials with SARS, and an American BSL-4 laboratory in Texas, lost a vial containing Guanarito (Venezuelan hemorrhagic fever virus):

> *Only one scientist worked with the virus, and Reyes said the lab suspects that scientist accidentally threw the vial away in November.*
> …
> *Galveston biolab requires the most stringent safety measures because it studies biosafetly level BSL-4 materials, or dangerous infectious diseases that have no vaccines or cures. BSL-4 materials include Guanarito, Ebola and smallpox.*

History knows other, much larger-scale leaks. For example, the "resurrection" of the H1N1 flu virus in 1977, which had previously been considered extinct. Yes, this is the virus of the famous "Spanish flu":

> *Human influenza H1N1 viruses appeared with the 1918 pandemic, and persisted, slowing accumulating small changes in its genome (with a major change in 1947), until the H2N2 "Asian" flu appeared in 1957, causing a worldwide pandemic. H1N1 influenza virus then apparently became extinct, and was not isolated for 20 years. In 1969 the "Hong Kong" H3N2 virus replaced the H2N2 virus, and is still circulating.*
>
> *In September 1977 an H1N1 influenza virus was isolated from human infections in the Far East region of the Soviet Union, and in early 1978 the Chinese reported they had isolated H1N1 virus in May of 1977 in northeast China adjacent to the Soviet outbreak. Using the early genetic tools available at the time, the 1977 H1N1 virus was found to be closely related to H1N1 human influenza viruses circulating in 1949–1950, but not to those circulating earlier or later.*

> …
> *Only since 2009–2010 did major pape*
> *H1N1 influenza was a laboratory relat*
> *laboratory strain is the re-emergent H1*
> *China in May of 1977 and in Russia sh*
> …
> *The speculation that the 1977 release n*
> *supported by the observation that in th*
> *isolates expressed "temperature sensitivity" (Kung 1978). Temperature sensitivity normally an uncommon trait, but one that was in the 1970s (and still is) a fundamental trait for making live attenuated influenza vaccines. Temperature sensitivity generally occurs only after a series of substantial laboratory manipulations and selections.*
>
> *Interestingly, further investigation indicated the circulating strains in 1977–78 were often comprised of mixed temperature-sensitive and normal components, and that temperature sensitivity apparently disappeared from the post-1978 H1N1 lineage rapidly. Escape of a mid-protocol population of H1N1 virus undergoing laboratory selection for temperature sensitive mutants would provide such a mixed population. In 1976–77 laboratory personnel in their late teens or early 20s would not have been exposed to pre-1957 H1N1 influenza viruses, and been susceptible to laboratory infections. The low severity of the 1977 pandemic might be in part due to the temperature sensitivity of the virus, a trait that limits virus replication in pulmonary tissues.*

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It seems that the creation of temperature-sensitive viral mutants to develop potentially attenuated vaccines was widespread at the end of the twentieth century. If you remember, in 1990, Ralph Baric himself also experimented with the creation of temperature-sensitive coronavirus strains.

Could something like this have caused the Covid-19 pandemic? Several options are possible — from a leak during development of a potential vaccine to fundamental research on laboratory recombination of the bat and pangolin viruses. Some particularly ambitious researcher could even decide to combine the two "fashionable research themes" — adding a furin site and transplanting RBM from a strain of one species (pangolin) to another (bats), so that later, confirming the increased virulence of the new chimeric virus, they can wax poetic about the dangers of the same recombination happening in Yunnan caves or wet markets. And if such a researcher could even pre-emptively develop a vaccine against this and other potential chimeras, all sorts of accolades could await.

Am I then saying this is what happened? Of course not, I do not claim to *know* what happened. Today, there is no evidence coincidences — for example, that the thousands of kilometers from Yunnan Virology. Or maybe not at the wet mar market. Plus, there are coincidences in which resemble manipulations that vi But coincidence is not proof.

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Moreover, coincidences happen, and CoV2 could obviously have arisen naturally. It is not yet clear exactly how — for this, the bat and pangolin strains must have met in the same cell of some animal in Wuhan, since the outbreak occurred there (otherwise we would have seen other outbreaks along the path that animal would have taken to get to Wuhan). Given that bats were not sold in the Wuhan market, and generally hibernate at this time of the year, and that no other carriers of ancestral strains have yet been identified, the exact scenario of natural emergence remains a mystery.

On the opposite side of the balance, giving credence to the lab hypothesis, there are reports that in 2018, American experts were quite alarmed after their visit to the Wuhan Institute of Virology and conversation with Shi Zhengli. Their "lab tour" resulted in two diplomatic dispatches to Washington in which they noted a number of safety weaknesses:

> *Sources familiar with the cables said they were meant to sound an alarm about the grave safety concerns at the WIV lab, especially regarding its work with bat coronaviruses. The embassy officials were calling for more U.S. attention to this lab and more support for it, to help it fix its problems.*
>
> …
>
> *"During interactions with scientists at the WIV laboratory, they noted the new lab has a serious shortage of appropriately trained technicians and investigators needed to safely operate this high-containment laboratory," states the Jan. 19, 2018, cable, which was drafted by two officials from the embassy's environment, science and health sections who met with the WIV scientists. (The State Department declined to comment on this and other details of the story.)*

The Chinese researchers at WIV were receiving assistance from the Galveston National Laboratory at the University of Texas Medical Branch and other U.S. organizations, but the Chinese requested additional help. The cables argued that the United States should

give the Wuhan lab further support, mainly because its research on bat coronaviruses was important but also dangerous.

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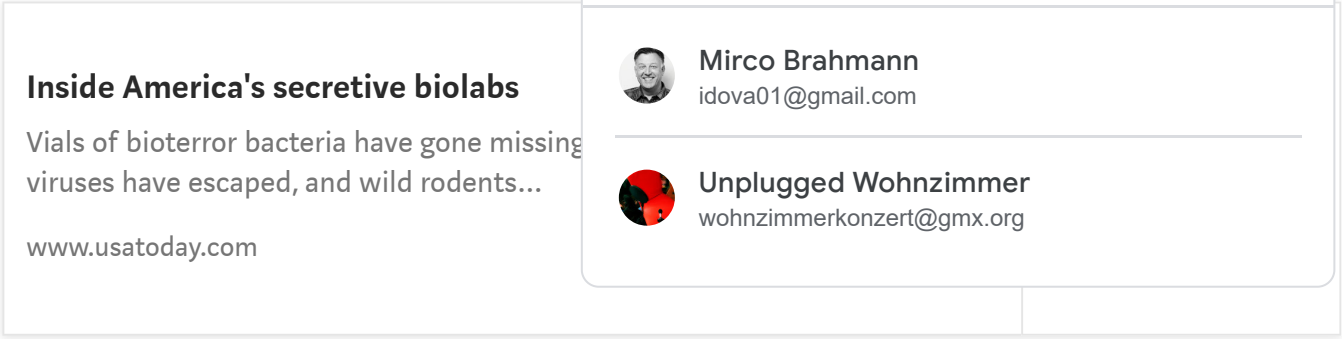
It is somewhat ironic the Wuhan lab r
Galveston, which at one time had itsel
specialists were trained at Galveston,
own newsletter (though, that publicat
it is still available at the Wayback Mac

A couple of final touches to the family portrait of laboratory leaks: in November 2019, an outbreak of brucellosis (a bacterial infection) occurred in two research centers in

Lanzhou, China, infecting over 100 researchers who worked there. American labs have also not been immune to outbreaks, a



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Possible Hallmarks of Lab Origin?

Let us now turn our attention back to the virus itself. Does it have any obvious signs of lab manipulation? First, a few words about what "obvious" means. Any mutation can arise naturally, and even if the amino acid insert that had created the furin site in CoV2 was not "PRRA" but "MADEINWVHANPRRA", there would still be a non-zero chance that it arose by accident. But for us, and for any court, I think this would be enough to prove lab origin *beyond a reasonable doubt*.

The main problem with such evidence is that even in a lab-made virus it simply may not exist. Basically, a good genetic engineer can create a synthetic virus that would be indistinguishable from a natural one. Moreover, often researchers deliberately introduce some synonymous mutations into their designs so that later they can discern their strain from natural ones. But if the creators choose not to reveal these markers, it is impossible to distinguish them from natural mutations.

But sometimes traces of manipulation may remain, especially if the creators do not try to hide them. First of all, I am talking about the spots in virus genome where its DNA is cut (recall that RNA virus manipulations are carried out in complementary DNA constructs). This occurs when virus creators need cut out a segment, or stitch together new segments. After all, DNA cannot be cut in arbitrary places (CRISPR aside), but only where its nucleotide sequence (usually 4–6 "letters") forms a sequence recognized by some restriction enzyme, that is, an enzyme that can cut a nucleotide chain. However, such an analysis is complicated by the fact that there are hundreds of different types of restriction enzymes used in genetic engineering. But let's try it anyways.

As a baseline, here is an example of the work of the Baric group from 2008, where they took Bat-SCoV and replaced its RBD by an RBD from human SARS. Here's how they

describe the creation of their chimera:

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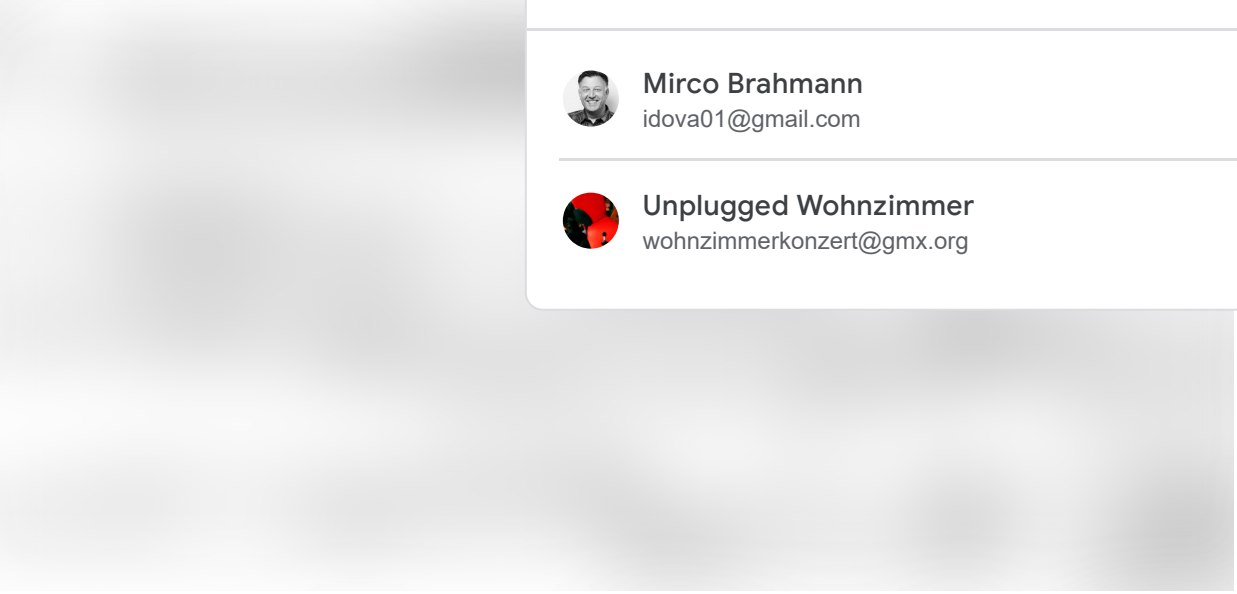

Schematic representation of SARS-CoV and Bat-SCoV variants.
(A) Schematic representation of SARS-CoV and Bat-SCoV (GenBank accession no. FJ211859) genomes and reverse genetics system. (Top) Arrowheads indicate nsp processing sites within the ORF1ab polyprotein (open arrowheads, papain-like proteinase mediated; filled arrowheads, nsp5 [3C-like proteinase] mediated). Immediately below are the fragments used in the reverse genetics system, labeled A through F. The fragments synthesized to generate Bat-SCoV exactly recapitulate the fragment junctions of SARS-CoV with the exception that the Bat-SCoV has 2 fragments, Bat-E1 and Bat-E2, which correspond to the SARS-E fragment.

As you can see, the Baric group first created a synthetic clone of Bat-SCoV in the same pattern as they used for their synthetic clone of SARS-CoV. That is, for the bat clone, they used the same 6 segments with the same restriction enzyme sites that they had previously used for SARS-CoV, which allowed them to swap virus segments between different strains like Lego pieces. Here is their detailed description:

> *Viruses containing PCR-generated insertions within the viral coding sequence were produced by using the SARS-CoV assembly strategy (24, 33, 53) with the following modifications. Briefly, for Bat-F virus, full-length cDNA was constructed by ligating restriction products from SARS-CoV fragments A–E and Bat-SCoV fragment F, which required a BglI-NotI digestion. For Bat-SCoV and Bat-SRBD, Bat-SRBM, and Bat-Hinge,* ***plasmids containing the 7 cDNA fragments of the Bat-SCoV genome were digested by using BglI for Bat-A, Bat-B, Bat-C, and Bat-D, BglI and AflII for Bat-E1 and Bat-E2, and BglI and NotI for Bat-F.*** *Digested, gel-purified fragments were simultaneously ligated together. Transcription was driven by using a T7 mMessage mMachine kit (Ambion), and RNA was electroporated into Vero cells (24, 53).*

All these three-letter abbreviations (BglI, AflII, NotI, etc.) in the sentence highlighted above are different types of restriction
the restriction enzyme sites in the spik
the genome of the original SARS-CoV

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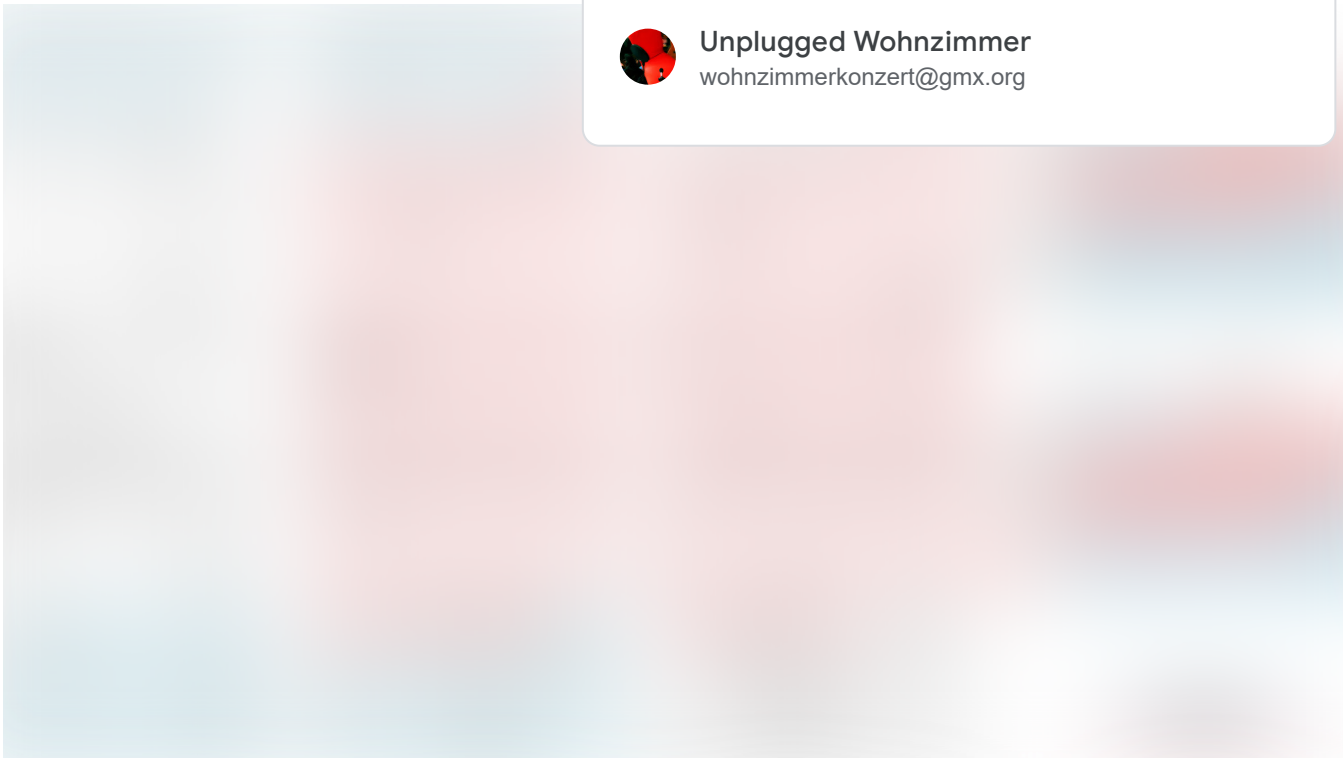

As can be seen, the restriction enzyme sites of the chimera are almost identical to those in the original sequences in Bat-SCoV or SARS from where they were taken. The only differences are noticeable at the “stitching” sites of the inserted SARS piece. Here, for example, is the left (5’-) edge of the insert:

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Here Bat-SCoV and SARS turned out to have a common identical region of nucleotides (the intersection of cyan and pink regions), and there are no new restriction enzyme sites at the stitching site of the two sequences, on the contrary, the SspI site from SARS disappeared. And here is the right (3'-) edge of the insert:

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Here, on the contrary, all the original restriction enzyme sites remain at the site of ligation, and even new ones appear, for example, EcoRII. Had I not known that the chimeric genome is the result of lab manipulations, could I deduce this by looking at these 3 sequences? Not really, and even if some suspicion did creep in, it would certainly not be beyond a reasonable doubt. Perhaps it would be obvious to specialists in genetic engineering by some other signs, and, if so, I hope they speak up.

But in any case, let's compare the RaTG13spike protein to CoV2 and pangolin-2019. Just in case something does jump out.

This is what the RBD (highlighted in light green) and RBM (yellow) look like for all three:

So, anything interesting? Well, I noticed some new restriction enzyme sites in CoV2 marked by red rectangles — they coin
sequence (also marked by red rectang
Just in case, I highlighted several othe
rectangle located in the region of the
CoV2 and pangolin-2019.

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Let's now compare the stretch around
CoV2 among those three strains:

Here, too, several new restriction sites have appeared (highlighted in blue) on both sides of the new insert. Could they have been used to create a furin site? Theoretically, yes. Alternatively, the insertion could have been made via existing sites or even using the "seamless" ligation method — i.e. by creation of segments with new restriction sites which disappear after the complementary ends are joined. You might remember that the Baric group have applied this technology in 2002 to create a synthetic clone of murine coronavirus:

> *The interconnecting restriction site junctions that are located at the ends of each cDNA are systematically removed during the assembly of the complete full-length cDNA product, allowing reassembly without the introduction of nucleotide changes.*

In 2003 they have used this approach again for a synthetic clone of SARS-CoV:

> *To rapidly assemble consensus clones, we used class IIS restriction endonucleases that cut at asymmetric sites and leave asymmet*
> *unique overhangs* ***that allow the seam***
> ***concomitant loss of the restriction si***

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Today, genetic manipulation techniqu
that the October 2019 Beijing paper w
chicken coronavirus, only devoted a c

> ***2.2. Generation of Recombinant Virus***
> *Recombinant rYN-S2/RRKR virus containing an S protein with the furin-S2′ site was generated by vaccinia recombination, as described previously [20,28]. Briefly,* ***plasmid with the furin-S2′ site was generated using the Seamless Assembly kit*** *(Invitrogen, Carlsbad, CA, USA) and transfected into CV-1 cells infected by vaccinia virus containing the genome of YN-ΔS-GPT. Furin-S2' site was introduced into the YN cDNA by homologous recombination using the transient dominant selection system [25].*

The pace of progress in genetic engineering is astounding. Here is a description of the above Seamless Assembly kit:

> *The GeneArt® Seamless Cloning and Assembly Kit enables the simultaneous and directional cloning of 1 to 4 PCR fragments, consisting of any sequence, into any linearized vector,* ***in a single 30-minute room temperature reaction****. The kit contains everything required for the assembly of DNA fragments, and their transformation into E. coli for selection and growth of recombinant vectors.*
>
> *•* ***Speed and Ease*** *— Clone up to 4 DNA fragments, with sequence of your choice, simultaneously in a single vector (up to 13 Kb); no restriction digestion, ligation or recombination sites required*
> *•* ***Precision and Efficiency*** *— Designed to let you clone what you want, where you want, in the orientation you want, and achieve up to 90% correct clones with no extra sequences left behind*
> *•* ***Vector Flexibility*** *— Use our linear vector or a vector of your choice*
> *•* ***Free Tools*** *— Design DNA oligos and more with our free web-based interface that walks you step-by-step through your project*
> *•* ***Diverse Applications*** *— Streamline many synthetic biology and molecular biology techniques through the rapid combination, addition, deletion, or exchange of DNA segments*

Up to 4 DNA fragments can be joined in a desired orientation in about half an hour, without having to deal with restrictio
“upload” your creation into *E. coli* to p

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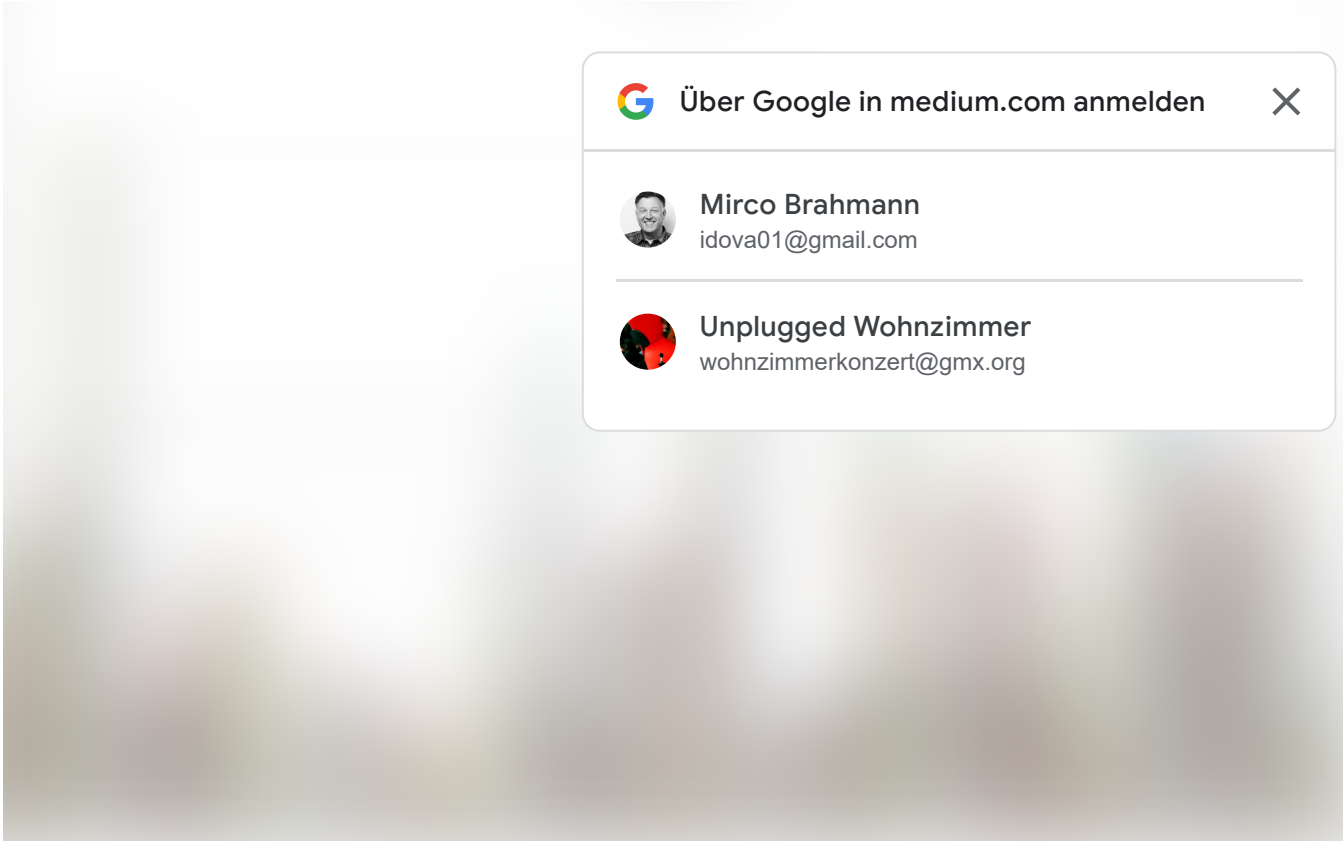
In summary, the restriction enzyme si
did, however, point out that not only
should continue digging into the origi

Codon Preferences

For these purposes, I decided to take a look at codon usage bias to check which strains look like CoV2 and RaTG13 the most. It is known that viruses tend to adapt their codon signature to the preferences of their hosts, so I expected to see RaTG13 exhibit a similar pattern to other bat viruses, and also hoped to see a difference from pangolin strains.

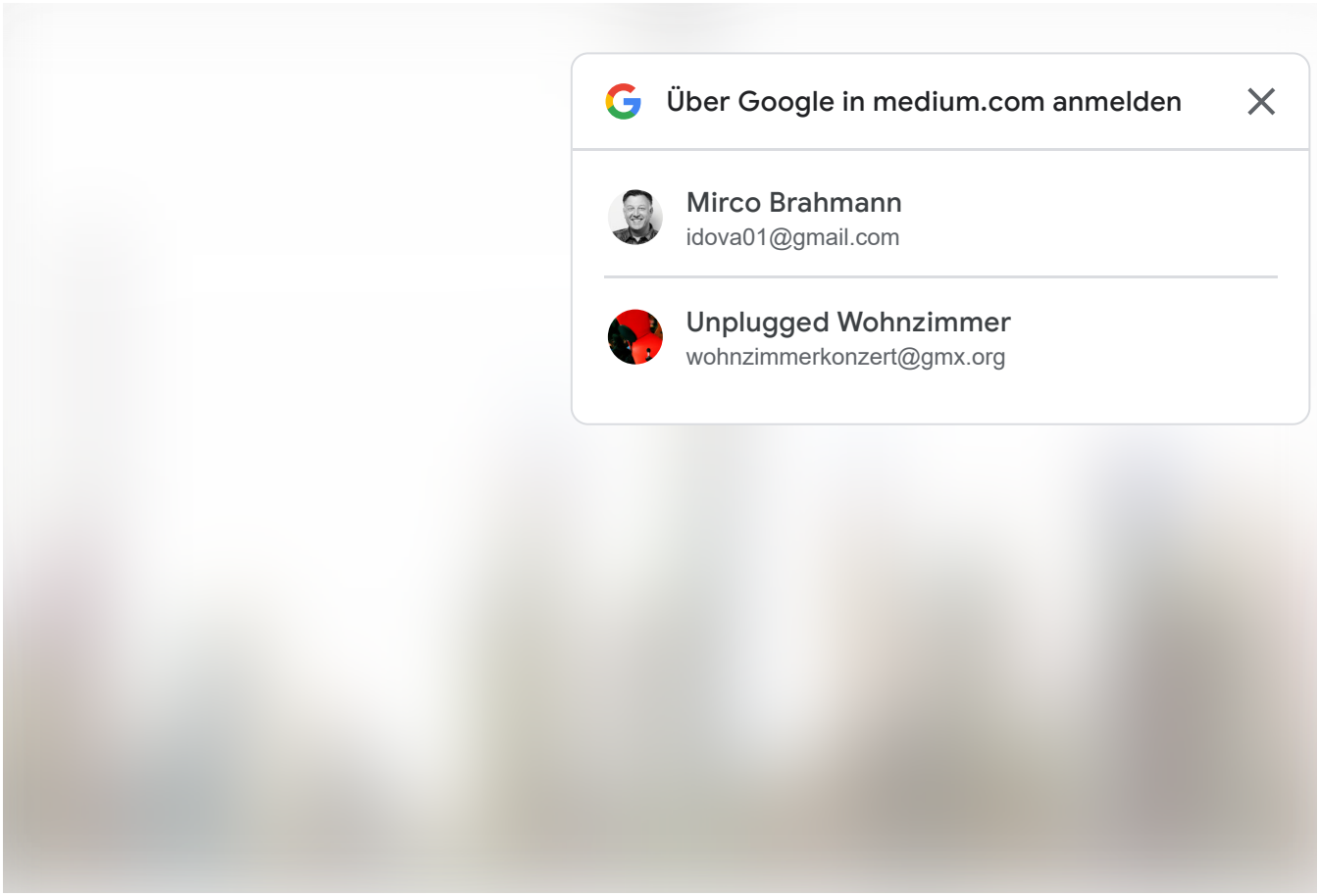
SARS-CoV, for example, is very similar to Rs3367 and RsSCH014, as one might expect:

Among themselves, by the way, SARS, MERS and CoV2 do differ:



RaTG13 is similar to CoV2, which is also to be expected:

But RaTG13 is actually not that close to the pangolin strains, and the pangolin strains are not exactly identical to each other:



RaTG13 also differs from ZXC21 and ZC45:

Looking at Yunnan strains, RaTG13 is quite distant from Rs3367 and RsSCH014, and closer to LYRa11, but also with notice

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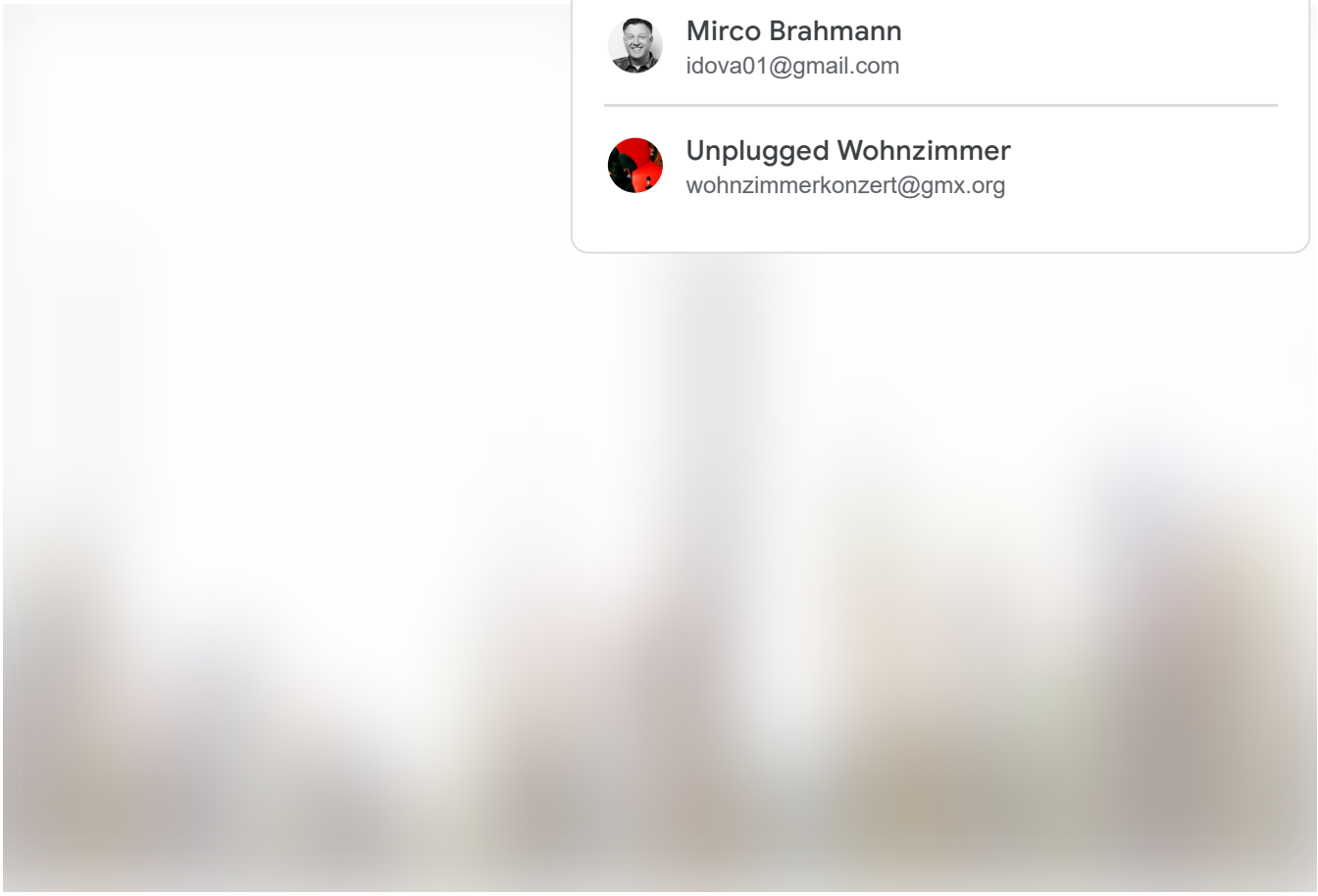

In general, as before, RaTG13 and CoV2 stand out in a class of their own. I was also intrigued by the AAA codon — they use it much more often than their fellow strains:

This is probably just another coincidence, but a similar proportion between AAA and AAG is observed in *E. coli*. Can the cDNA codon signature change if it is being

cultivated for a long time in cell culture? Maybe, but I haven't yet dug into this topic very deeply.

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[UPDATED] I also decided to check co
Ra strains collected from the same aba
Zhengli's group found strain RaBtCoV
bp RdRp segment with RaTG13. Unfo
gene were available for the other Ra s
I extracted the corresponding 816-bp segment from RaTG13 for the purposes of codon usage comparison. RaTG13 again differed substantially, while the other two strains clustered together:

So codon analysis also did not reveal any obvious signs of lab origins, but once again confirmed the uniqueness of CoV2 and RaTG13. What does this leave us with? So far, just a number of oddities, which, as scientists like to say, *taken together*, do not allow us to reject the lab origin hypothesis of CoV2.

The Nature Paper vs. the Lab-Made Hypothesis

But didn't that Nature article refute the lab-made hypothesis? No, not really. There is no irrefutable evidence against it in the paper, just a loud "we don't believe so" based

on a shaky foundation. Judge for yourself — here are the authors' key arguments in support of their conclusions:

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> *While the analyses above suggest that S affinity, computational analyses predic sequence is different from those shown Thus, the high-affinity binding of the S likely the result of natural selection on optimal binding solution to arise. This is strong evidence that SARS-CoV-2 is not the product of purposeful manipulation.*

In the original paper, the quoted sentences are just below the diagram showing identical RBMs between CoV2 and pangolin-2019. So I am puzzled as to what "computational analysis" has to do with anything. Obviously, the most likely scenario for the lab-made hypothesis is the transfer of RBM from one strain to another — which virologists have done many times before. Therefore, the author's chain of arguments does not make sense: "computer says binding is not ideal, thus CoV2 must be the result of natural selection. Ergo, this is strong evidence that CoV2 is not lab-made." Wait, just because CoV2 differs from some "optimal" virus, doesn't mean it could not have been created in a lab. Not the lab trying to create "optimal" bioweapons, but a lab creating chimeras of naturally found strains, say, in bats and pangolins.

The authors continue to surprise:

> *Furthermore, if genetic manipulation had been performed, one of the several reverse-genetic systems available for betacoronaviruses would probably have been used. However, the genetic data irrefutably show that SARS-CoV-2 is not derived from any previously used virus backbone.*

Again, the same questionable logic dressed in categorical adjectives: "genetic analysis irrefutably proves that CoV2 was not created on the basis of previously known strains!" Well thanks, Captain Obvious. But why couldn't potential creators of CoV2 make a cDNA backbone from unpublished strains related to or even derived from RaTG13? Then they could easily insert the pangolin RBM into it, as well as add a furin site (or maybe the cDNA backbone already had one). Virologists have been doing things like this for 20 years, and modern genetic engineering tools make such manipulations accessible even to a grad student.

As for the chances of the furin site arising in cell culture, the authors also express strange ideas:

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> *The acquisition of both the polybasic cl*
> *argues against culture-based scenarios.*
> *only after prolonged passage of low-pat*
> *Furthermore, a hypothetical generatio*
> *would have required prior isolation of* ***similarity, which has not been described.*** *Subsequent generation of a polybasic cleavage site would have then required repeated passage in cell culture or animals with ACE2 receptors similar to those of humans, but such work has also* ***not previously been described****.*

First off, the authors themselves cite previous works where the furin site arose *in vitro* as viruses were cultured in cells. And second, what do they mean, a strain with high genetic similarity has not been described — what about RaTG13? If it had its RBM replaced by one from the pangolin strain, and then the chimeric strain was cultured *in vitro*, then the furin site could well have arisen in this matter. Additionally, the new strain could thus acquire other mutations that distinguish CoV2 from RaTG13 and pangolin-2019.

But in terms of the potential lab-based origin of the furin site, I am more inclined to hypothesize a specific insertion — as in the Beijing paper from October 2019 with chicken coronavirus. After that, the synthetic strain could have acquired new mutations by subsequent culturing *in vitro* or *in vivo* — like the MA15 murine strain in 2007, for example. Or maybe even using the same mouse model with humanized lung tissues and immune system that was created at UNC by Baric's and other groups in 2018, in which they reported testing several viruses including MERS:

> ***The human innate and adaptive immune system of BLT-L mice***
> *We generated an in vivo model with human lung implants and an autologous human immune system by constructing BLT mice with autologous human lung implants (BLT-L humanized mice).*

Finally, even if CoV2 is the product of selection rather than intelligent design, that does not rule out a lab leak either — selection can happen in the lab just as well, both natural and artificial kinds. Different strains can recombine in research animals or *in vitro* by design or by chance.

On the 4% Genome Difference between RaTG13 and Cov2

Some critics of the lab-made hypothes
difference between RaTG13 and CoV2
RaTG13 itself was used as a backbone
widely — from 10^{-6} to 10^{-4} nucleotide
seems to mutate at a rate of 25 mutati
years, if not decades, for two strains to
are several issues with that line of reasoning.

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First, *in vitro* mutation *speeds* (i.e. per unit of time) are much higher, as you can passage cells much more often than infect new animals. As SARS and MERS *in vitro* experiments showed, significant mutations might be observed after only a few passages. For example, the 2004 paper reported that only after 600 passages there already was a 2.1% difference in the genomic sequences of spike proteins between the original strain and its progeny:

Moreover, in the presence of some antiviral compounds, such as nucleoside analogs (e.g. ribavirin or remdesivir), mutation rates in RNA viruses can increase even further:

> *We obtained an estimate of the spontaneous mutation rate of ca. 10^{-4} substitutions per site or lower, a value within the typically accepted range for RNA viruses.* ***A roughly threefold increase in mutation rate and a significant shift in mutation spectrum were observed in samples from patients undergoing 6 months of interferon plus ribavirin treatment.*** *This result is consistent with the known in vitro mutagenic effect of*

> *ribavirin and suggests that the antiviral effect of ribavirin plus interferon treatment is at least partly exerted through lethal mut*

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So if ancestral CoV2 was being lab-tes
the efficacy of potential vaccines or ar
mutations at a much higher rate.

But possibly, the biggest problem with
RaTG13 being exactly what WIV says it is. If we are to seriously consider the lab leak hypothesis, we must concede that it does not make sense to blindly trust the data released by the very lab suspected of the leak. If the leak did occur, as is the premise of the lab hypothesis, then the description of what RaTG13 is could be furthering the goal of covering up the leak.

Again, I am not claiming with certainty that is what is happening here. All I am saying is that this is what *could* have happened, and we need a lot more evidence before we can reach a definitive conclusion. One thing that could help rule out tampering with RaTG13 is having independent labs sequence the 2013 Yunnan samples that She Zhengli extracted RaTG13 from. WIV must still have them if they re-sequenced RaTG13 in 2020.

Shi Zhengli-2020

As I was writing this post, a fresh paper co-authored by Shi Zhengli came out, in which the authors tested a peptide which they have been studying for some time before against CoV2. That peptide was meant to be a pan-coronavirus inhibitor, and its designed mode of action was to block the fusion of a spike protein with a cell membrane. The authors, of course, mention the new furin site of CoV2, and suggest that it may play an important role in the much more efficient penetration of CoV2 into the cell:

> *In this study, we have shown that SARS-CoV-2 exhibits much higher capacity of membrane fusion than SARS-CoV, suggesting that the fusion machinery of SARS-CoV-2 is an important target for development of coronavirus fusion inhibitors.*
> …
> *Generally, β-B coronaviruses lack the S1/S2 furin-recognition site, and their S proteins are uncleaved in the native state. For example, SARS-CoV enters into the cell mainly via the endosomal membrane fusion pathway where its S protein is cleaved by endosomal cathepsin L and activated. Inducing the S1/S2 furin-recognition site could significantly*

> *increase the capacity of SARS-CoV S protein to mediate cellular membrane surface infection.*

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In this context, I wonder whether the
on how adding a furin site can alter th
vaccines) against a given coronavirus.

Not to be outdone, Ralph Baric also jo
understand, he and co-authors took data on the effectiveness of their nucleoside analogue (β-D-N4-hydroxycytidine, NHC) against SARS-CoV and MERS that they already had, added some *in vitro* data on CoV2, and sent off the paper to print. Nucleoside analogues (such as the famous remdesivir) are a fundamentally different approach than Shi Zhengli *et al.* Here, the authors try to prevent viral replication by giving "defective" letters of the genetic alphabet to virus' copying machine, while Shi Zhengli and coauthors try to prevent the virus from entering the cell altogether. Theoretically, these approaches could be combined.

This is the End, Beautiful Friend

If you made it here by reading rather than scrolling, mad props to you. Hey, even if you scrolled, that's cool too, and I apologize for the verbosity. I just didn't anticipate that the rabbit hole would turn out to be a whole underground cave system. I hope that you found this deep dive into the world of virology interesting and enjoyed the exploration of the lab-made CoV2 hypothesis. In my opinion, the data I have presented, taken together, do not allow us to reject this possibility.

Let me be clear: this does NOT prove that CoV2 was synthesized in the laboratory. Yes, as we have seen above, from a technical standpoint, it would not be difficult for a modern virologist to create such a strain. But there is no direct evidence that anyone did this, and strange coincidences cannot pass for circumstantial evidence. On balance, the current chances against this are still higher than for the natural origins of CoV2. Moreover, even if CoV2 was indeed an unfortunate lab leak, the scientists themselves are not to blame, as they were working within the established international laws and guidelines on such research. Now, those who might be trying to cover up that leak, that's a different story.

The opposite point is worth repeating too: the inverse hypothesis about the exclusively natural origin of the virus does not yet have strong evidence either. Until intermediate ancestors between RaTG13, pangolin-2019 and CoV2 are found, in whom we could

trace the mosaic recombination that we observe in CoV2, the question of its origins remains open. In closing, there is no o

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Baric himself:

> ***What is the reservoir species of SARS***
> *They have not identified the actual rese*
> *potentially the intermediate host, but p*
> *CoV-2. In comparison, civet and racoon*
> *identical to SARS-CoV from 2003. In other words, we are talking about a handful of mutations between civet strains, racoon dog strains and human strains in 2003. Pangolins [strains of CoV2] have over 3000 nucleotide changes, no way they are the reservoir species. Absolutely no chance.*

So there you have it. It remains possible that the mysterious virus host was a lab:

Bad pun? Sorry, last one.

How I Learned to Hate the GOF

Ich hoffe, dieser Beitrag wird nicht dazu verwendet, vorzeitig Schuld zuzuweisen oder einseitige Theorien zu verbreiten. Was ich *tun* hoffe , es unterstreicht die Skala von gefährlicher gain-of-function Forschung , die war und *ist* los in der Virologie. Die Covid-19-Pandemie hat ihre enormen Risiken angesichts weniger Vorteile deutlich gemacht: Die GOF-Forschung hat uns nicht vor diesem Ausbruch geschützt, uns keine wirksamen Behandlungen oder Impfstoffe rechtzeitig zur Verfügung gestellt, um Hunderttausende von Menschenleben zu retten CoV2, und wenn es sogar eine 0,1% ige Chance gibt, dass GOF-Forschung das Ganze verursacht hat, ist diese Chance zu hoch.

Covid-19 SARS-CoV-2 Virologie Wuhan Coronavirus

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