Feline infectious peritonitis (FIP) is caused by feline coronaviruses (FCoVs) and represents one of the most important lethal infectious diseases of cats. To date, there is no efficacious prevention and treatment, and our limited knowledge on FIP pathogenesis is mainly based on analysis of experiments with field isolates. In a recent study, we reported a promising approach to study FIP pathogenesis using reverse genetics. We generated a set of recombinant FCoVs and investigated their pathogenicity in vivo. The set included the type I FCoV strain Black, a type I FCoV strain Black with restored accessory gene 7b, two chimeric type I/type II FCoVs and the highly pathogenic type II FCoV strain 79–1146. All recombinant FCoVs and the reference strain isolates were found to establish productive infections in cats. While none of the type I FCoVs and chimeric FCoVs induced FIP, the recombinant type II FCoV strain 79–1146 was as pathogenic as the parental isolate. Interestingly, an intact ORF 3c was confirmed to be restored in all viruses (re)isolated from FIP-diseased animals.

FCoVs can be classified into two biotypes according to their pathogenicity. Feline enteric coronavirus (FECV) is known to cause mild enteritis or an often symptomless persistent infection whereas feline infectious peritonitis virus (FIPV) is the causative agent of the fatal, systemic granulomatous disease feline infectious peritonitis (FIP). In accordance with the internal mutation theory, FIPVs arise through mutations from FECVs in approximately 5% of the persistently infected cats. So far, the responsible mutations for biotype switch from FECV to FIPV have not been identified, although mutations in the accessory genes 3c, 7a, and 7b as well as in the S gene are thought to be associated with the development of FIP. Lately, Chang and colleagues speculated about the possible role of 3c in the pathogenesis of FIP. Upon extensive sequence analysis they reported that more than two thirds of the FIPV isolates harbored mutated 3c genes, while all of the FECV isolates contained intact 3c genes. Consequently, the authors suggested that an intact 3c gene is essential for the replication of FECV in the gut, but it is nonessential for systemic replication of FIPV. Additionally, as pointed out by Chang and colleagues, it is still not known to which extent other regions within the 29 kb genome might...
contribute to the biotype switch. It is important to note that usually the collected samples in various studies represent random field cases, which makes it impossible to follow with which FCoVs the animals have originally been infected. Therefore, there is still an urgent need to experimentally verify at the molecular level the mutations that contribute to the development of FIP and to investigate whether such mutations are the same for type I and type II FCoVs. For such experiments, the use of well-defined viruses is indispensable. Nevertheless, one major obstacle hampering the research on FCoV is the fact that FECV field viruses do not grow in cell culture. There are only few FIPV isolates that can be cultured in vitro, however most of them lost the ability to induce FIP in animals due to cell culture adaptation.\textsuperscript{2,16}

The most compelling way to tackle the molecular pathogenesis of FIP and experimentally validate the responsible mutations that lead from the harmless FECV to the fatal FIPV is the usage of well-defined and characterized viruses generated by reverse genetics followed by animal experiments. For FCoVs three different types of reverse genetic systems exist. The first system is based on targeted RNA recombination and was established for type II FIPV strain 79–1146.\textsuperscript{20} Although this system is a very useful tool to manipulate the 3′-third of the viral genome, its major disadvantage is the inability to target the 5′ untranslated region (UTR) of the genome and the entire replicase gene. A further reverse genetic system, based on bacterial artificial chromosome (BAC), has recently been developed for type II FIPV strain DF2.\textsuperscript{21} Although this system enables one to alter the entire FCoV genome, instability of the cloned cDNA in the BAC often makes the efficient generation of recombinant FCoVs difficult. The third type of reverse genetic system for FCoVs eliminates the disadvantages of the prior two systems. This was engineered in our laboratory and is based on the full-length cDNA of the type I FCoV strain Black inserted into the vaccinia virus genome. The full-length type I FCoV strain Black cDNA was assembled and inserted into the thymidine kinase (tk) gene of the vaccinia virus \textit{Not I}tk genome using a single \textit{Not I} cleavage site. The FCoV cDNA is preceded by T7 DNA-dependent RNA polymerase promoter and followed by synthetic poly-(A) tail, a \textit{Cla I} site, and hepatitis delta ribozyme (HDR) sequence (Fig. 1A) as described previously.\textsuperscript{22} The generation of recombinant FCoVs with our vaccinia virus-based reverse genetic system comprises the following steps: (1) manipulation of the FCoV cDNA using vaccinia virus-mediated homologous recombination (Fig. 1B) and (2) recovery of the recombinant FCoVs (Fig. 1C). The modification of the FCoV cDNA is performed by two rounds of vaccinia virus-mediated homologous recombination using guanosine phosphoribosyl transferase (gpt) as a positive and negative selection marker as described previously.\textsuperscript{22,14} This vaccinia virus-based reverse genetic system opened up new ways to study the molecular pathogenesis of FIP.

Recently, we described infection studies in cats using cloned recombinant FCoVs. In the first round of animal experiments, we assessed whether cell culture adapted type I FCoV Black virus and its recombinant counterpart (recFCoV) generated via reverse genetics, are capable of inducing FIP under experimental conditions. This is of importance, since the few type I FIPVs that can be grown in cell culture usually tend to lose their ability to induce FIP in cats due to cell culture adaptation.\textsuperscript{2,16} For the infection of cats with FCoVs, specific pathogen free (SPF) cats were used at an age of 4–5 mo. Groups of two kittens were infected intra-peritoneally (i.p.) with type I FCoV Black and recFCoV, respectively. The kittens were monitored for clinical signs of FIP on a daily basis. Blood and fecal samples were collected weekly over a period of 8 wk. Although productive FCoV infection could be confirmed via detection of FCoV-RNA in the feces as well as virus-specific antibodies in the blood for both groups, no FIP developed in any of the cats during the experiment. These results suggest that cell culture adaptation has turned this isolate from a cat with FIP into a non-pathogenic virus. It has been speculated that the presence of an internal stop codon or deletion of nucleotides in accessory gene 7b of FIPVs might be a sign of cell culture adaptation and could lead to loss of pathogenicity.\textsuperscript{2,16} In order to clarify whether the presence of an internal stop codon in 7b gene of our type I FCoV Black and recFCoV indeed contributes to the loss of pathogenicity, we removed the internal stop codon and restored the 7b gene. The newly engineered recombinant virus was designated recFCoV-\textit{ΔStop}-7b (Fig. 2) and used for infection of cats as described above. However, in spite of an intact 7b gene, the recombinant virus did not gain a capacity to induce FIP in kittens, suggesting that the internal stop codon in 7b gene cannot be the only contributing factor to a non-pathogenic phenotype and recFCoV-\textit{ΔStop}-7b genome harbors further attenuating mutations.

In order to generate a recombinant FCoV that is able to reproducibly induce FIP, we decided to replace additional portions of the recombinant Black genome with corresponding regions derived from the highly pathogenic type II FCoV 79–1146.\textsuperscript{23} In an initial step, the spike (S) gene and accessory genes 3a, 3b and 3c of the type II isolate were introduced into the background of type I FCoV Black. Accordingly, this virus encodes the structural gene S and the accessory genes 3a, 3b, and 3c from type II FCoV strain 79–1146, while the remainder of the genome is derived from type I FCoV strain Black. This chimeric virus was called recFCoV-S-3abcII (Fig. 2). In following experiments the latter virus was further modified and designed to result in a type II FCoV genome organization with regions of type I and type II-derived sequences similar to natural type II FCoVs that arose via recombination between type I FCoV and CCoV.\textsuperscript{11,12,24} Accordingly, we replaced the majority of type I FCoV ORF 1b with the corresponding sequence of type II FCoV strain 79–1146 in the recombinant virus recFCoV-S-3abcII. The new recombinant virus was designated recFCoV-1b-3abcII (Fig. 2). Finally, the remaining type I FCoV regions of this virus were replaced with the corresponding parts (5′UTR, ORF1a, remaining part of ORF1b, E, M, N, ORF7a, ORF7b, and 3′UTR) of the type II 79–1146 in order to generate a full-length recombinant type II FCoV 79–1146 (recFCoV-II). To assess
the pathogenicity of the type I and/or type II chimeric viruses and of the recFCoV-II and/or original type II FCoV 79–1146 cats were infected as described above. In spite of productive infection, none of the chimeric viruses induced FIP, suggesting that the type II parts were not sufficient to turn the non-pathogenic virus into FIP-inducing virus. This further implies that the remaining type I regions of the chimeric viruses contain attenuating cell culture adaptations. Another possible explanation is that the particular combination of type I FCoV strain Black and type II FCoV strain 79–1146 genomic regions do not lead to a pathogenic type II like FCoV capable of inducing FIP. To gain clarity in this regard, further experiments are needed.

In contrast, infection of cats with recFCoV-II and its corresponding original isolate type II FCoV 79–1146 robustly induced FIP. The animals displayed typical clinical signs of FIP, and the diagnosis was further confirmed by pathological examination. Finally, we successfully determined the entire FCoV genome sequence forms ascites of the type II FCoV 79–1146 diseased cat. For the determination of the FCoV sequence from cats infected with recFCoV-II, spleen and kidney tissues were used. After comparing the full-length sequences originating from ascites and/or tissues with the input virus sequence, we found one striking difference. In all sequences derived from diseased cats, we could detect the same single nucleotide change in the 3c gene. This mutation led to the restoration of a full-length ORF 3c that was neither present in the type II FCoV 79–1146 nor in the recFCoV-II input viruses. There is growing evidence that the 3c protein is associated with FCoV pathogenicity, and two studies showed that in most FIPVs the 3c gene is mutated. 17,25 In contrast, FECVs that replicate in the gastrointestinal tract contain an intact 3c gene. This led to the hypothesis that a functional 3c...
proteins are crucial for FECV replication in the gut but dispensable for systemic FIPV replication. At the first glance, our observation with restored ORF 3c in FIP-diseased cats seems to be in conflict with other previous findings. However, it is important to emphasize that our knowledge is very limited with regard to target cells and the time period during the course of infection where the presence of 3c might be beneficial for the virus. Furthermore, it is believed that FIPVs are not horizontally transmitted via the feces, since these viruses do not primarily replicate in the gut, which might be the consequence of disrupted ORF 3c in FIPVs. Interestingly, we were able to detect FIPV RNA in the feces samples shortly before the cats succumbed, suggesting that prior to death of the animals FIPV replication took place in the gut. In our case, this observation could be explained by an effect of the restored ORF 3c, which further supports the hypothetical role of an intact 3c protein in the replication of FCoVs in the gut. However, it needs to be further investigated whether all FIPVs with intact ORF 3c can replicate in the gut and are shed in the feces.

In summary, FIP is a lethal disease with essentially no treatment options. Our knowledge on FIP pathogenesis is still rudimentary and mainly based on studies of field isolates. Here, we describe an alternative approach to study FIP pathogenesis using reverse genetics. Based on our recently described full-length cDNA clone for cell culture-adapted type I FCoV strain Black, we have assessed the pathogenicity of a set of recombinant FCoVs. One major conclusion from our data are that the induction of FIP in cats by type II FCoV 79–1146 (recFCoV-II) establishes a novel experimental approach to study FIP pathogenesis with two main advantages: (1) the entire FCoV genome is amenable to mutagenesis, and (2) the recombinant FCoV used for infection is derived from cloned cDNA, and thus the genome sequence is precisely defined. In the future, it is of importance to extend reverse genetics in order to investigate the molecular pathogenesis of FIP induced by the predominant type I field isolates. However, a major obstacle is that there is no conventional culture system available to efficiently propagate type I field isolates. Thus, the establishment of such a cell culture system suitable to allow propagation of field isolates in combination with full-length cDNA clones of type I FCoVs is desired. It will also be important to obtain recombinant FECV/FIPV virus pairs in order to identify and characterize the basic molecular determinants of FIP pathogenesis and to map the mutations that are responsible for the biotype switch. These future developments will be instrumental to improve FIP diagnostic tools and to tackle important questions of FCoV biology and pathogenesis, such as identity of the so far unknown cellular receptor for type I field isolates, changes in the cellular tropism during the development of FIP, and the role of the host immune system during persistent FCoV infection.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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